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APPLICATION DE L'IMMUNOLOCALISATION A LA RECHERCHE DE LA CELLULE SOUCHE ENDOTHELIALE CORNEENNE HUMAINE

Présentée et soutenue publiquement par

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Le 28 octobre 2011

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AVANT-PROPOS

L'endothélium cornéen, monocouche cellulaire spécialisée dans le contrôle de l'hydratation du stroma, est indispensable au maintien de la transparence de la cornée. Pour assurer ses fonctions, l'endothélium doit être composé de cellules endothéliales (CE) parfaitement jointives, sans solution de continuité, et en nombre suffisant pour que les sites enzymatiques responsables des mouvements ioniques soient assez nombreux. La gamme de densité cellulaire endothéliale (DCE, exprimé en cellules/mm^2) compatible avec un fonctionnement normal, c'est à dire capable de maintenir transparente la cornée est étonnamment large s'étendant de 400 à 4000 cellules/mm^2. La surface des CE peut donc varier d'un facteur 10 (250 µm^2 pour les plus petites à 2500 µm^2 pour les plus grosses) sans que leur fonction ne soit en apparence perturbé. De façon physiologique, la DCE décroit très lentement tout au long de la vie mais reste toujours largement suffisante pour que la cornée reste transparente même aux âges les plus avancés. Chez l'homme, cet endothélium est dépourvu de capacité régénérative significatives in vivo. Aussi, les pathologies endothéliales primitives ou secondaires qui font disparaître les CE en place sans possibilité de remplacement efficace par de nouvelles CE, aboutissent à un dysfonctionnement endothélial permanent responsable d'un œdème de cornée irréversible et d'une cécité dite « cornéenne ». Dans ces pathologies de différentes origines, le traitement actuel est uniquement chirurgical : l'allogreffe de cornée. La greffe peut remplacer soit toute l'épaisseur du tissu soit sélectivement l'endothélium si l'œdème stromale ne s'est pas encore compliqué de phénomènes fibrosant formant des cicatrices irréversibles. Dans les deux cas le greffon doit apporter un maximum CE au receveur pour garantir un fonctionnement durable du greffon. Or, si les CE ont une longévité « fabuleuse » in vivo (si l'on admet que les mêmes CE survivent de la naissance à la mort de l'individu), il en est tout autrement après le décès. Certaines CE du greffon meurent rapidement quel que soit le procédé de conservation, à 4°C dans le système de conservation à court terme (2-8 jours) des américains ou à 31°C en organoculture selon la méthode « européenne ». A titre d'exemple, on estime que la perte cellulaire quotidienne en organoculture est 600 fois plus élevée que la perte cellulaire physiologique. Toute la problématique de la conservation des greffons par les banques de cornée est donc de préserver au mieux la viabilité des CE et de contrôler de façon précise leur nombre afin de sélectionner uniquement les meilleures greffons Aussi, au moment de la cession d'une cornée par une banque, une DCE supérieure à 2000-2400 cellules/mm^2 est indispensable pour assurer une survie durable de la cornée chez son receveur. La sélection est telle que 20% à 30% des 100 000 à 150 000 greffes conservées chaque année en Europe en organoculture sont exclues de la greffe en fin de conservation pour qualité endothéliale insuffisante. [Peh 2011]. Un partie de notre travail expérimental de Thèse a consisté à développer un outil innovant permettant une meilleure formation des techniciens des banques de cornées et un contrôle de la fiabilité de leurs analyse en terme de précision et de reproductibilité. Cet outil, nommé kératotest, consiste en un procédé de microlithogravage sur lame de verre d'une mosaique endothéliale photographiée sur des cornées humaines de différentes DCE. Le procédé est en cours de publication dans Optics Letter (Article 2). Et va être utilisé en cours d'année pour analyser la fiabilité de l'analyse endothéliale dans 100 banques de cornées européennes (PHRC inter régional 2011).

Un autre enjeu, encore futuriste mais néanmoins réaliste, pour les banques de cornées, sera de proposer des greffons « enrichis » en CE, par thérapie cellulaire ou génique ex vivo durant la conservation, voire des greffons endothéliaux crées « ex nihilo » par bioengineering, afin de subvenir aux besoins croissants de greffons à travers le monde. Cette perspective nécessite de franchir deux étapes majeures : comprendre ce qui régule la non prolifération de la majorité des CE chez l'homme et identifier s'il persiste des CE qui possèdent des capacités prolifératives résiduelles exploitables, que ce soit des cellules d'amplification transitoire ou de réelles cellules souches endothéliales adultes. Aussi, nous avons organisé notre travail expérimental de Thèse autour de trois parties principales qui contribuent à répondre à ces questions.

La première partie a consisté à mettre au point une technique d'immunolocalisation sur l'endothélium de cornées humaines montées à plat qui présente de nombreux avantages par rapport à l'histologie classique sur coupe transversale mais qui n'était, jusqu'à présent utilisée que de façon anecdotique et non systématisée. La visualisation de l'ensemble des CE de l'endothélium intact, « vu d'avion », permet l'étude de disparités régionales dans l'expression des protéines et le repérage d'éventuelles cellules rares ayant un profil d'expression différent au sein d'une population en apparence homogène. L'observation de la CE en face, permet par ailleurs une parfaite localisation intracellulaire de protéines ciblées. Avec cette technique, nous avons analysé l'expression de protéines impliquées dans le contrôle du cycle cellulaire : cyclin D1 et E et inhibiteurs des kinase dépendante des cyclines p21, p27 et p16 de la phase G1, cycline A de la phase S,

MCM2, PCNA, Ki67, 3 marqueurs usuels de prolifération, et ZONAB impliqué dans l'arrêt de prolifération à confluence. (ARTICLE 1)

L'approche génique est indispensable pour appréhender les mécanismes de régulation du cycle cellulaire, en particulier les approches de sur et de sous expression géniques. La transfection des CE est de plus une méthode potentielle pour une future thérapie du greffon visant à réduire la perte de CE durant la conservation, ou du patient pour traiter des patients porteurs de stades précoces de pathologies endothéliales comme la dystrophie de Fuchs. Cette approche est ici facilitée par la structure en monocouche des CE. Deux méthodes sont disponibles actuellement : les vecteurs (vecteur viraux et synthétiques) et l'électroporation. Bien que des études portant sur les techniques de transfection par vecteurs, en particulier rétroviraux aient montré un meilleur taux de transfection, leur utilisation en pratique clinique est sujette à caution, devant leur absence d'innocuité totale à cause du risque éventuel d'immunogénicité et de mutation. A l'inverse, la transfection par électroporation n'introduit aucune matière exogène autre que le gène d'intérét. Elle n'avait pas été étudiée pour l'endothélium cornéen humain. La deuxième partie de notre thèse a consisté à mettre au point une technique de transfection de gène par électroporation sur l'endothélium de cornées humaines conservées en organoculture. (ARTICLE 3)

La recherche de la cellule souche endothéliale a constitué la dernière approche de notre travail. Il existait dans la littérature de nombreuses pistes pour l'existence, à la périphérie de la cornée humaine d'une zone au potentiel prolifératif, sans toutefois que n'aient jamais été identifiées de CE en division, de cellules souches clairement repérables ni de structures anatomiques correspondant à des niches des cellules souches. En s'inspirant des nombreux travaux réalisés depuis 20 ans sur la régénération épithéliale cornéenne à partir du limbe scléro-cornéen, nous avons analysé de façon systématique une grande série de cornée en combinant plusieurs techniques dont l'immunolocalisation mise au point plus tôt au cours de notre Thèse. Le choix d'analyser des cornées immédiatement après le décès des donneurs du don du corps à la Science nous a permis d'avoir une source inestimable de tissus non modifiés par la conservation ex vivo. Cette originalité nous a permis de revisiter la microanatomie de la périphérie de l'endothélium cornéen humain et de montrer des preuves indirectes d'une migration continue des CE de la périphérie vers le centre. Nous avons ainsi pu proposer un modèle d'homéostasie endothéliale intégrant l'existence de cellules souches périphériques dont la niche reste à préciser et la migration en colonne centripète vers l'axe optique de la cornée (ARTICLE 4).

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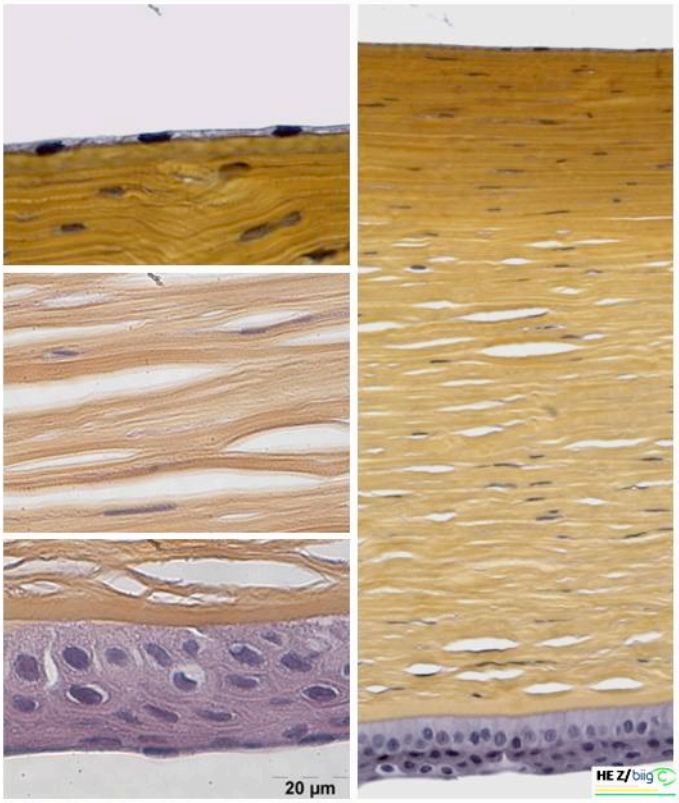

Figure 1. Histologie de la cornée humaine en coupe transversale. ***(Images de BiiGC/He et Dumollard)***

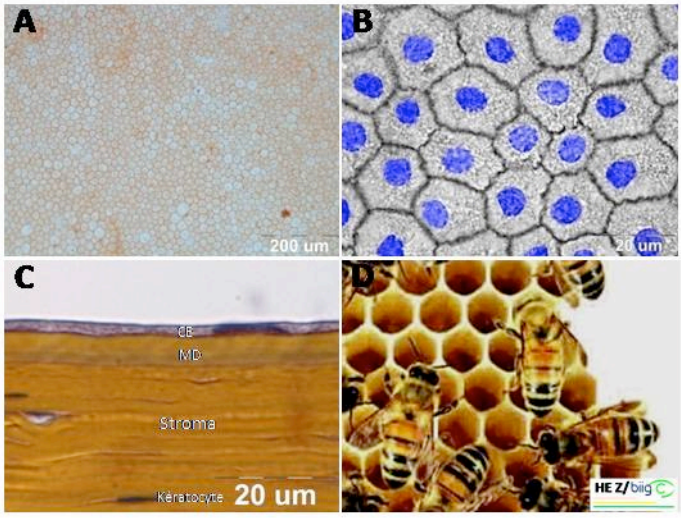

Figure 4. Morphologie de l'endothélium cornéen humain. A) Contours cellulaires colorés par le rouge alizarine sur une cornée montée à plat; B) Contours cellulaires colorés par le rouge alizarine associés à un marquage des noyaux par Hoechst sur une cornée montée à plat; C) Histologie d'une cornée en coupe transversale utilisant la coloration HES D) Modèle « Nid d'abeille» de morphologie similaire aux CE cornéennes. ***(Images de BiiGC/He et Dumollard)***

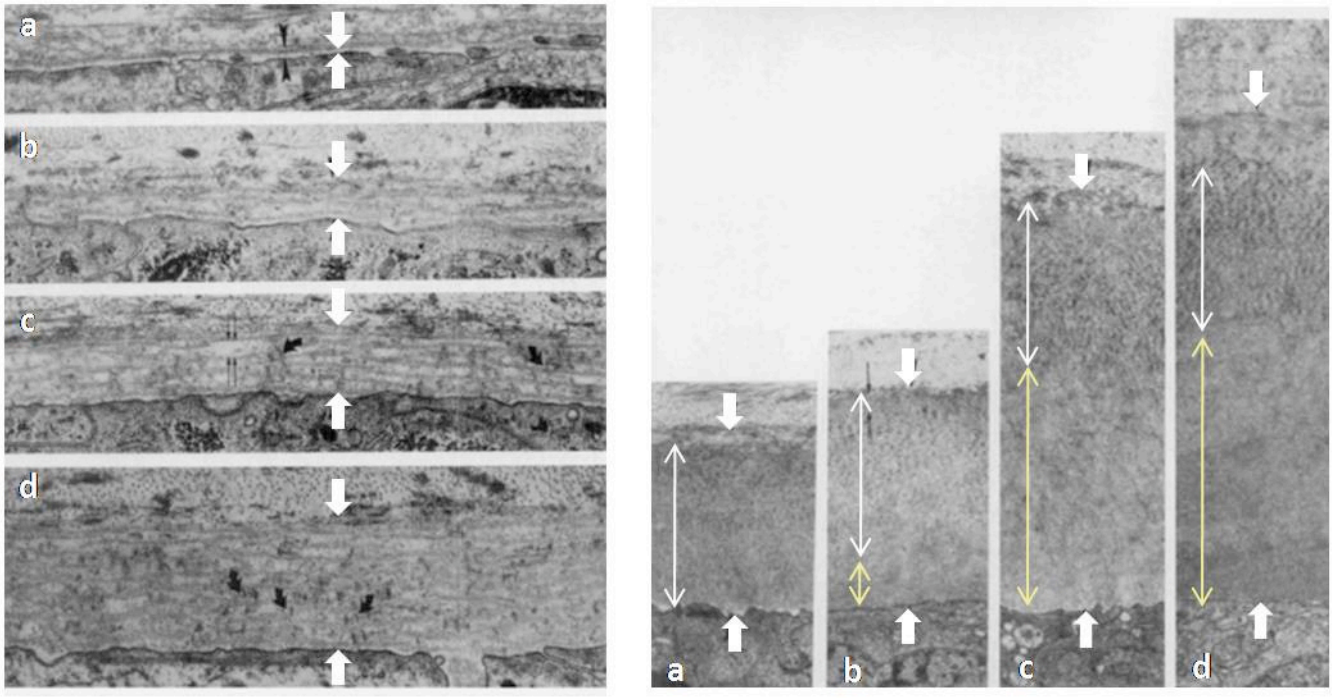

Figure 2. Croissance continue de la MD chez l'humain. L'observation a été faite par MET. Le versant endothélial est en bas et le versant stromal est en haut. L'épaisseur de la MD est indiquée par deux flèches opposées. A gauche, a, b, c, d montrent la MD à la 12ème,16ème, 19ème, et 26ème semaine de la gestation (grossissement x20750). A droite, a, b, c, d montrent la MD à 0, 11, 29 et 63 ans. Les doubles flèches en blanc indiquent la partie striée et celles en jaunes montrent la partie non striée (grossissement x 16500). ***(d'après Murphy, 1984)***

La cornée est le premier et plus puissant dioptre oculaire, elle permet, avec le cristallin, l'obtention d'une image ponctuelle rétinienne. Pour cela, la cornée doit avoir une transparence et un pouvoir dioptrique appropriés.

Histologiquement, elle est composée de 3 parties distinctes et parallèles entre elles : l'épithélium (environ 50 µm d'épaisseur), formé de 4 à 7 couches de cellules et de la membrane de Bowman, le stroma cornéen (500 µm) qui représente à lui seul environ 90% de la cornée, et l'endothélium et sa membrane basale formant un complexe appelé endothélio-Descemet (10 µm) **(Fig.1)**.

A l'état normal, la cornée est transparente grâce au parfait arrangement - unique dans le mode du vivant- de ses fibres de collagènes. Elle le reste grâce à la fonction de « pompe endothéliale » qui lutte en permanence contre l'hydrophilie spontanée du stroma : la cornée reste en état de perpétuelle déturgescence, garde une épaisseur constante et une organisation stable de son stroma. Le rôle de l'endothélium, qui ne représente que 2% de l'épaisseur d'une cornée adulte est donc tout à fait essentiel à la fonction visuelle.

I) Anatomie et histologie de l'endothelio-Descemet

I-1) Membrane de Descemet (MD)

Membrane basale transparente de l'endothélium cornéen qu'elle sépare du stroma cornéen, la MD est une membrane amorphe et élastique. Elle est constituée de fibrilles de collagène de petit diamètre réparties dans une matrice glycoprotéique. Les fibres collagènes du stroma postérieur sont entremêlées avec celles de la MD.

Feuillet antérieur et postérieur de la MD

La MD humaine qui est formée de molécules de la matrice extracellulaire (MEC) secrétée par les CEs, n'est pas une membrane inerte mais au contraire une structure qui fait l'objet de modifications constantes tout au long de l'existence **(Fig. 2)**[Murphy 1984a]. Elle apparaît à partir de la 12ème semaine de gestation et croît rapidement durant la période prénatale. A la naissance, la MD est épaisse d'en moyenne 3 µm et occupe la même surface qu'à l'âge adulte. Après la naissance, l'apposition de MEC sur la MD par les CE se ralentie et la croissance de la MD se fait principalement en épaisseur sans pratiquement étendre sa surface. Son épaisseur est en moyenne de 6 à 8µm au centre de l'endothélium chez sujets âgés de 60 à 90 ans. Dans la dystrophie de Fuchs, son épaisseur peut atteindre 20 µm au centre.

L'étude de son ultrastructure en microscopie électronique à transmission (MET) permet de distinguer deux feuillets **(Fig. 2)**[**Murphy 1984a**]. Le feuillet antérieur au contact du stroma, se caractérise par une structure striée produit par les CEs immatures avant la naissance, il est appelé « partie striée » ou « partie embryonnaire ». Le feuillet postérieur au contact de l'endothélium, est produit quant à lui par des CE matures et se forme après la naissance, il n'a pas de structure striée et est qualifié de « partie non striée » ou « partie adulte ».

La coloration HES sur coupe cornéenne différentie, par leur couleur, les feuillets antérieurs et postérieurs de la MD. Ceci témoigne de l'hétérogénéité des composants chimiques de ces deux structures. Le feuillet antérieur comprend les chaines alpha 1 et alpha 2 de collagène IV ainsi que de la fibronectine qui semble jouer un rôle dans l'adhésion avec le stroma. Le feuillet postérieur contient les chaines alpha 3 et alpha 5 de collagène IV, de l'entactine/nidogène, de la laminine-A et du perlécane [Ljubimov 1995].

Le feuillet postérieur de la MD peut se détacher facilement dans certaines circonstances, notamment sur les cornées de personnes âgées, qui possèdent en effet une MD non striée épaisse. Si cette propriété doit faire l'objet d'une attention particulière lors d'interventions chirurgicales intraoculaires telles que l'implantation d'un cristallin artificiel, elle a aussi pour avantage de permettre de mieux dissocier les CE sans les léser au cours de kératoplasties endothéliales, ou, pour nous chercheurs, de faciliter l'obtention des CE en vue d'une culture primaire au laboratoire.

Centre et périphérie de la MD

Chez le sujet âgé, nous avons constaté des différences remarquables de la MD entre le centre et la périphérie : constamment fine et lisse au centre, épaisse et irrégulière en périphérie **(voir article 4, partie expérimentale)**. De plus, l'épaisseur et l'irrégularité de la MD augmentent progressivement en périphérie, et les

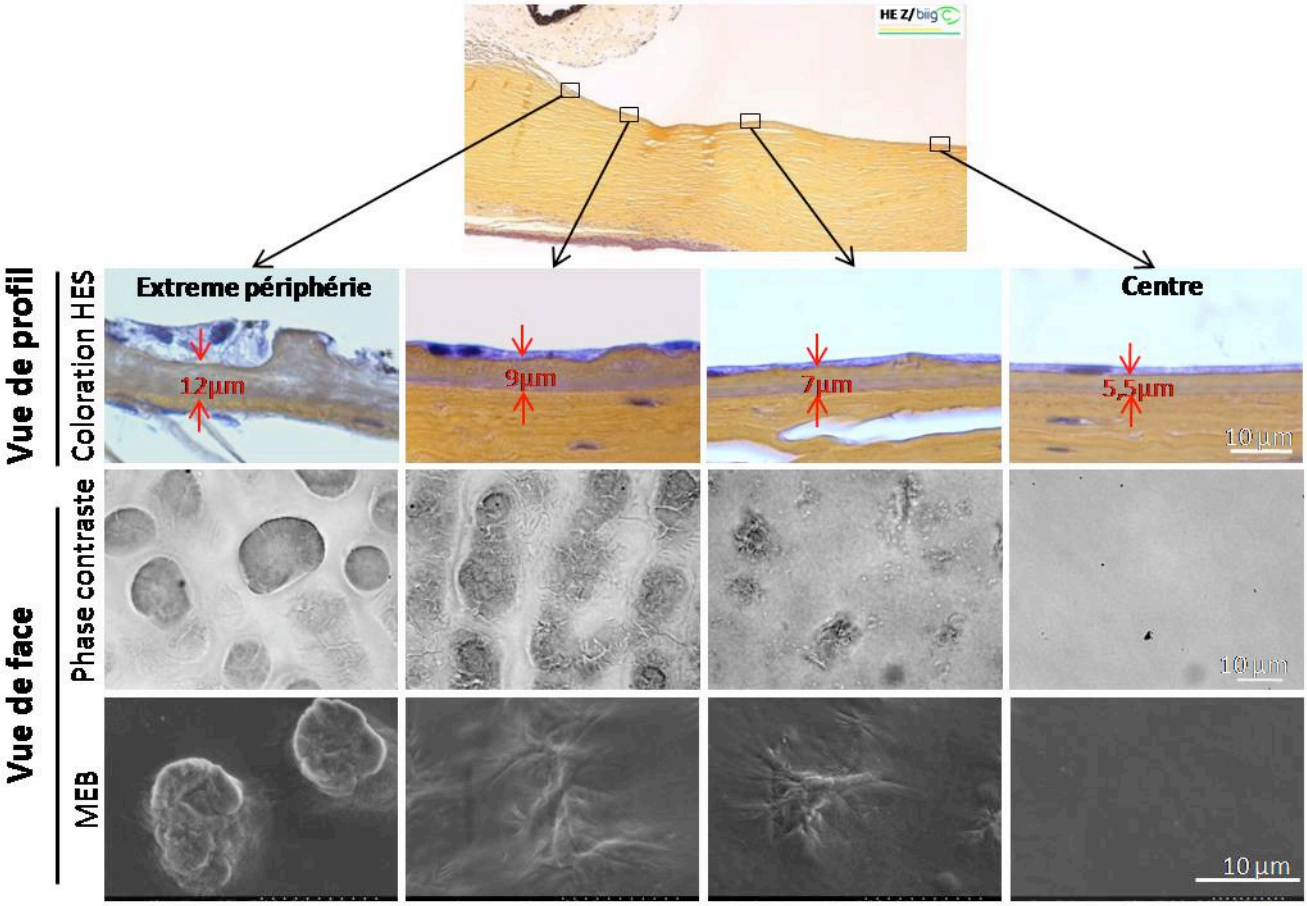

Figure 3. Variations de l'épaisseur et de l'irrégularité de surface du centre à l'extrême périphérie de la MD. ***(Propre observation de BiiGC et de CMES/He, Dumollard et Thuret)***

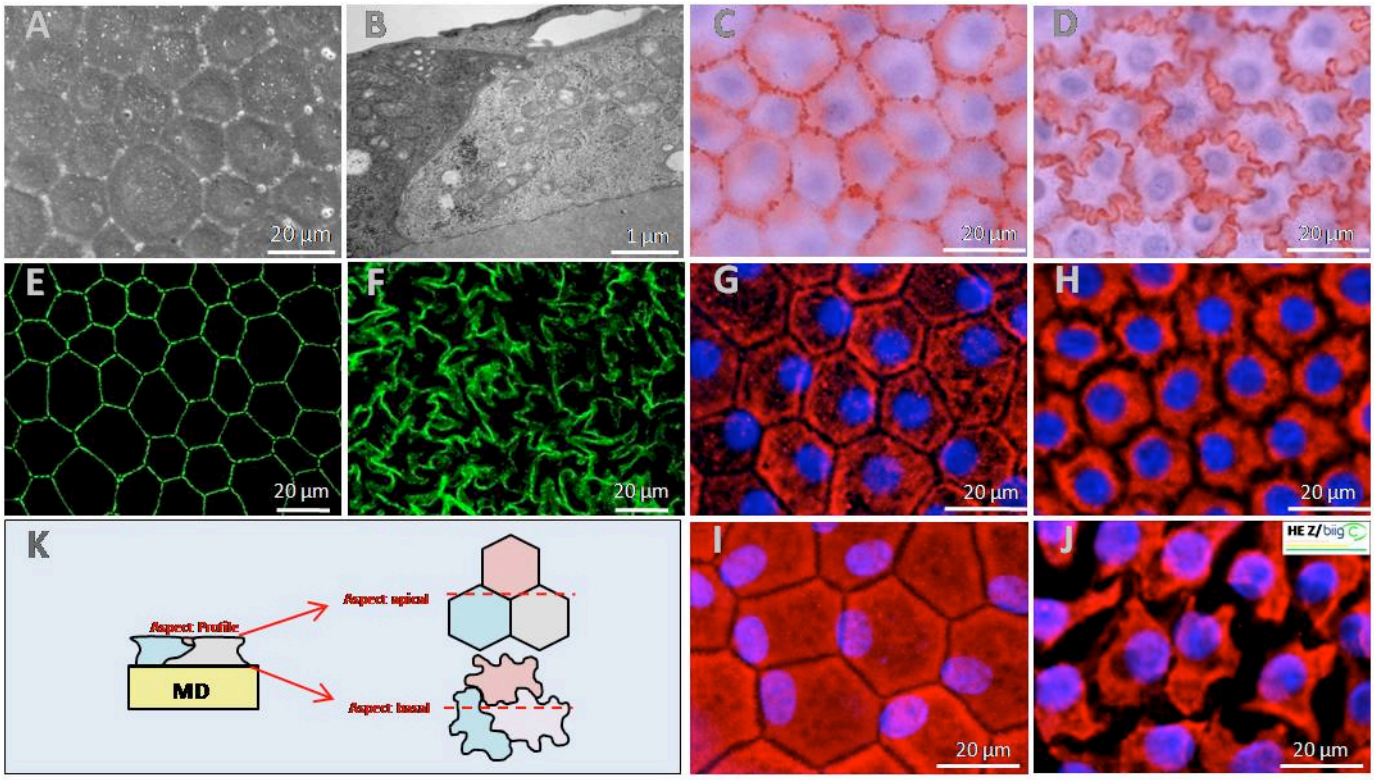

Figure 5. Pôles apical et basal des CE in situ et organisation spatiale intracellulaire. **A)** CEs observées de face par MEB; **B)** Organisation inter-celluiare observée de profil par MET; **C)** Pôle apical et **D)** Pôle basal en utilisant la double coloration rouge alizarine et Bleu Trypan ; Observation par immunolocalisation sur cornée montée à plat: **E)** Contours cellulaires du pôle apical visualisés par ZO-1; **F)** Membrane latérale visualisée par d'ATPase; **G** et **I)** Actine et myosine IIa participent à la formation hexagonale de la face apicale du cytoplasme; **H** et **J)** coxIV et histone H4 se localisent dans le cytoplasme sous apical et basal; **K)** Schéma explicatif de l'aspect des pôles apical et basal. ***(Observations de BiiGC et de CMES/He)***

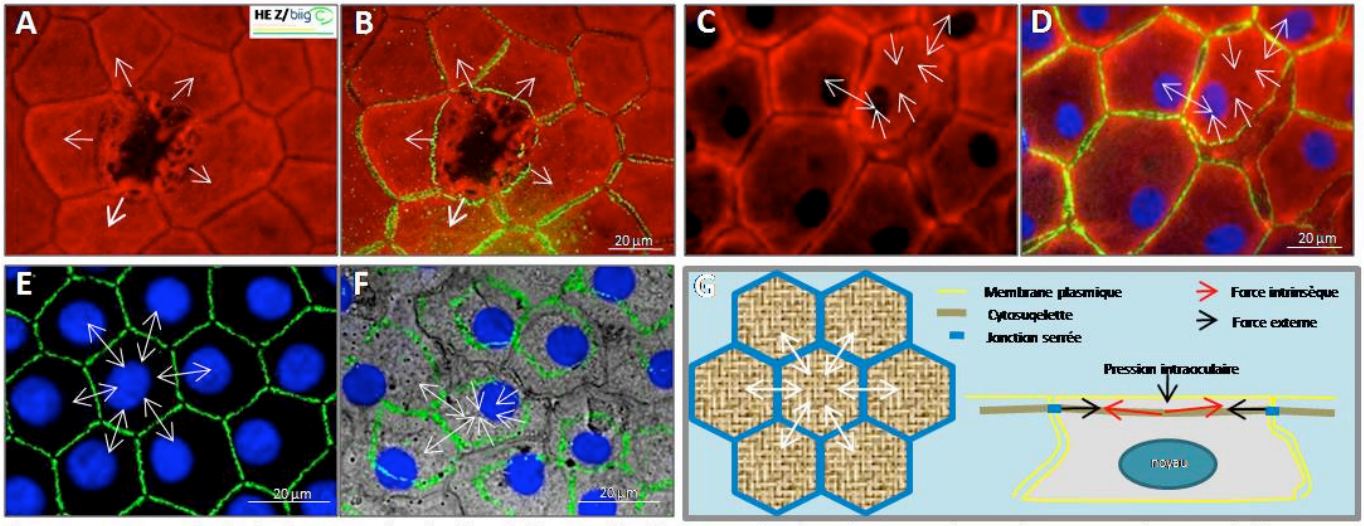

Figure 6. La morphologie hexagonale résulte de la coopération entre les jonctions serrées et le cytosquelette spécifique au pôle apical. A ,B, C, D) Co-localisation de ZO-1 (vert) et de la myosine IIa (rouge); A et B) La destruction de la myosine IIa, protéine cytosquelettique apicale, induit la disparation de la forme hexagonale; C et D) En cas d'hypertonie associée à une DCE basse, la rupture des jonctions serrées apparaît entre les CE voisines conjointement avec la contraction de la myosine IIA; E) La forme hexagonale régulière est liée à l'organisation de ZO-1; F) Contraction et perte de la forme hexagonale après la destruction des jonctions serrées par traitement à l'EDTA : Zo-1 (verte) combiné avec la phase contraste. Les flèches simples indiquent les directions de la contraction cytosquelettique en situation non physiologique; les doubles flèches montrent l'équilibre la contraction cytosquelettique entre deux cellules voisines permettant la formation de la forme hexagonale au pôle apical. G) schéma explicatif de la morphologie hexagonale au pôle apical des CE : fonction de lutte contre la pression intraoculaire résultant de l'équilibre entre la force intrinsèque du cytoquelette apical et des jonctions serrées et de la pression intraoculaire. ***(Propre observation de BiiGC/HE)***

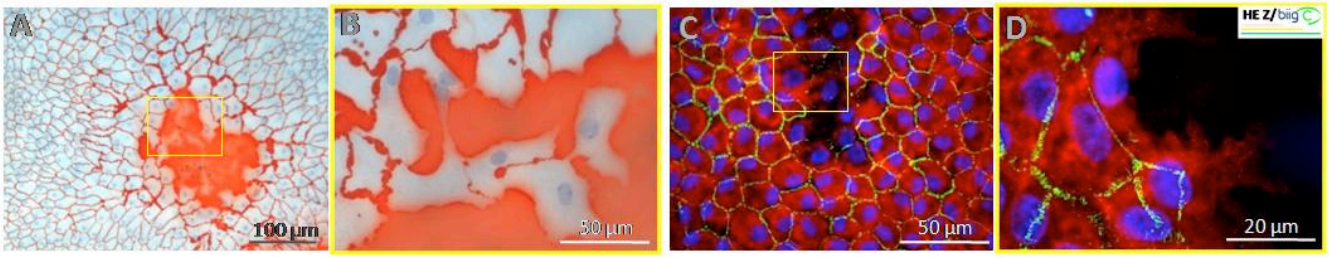

Figure 7. Réparation de l'endothélium cornéen humain ex vivo en cas de lésion et différences de mobilité entre les pôles cellulaires apical et basal. A et B) Migration et élargissement des CE au cours d'un processus de réparation d'une lésion au sein de l'endothélium; le contour cellulaire et la MD sont colorés par le rouge alizarine et les noyaux sont colorés par le bleu trypan. C et D) Différences de mobilité entre les pôles apical et basal pendant la réparation d'une micro-lésion par immunolocalisation sur cornée montée à plat : la partie basale du cytoplasme est marquée par histone H4 (rouge) et la partie apicale est marquée par Zo-1 (vert). ***(Observation de BiiGC/He)***

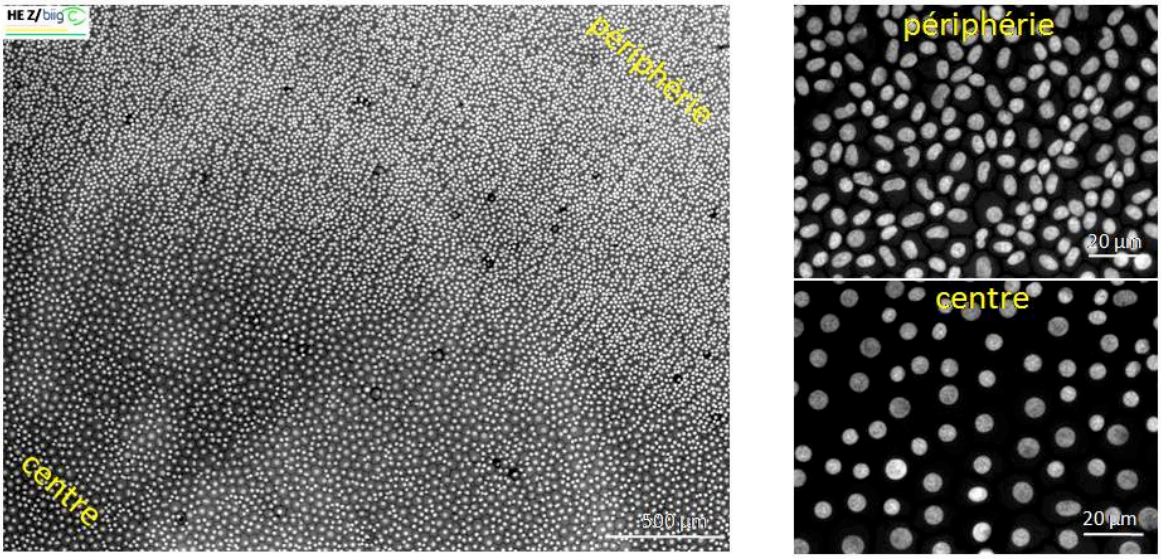

Figure 8. Différentes DCE et morphologie nucléaire entre la périphérie et le centre de l'endothélium humain d'une cornée fraîche. Les noyaux sont marqués par Hoechst. ***(Images de BiiGC/He)***

corpuscules de Hassal-Henlé se forment à l'extrémité de la MD **(Fig. 3)**. Les corpuscules de Hassal-Henlé de même que des structures en boucles (curly structures, plus rares) [Inaba 1986] sont des structures particulières situées en extrême périphérie de la MD, ne dépassant pas à l'état normal une couronne de plus de 100 µm de la périphérie. Elles sont absentes chez les sujets de moins de 30 ans et leur incidence et leur surface augmentent lentement avec l'âge pour être pratiquement constantes chez les sujets très âgés. Leur genèse et leurs fonctions physiologiques restent, à ce jour, mystérieuses.

I-2) Endothélium

L'endothélium cornéen humain forme une monocouche d'environ 300 000 à 500 000 CE plates, hexagonales, tapissant la face postérieure de la cornée **(Fig. 4)**. Le pôle apical des CE est en contact direct avec l'humeur aqueuse. Leur pôle basal adhère à la MD. Durant la dernière semaine de gestation, la densité cellulaire endothéliale (DCE) est d'environ 6000 CE/mm^2. Après la naissance, la DCE diminue avec l'âge au profit d'une augmentation du diamètre cellulaire et d'un aplatissement de CE sans possibilité de renouvellement direct par mitose. Chez l'adulte, les CE ont un diamètre moyen de 20 à 25 µm (surface moyenne de 240 à 350 µm$^{2)}$, une épaisseur de 3 à 5 µm. Dans une étude du BiiGC, portant sur 48 cornées prélevées chez 24 donneurs âgés de 51±18 ans, la DCE mesurées par analyse d'images [Gain 2002] était très comparable d'un œil à l'autre : 2727±272 CE/mm^2 à droite contre 2720±256 CE/mm^2 à gauche.

La distribution et la morphologie des CEs n'est pas uniformes sur toute la surface endothéliale, surtout entre la zone périphérique et centrale. La structure de la CE se révèle beaucoup plus complexe qu'une simple mosaïque de cellules en «nid d'abeille».

Endothélium du pôle apical au pôle basal

La conformation hexagonale, caractéristique des CE in situ, n'existe que pour leur pôle apical, celui directement en contact avec l'humeur aqueuse. A l'inverse, le pôle basal au contact de la MD, est irrégulier, fait de nombreuses expansions et interdigitations **(Fig. 5)**. La localisation cytoplasmique de différentes protéines révèle ces différents aspects morphologiques de la CE **(Fig. 5)**.

La conformation hexagonale est obtenue par l'interaction des jonctions serrées et au moins deux protéines du cytosquelette, l'actine et la myosine IIa. Elles forment une interface élastique liée directement à la structure de la jonction serrée. La force résultante de cette ultrastructure cytosquelettique contribue à la mise en tension des deux CE voisines, formant alors une ligne intercellulaire rigide. La perte de cette force de contraction induite par le cytosquelette apical dans une cellule se répercute sur la morphologie des cellules voisines **(Fig. 6A,-B)**. Expérimentalement, la rupture de cette jonction serrée par l'EDTA (acide éthylène diamine tétracétique), chélateur de Mg^{2+} et Ca^{2+}, induit la perte immédiate de la forme hexagonale **(Fig. 6E,-F)**. Cette structure peut être perturbée in vivo dans des cas pathologiques, telle que DCE basse et pression intraoculaire (PIO) élevée **(Fig. 6C,-D)**, et ceci semble indiquer que cette organisation spécifique pourrait être impliquée dans le système cellulaire de résistance à la PIO et aux flux d'humeur aqueuse **(Fig. 6G)**. La disposition hexagonale est une caractéristique très importante des CEs, non seulement morphologique mais aussi fonctionnelle : elle participe à l'inhibition de contact qui bloque la prolifération, probablement à la résistance contre la PIO, mais aussi aux mécanismes de réparation des lésions endothéliales par migration des cellules voisines **(Fig. 7A,-B)** car les CE forment une barrière à perméabilité contrôlée dont la forte cohésion ne permet pas la migration directe de cellules distantes, mais seulement une redistribution des cellules immédiatement voisines.

L'interface MD/CE ne comporte pas de dispositif ponctuel d'attachement habituel, en particulier pas d'hémi-desmosome (à l'inverse des cellules basales de l'épithélium cornéen). Cette particularité doit permettre une fluidité accrue du pôle basal des CE par rapport au pôle apical immobile **(Fig. 7C et -D)**. En cas de lésion endothéliale, la réparation endothéliale se fait par migration/élargissement des cellules voisines. La migration de la cellule nécessite le détachement des jonctions serrées apicales. Il en résulte un élargissement des CE redistribuées par rapport aux CE normales **(Fig. 7A, -B)**.

Endothélium central et périphérique

La DCE est chez l'humain plus élevée en périphérie qu'au centre [Schimmelpfennig 1984, Amann 2003]. Dans une série personnelle de 20 cornées fraîches, pour des donneurs d'âge moyen de 82 ans, observées juste après prélèvement et donc sans aucune conservation, la DCE périphérique était supérieure de 45% par rapport au centre, et cette différence diminue sur les cornée conservées en organoculture (OC) **(Tableau 1 et Fig. 8)**. Dans des cas particuliers, nous avons observé que la DCE peut atteindre en périphérie plus de 7000 CE/mm^2.

	Âge (ans)	PM (heures)	Durée de l'OC (jours)	DCE centrale Ø=8,25mm (cellules/mm²)	DCE périphérale (cellules/mm²)	Ration de la périphérie /centre
En OC (n=18)	78±11 (60 à100)	16±9 (7,5 à 46)	34±46 (3 à150)	1835±831 (685 à 3507)	2105±879 (784 à 4391)	1,20±0,27 (0,73 à 1,83)
Fraîche (n=20)	82±9 (59 à 92)	21±16 (4,5 à 63)	-	2389±437 (1775 à 3101)	3362±645 (1944 à 4348)	1,45±0,41* (1,07 à 2,45)

Tableau 1. Comparaison de la DCE entre la zone centrale et la périphérie sur des cornées fraîches et en OC prélevées chez des donneurs âgés. Le comptage a été fait sur des cornées montées à plat et est basé sur le nombre de noyaux par unité de surface. * différence significative ***(Observation de BiiGC/HE et Thuret)***

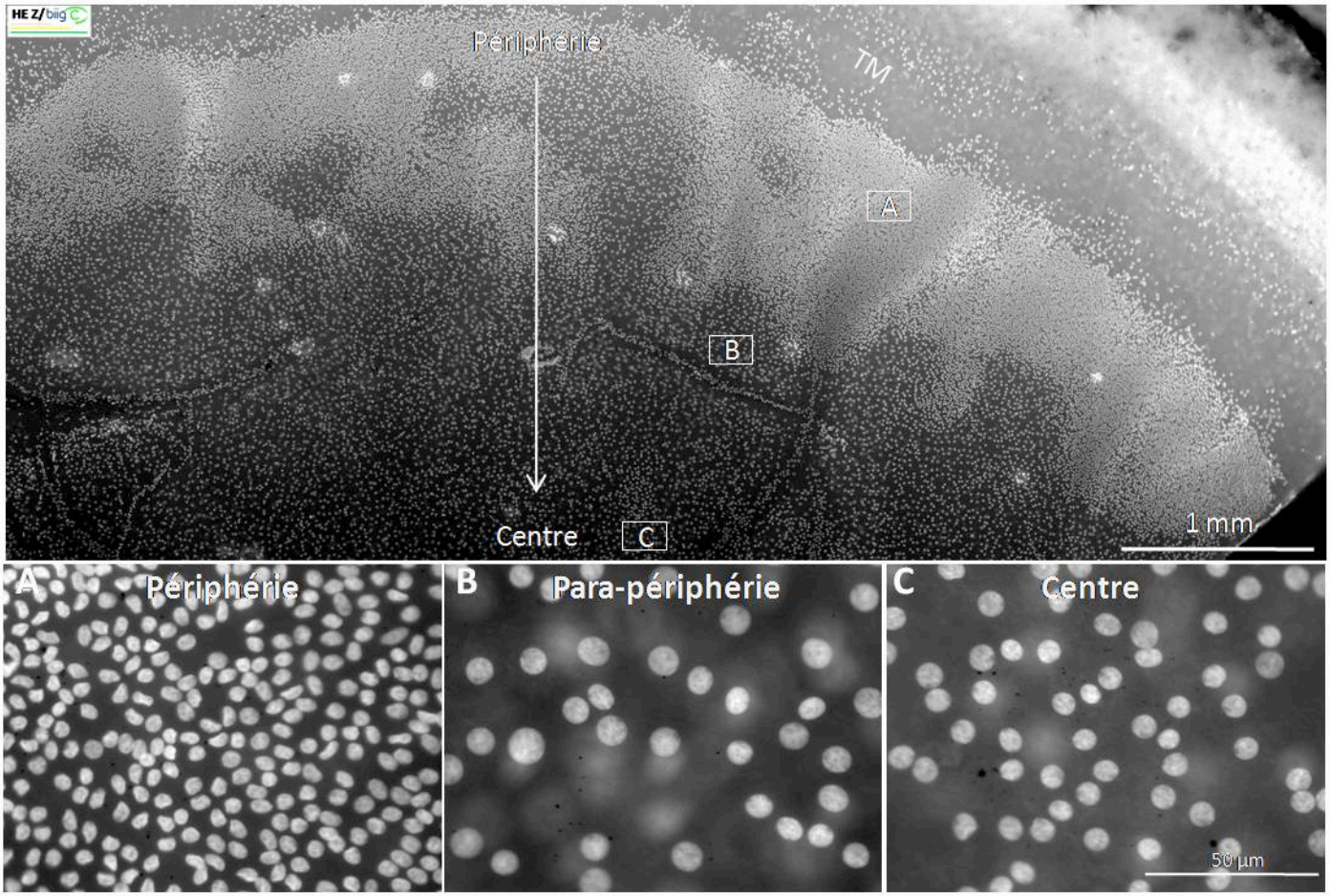

Figure 9. Cas d'une différence extrême de DCE entre les différentes zones de l'endothélium d'une cornée humaine provenant d'une personne âgé de 81 ans. Les noyaux sont marqués par Hoechst. La DCE (cellules /mm²) est de **A) 7150, B) 975, C) 1490**. les trois photos de forte grossissement (A, B et C) sont prises avec le même grossissement (échelle marquée sur la dernière photo). ***(Observation de BiiGC/He)***

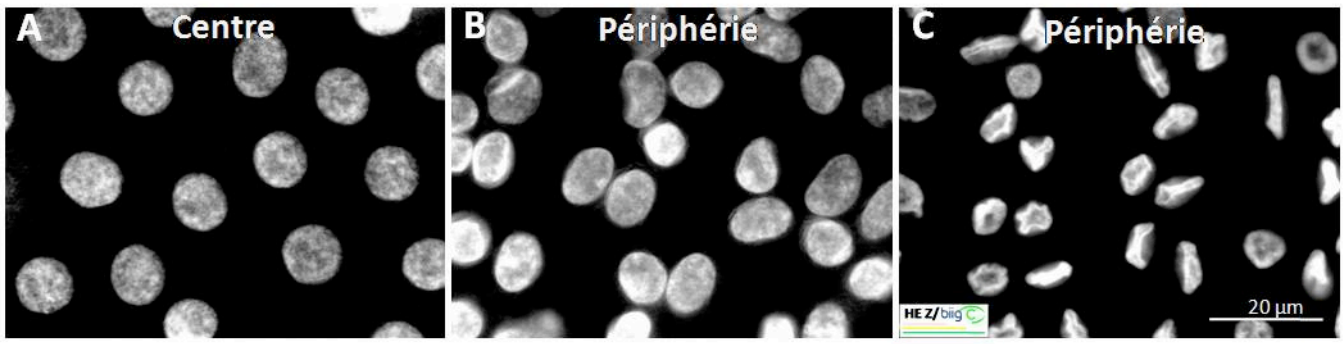

Figure 10. Différence de forme et de distribution des noyaux entre la zone centrale et périphérique. Les noyaux sont marqués par Hoechst; les trois photos sont prises avec le même grossissement (échelle marquée sur la dernière photo). ***(Images de BiiGC/He)***

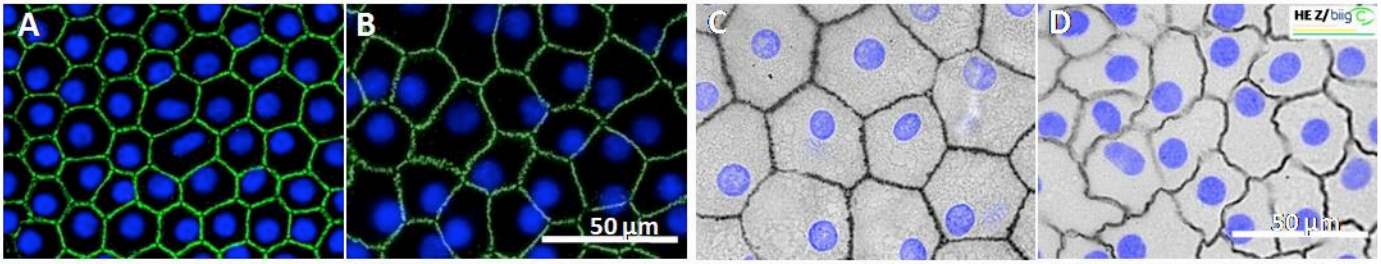

Figure 11. La conservation en OC fait perdre leur morphologie hexagonale typique aux CE. A et C): boutons cornéens à 15 minutes du prélèvement; B et D) cornées conservées en OC pendant 3 semaines; A et B) la morphologie apicale est montrée par immunomarquage utilisant ZO-1; C et D) le contour cellulaire est marqué par le rouge alizarine. Les noyaux sont tous colorés par Hoechst. Tous ces images sont prises avec le même grossissement (barres d'échelle indiquées sur les images de droite). **(Observation de BiiGC/HE)**

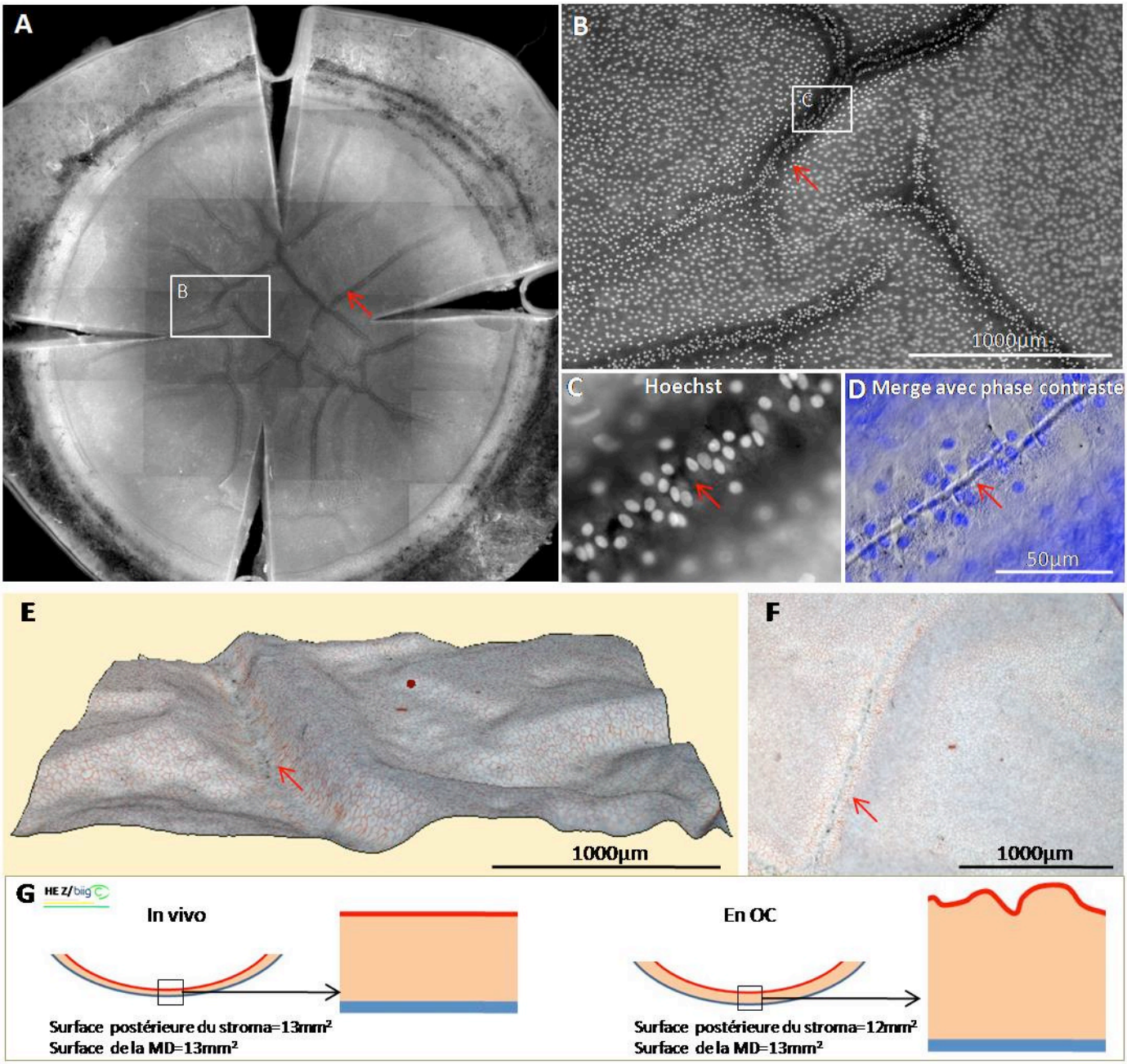

Figure 12. Plis endothéliaux après OC. Après OC, des plis se forment sur l'endothélium. A, B, C et D): Le noyau des CE sont colorés par Hoechst; E): image 3D reconstituée à partir de l'image F) où une série de photos en axe Z de l'endothélium coloré par le rouge alizarine et le bleu trypan ont été prises. La flèche rouge montre le fond d'un pli. G): Schéma explicatif de la formation de plis causés par un œdème stromal de la cornée en OC. ***(Images de BiiGC/HE et Thuret)***

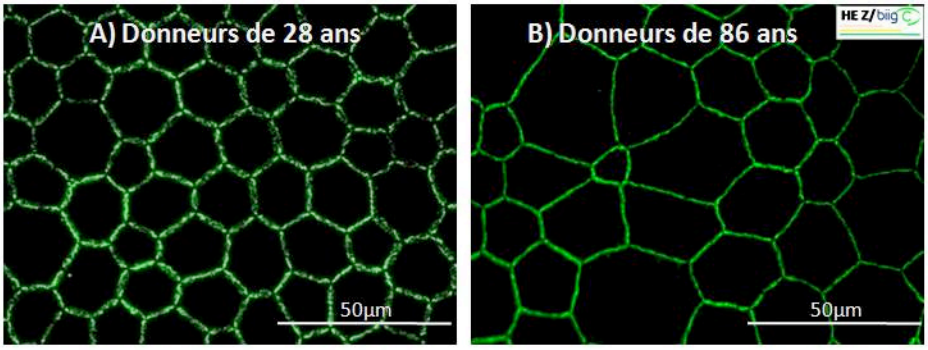

Figure 13. La perte de la forme hexagonale et de l'homogénéité s'accentue avec l'âge en s'accompagnant d'une diminution de la DCE. La forme hexagonale est représentée par immunolocalisation de Zo-1. ***(Images de BiiGC/HE)***

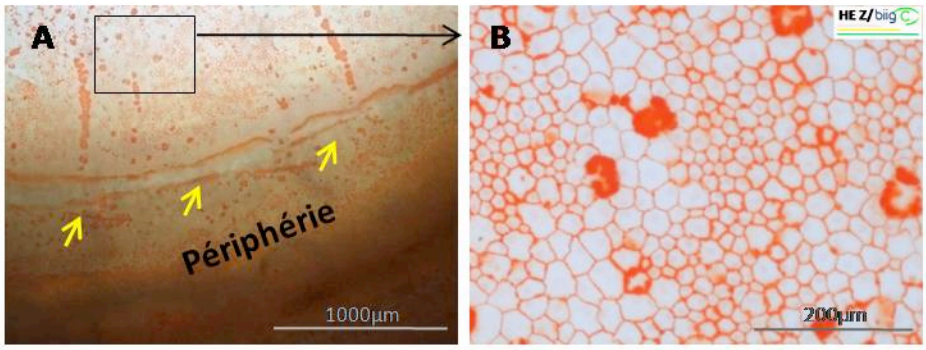

Figure 14. L'opération de la cataracte induit une baisse de la DCE ainsi qu'une perte de l'homogénéité. Le contour des CE est coloré par le rouge alizarine. La cicatrice endothéliale est indiquée par des flèches jaunes. L'image B est prise dans une zone intermédiaire entre la cicatrice et le centre de l'endothélium. ***(Images de BiiGC/HE)***

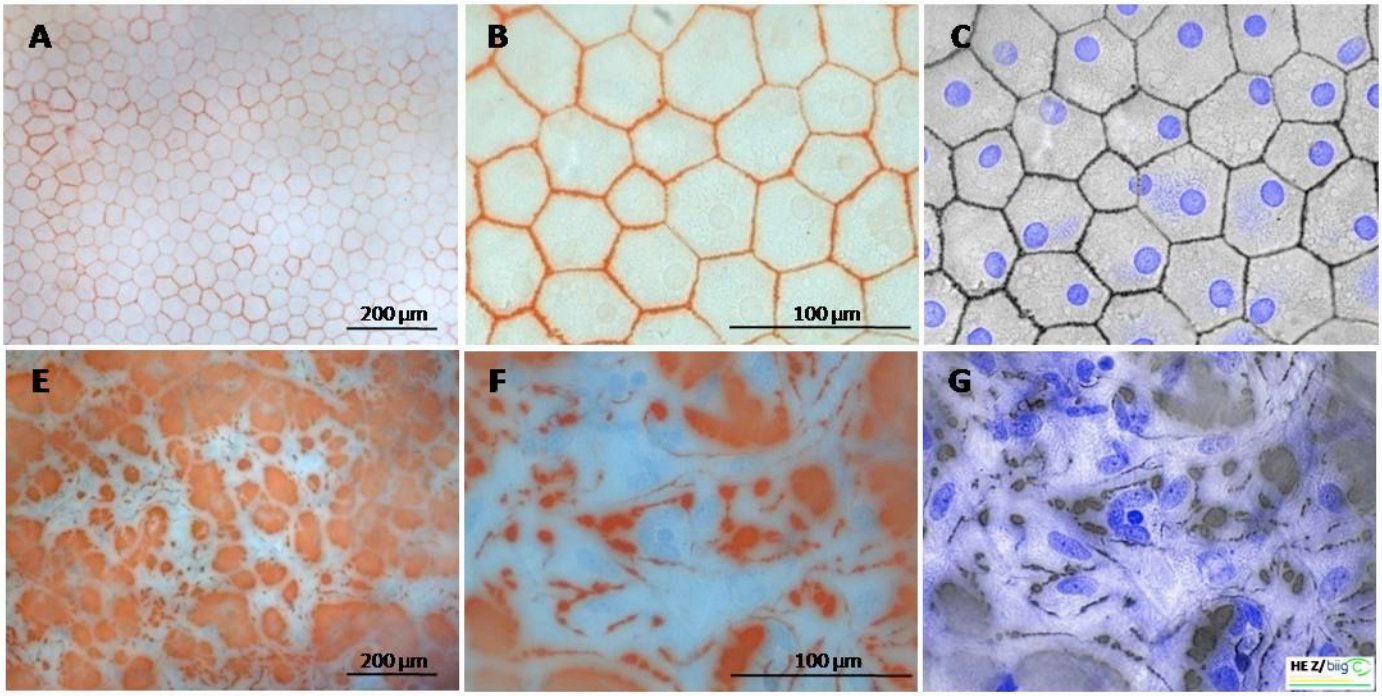

Figure 15. Etat de l'endothélium sur les boutons cornéens de la regreffe. A,B et C: Sur certains boutons de la regreffe, l'endothélium gardent parfaitement sa morphologique hexagonale, malgré une DCE d'environ 500 cellules /mm²; E, F et G: Sur d'autres boutons , l'endothélium a complètement perdu sa morphologie hexagonale. A, B, E et F: Seule coloration par le rouge alizarine (caméra couleur); C et G: Rouge alizarine combiné avec Hoechst (caméra noir et blanc). ***(Observation de BiiGC/HE)***

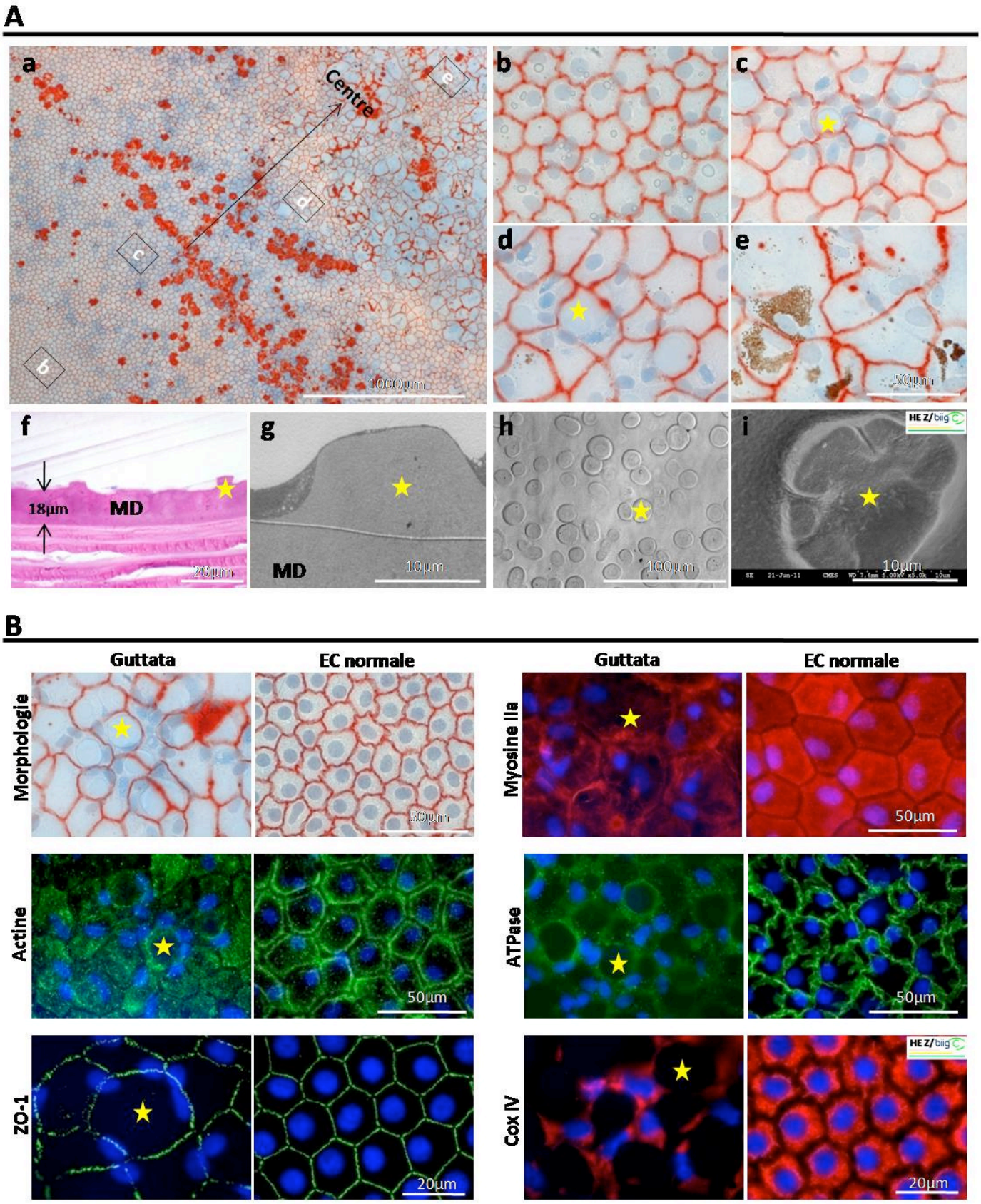

Figure 16. Guttata/dystrophie de Fuchs. En présence de cornée guttata, une des gouttes est indiquée par une étoile jaune.
A) Evolution de la gravité de la périphérie vers le centre et le changement de la MD. a, b, c, d et e): Un bouton cornéen atteint de la dystrophie de Fuchs est coloré par le rouge alizarine combiné au bleu trypan; a) montre une vue d'ensemble des différentes zones de la cornée; b, c, d, e) montrent l'aggravation de la pathologie de la périphérie au centre; f et g) montrent en vue de profil l'épaisseur de la MD et les gouttes de la guttata; h et i) montrent les gouttes au sein de la MD de face; g) MET et i) MEB. ***(Images de BiiGC et de CMES/He)***
B) Différence de morphologie et des différentes protéines caractéristiques des CE entre des CE normales et atteintes de Guttata. ***(Observations de BiiGC/He)***

Cette DCE extrême a été rapportée en périphérie d'une paire de cornées fraiches d'une donneuse de 83 ans, ainsi que sur une paire d'une donneuse de 81 ans avec une durée entre décès et prélèvement court (de 3h30) et après seulement 9 jours d'OC. Ces cas extrêmes ne sont pas inclus dans le Tableau 1. La DCE centrale de ces deux paires était respectivement de 2900 et de 1500 (soit de l'ordre de 5 fois moins) **(Fig. 9)**.

La morphologie nucléaire diffère aussi entre le centre et la périphérie de l'endothélium humain. Les noyaux au centre sont généralement de forme ronde et la distance entre deux noyaux voisins est relativement constante **(Fig.10A)**. Au contraire, en périphérie, leur forme varie : ronde, allongée, parfois un peu irrégulière et la distance internucléaire est souvent variable **(Fig. 10B,-C)**.

Endothélium in vivo et en organoculture

In vivo, la perte endothéliale physiologique chez l'adulte est de 0,56% par an [Murphy 1984b]. Lorsque la cornée est conservée en OC, cette vitesse s'élève à 1% par jour (près de 500 fois plus). Le **Tableau 1** compare la DCE d'une série de cornées conservée en OC pédant 34 jours en moyenne et d'une série de cornées fraîches avec un délai post-mortem moyen de 21 heures. Nous avons observé que la perte de cellules est beaucoup plus importante à la périphérie qu'au centre. De plus, La forme hexagonale apicale se détériore partiellement en OC **(Fig. 11)**. Un autre changement important sur l'endothélium en OC est la présence de plis endothéliaux **(Fig. 12A, -D)**. L'OC induit un œdème stromal responsable d'un doublement de l'épaisseur cornéenne, passant au centre de 550 à plus de 1000 µm. L'œdème est plus important dans le stroma postérieur. Il fait apparaître des plis dans le stroma postérieur et la MD inextensible **(Fig. 12G)**. Ces plis endothéliaux peuvent avoir une profondeur de plus de 200µm et accentuent la difficulté de mesure de la DCE sous microscopie optique et créant des erreurs de parallaxe qui sur-estime la DCE **(Fig. 12E, -F)**. De plus, la formation de plis endothéliaux induit une surmortalité des CE, probablement en raison de contraintes mécaniques anormales.

Endothélium et âge

L'organisation classique en «nid d'abeille» composé de CE parfaitement hexagonales s'avère en réalité rarement respectée in vivo. Certes, chez le sujet jeune dont la DCE est très élevée, la forme hexagonale et la surface des CE est plus homogène **(Fig. 13A)** : le polymorphisme et le polymégéthisme sont faibles. Avec l'âge et la diminution de la DCE, ces deux paramètres augmentent **(Fig. 13B)**.

Endothélium et circonstances particulières : explant cornéen, opéré de cataracte, dystrophie de Fuchs

L'endothélium cornéen possède normalement une réserve suffisante de CE pour maintenir sa fonction primordiale de maintien de la transparence de la cornée tout au long de la vie, car la perte de CE en condition physiologique est très lente. Cependant, dans des circonstances particulières, la perte de CE peut être accélérée et induire une diminution de la DCE et une altération de la morphologie des CE. En dessous d'un certain seuil de DCE (300 à 500 cell/mm^2 selon la dynamique du processus pathologique), une dysfonction endothéliale irréversible s'installe.

La chirurgie de la cataracte est l'intervention chirurgicale la plus pratiquée dans le monde. L'incision cornéenne induit localement la mort des CE autour de l'effraction Descemétique. Nous avons observé régulièrement des cornées provenant de donneurs pseudophaques. La DCE est toujours très basse autour de la cicatrice. Une DCE basse et une morphologie altérée ont été également observées dans la bande radiaire entre la cicatrice et le centre cornéen **(Fig. 14)**.

Les CE d'un greffon cornéen transfixiant meurent beaucoup plus rapidement que dans les conditions physiologiques, même en absence de toute pathologie spécifique (réaction de rejet, élévation de la PIO principalement). Ce phénomène mal compris actuellement conduit à l'épuisement endothélial tardif du greffon. La perte des CE d'un greffon parait être très régulière, comme en témoigne l'observation de greffons explantés pour des raisons non endothéliales (récidive de dystrophie stromale, astigmatisme géant, etc..) qui montre le plus souvent une mosaïque de grandes CE hexagonales très régulières **(Fig. 15A,-B et -C)**. Une invasion par des cellules fibrogliales probablement d'autres origines, peut aussi être retrouvée sur les greffons œdémateux explantés lors de regreffe (**Fig. 15D, -E et -F)**.

La dystrophie de Fuchs (et ses stades précoces de *cornea guttata*) est une pathologie primitive de l'endothélium cornéen rapportée pour la première fois en 1910 par Ernst Fuchs. Son étiologie reste encore mal élucidée. La lésion élémentaire est une excroissance ronde de collagène (verrucosité ou goutte) apparaissant sur une MD plus épaisse (**Fig. 16A)**. Le nombre de ces lésions augmentent avec le temps et s'accompagne à terme d'une dysfonction endothéliale et d'une apoptose de certaines CE. Habituellement, on désigne sous le terme de «guttata» les gouttes de la MD au centre de la cornée observées en microscopie spéculaire. La plupart des *cornea guttata* n'évolue pas vers le stade d'œdème cornéen irréversible (stade de la dystrophie

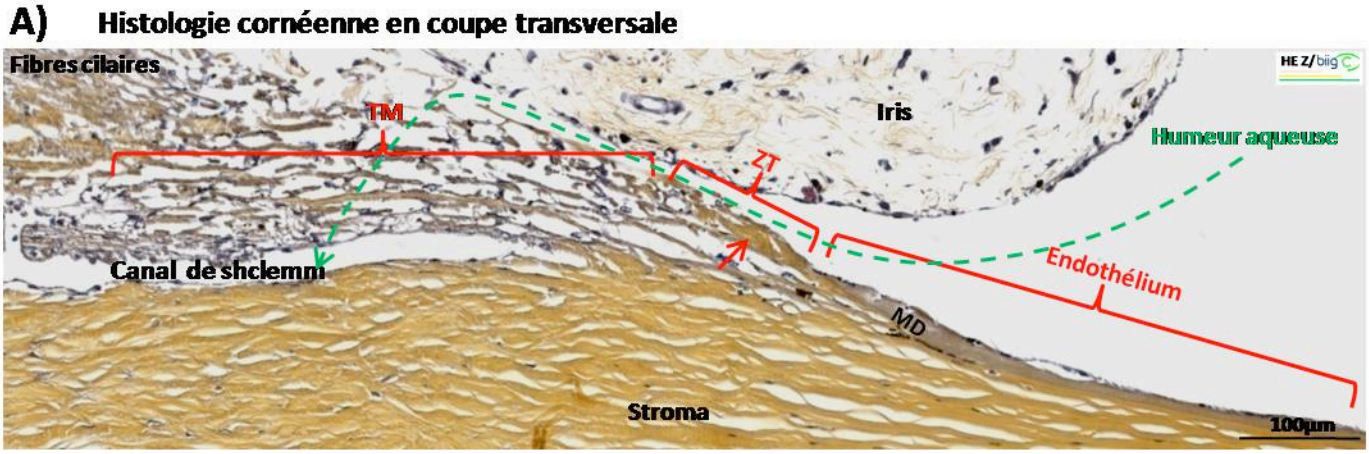

Figure 17. Trabéculum (TM) et zone de transition (ZT) . A) Coloration HES, la flèche rouge indique l'anneau de Schwalbe qui se trouve au dessous de la ZT; B) Observation du TM et de la ZT sous MEB (les cellules ont été détruites chimiquement); C) Coloration par le rouge alizarine combiné avec le bleu trypan; D) L'immunomarquage du TM sur cornée montée à plat montre la différence entre les CE et les cellules située dans le TM. ***(Images de BiiGC et de CMES/ HE, Dumollard et Thuret)***

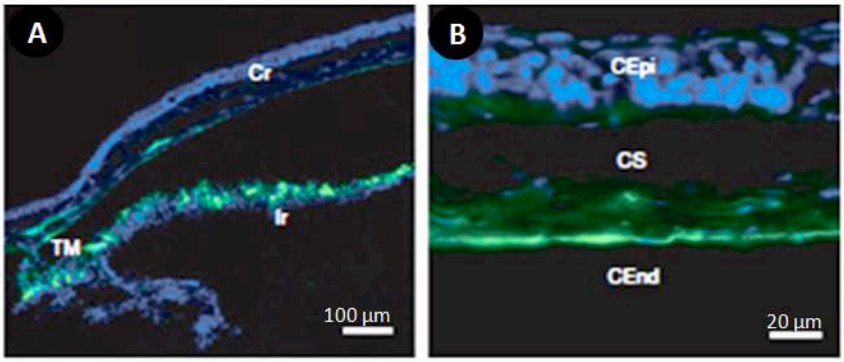

Figure 18. L'endothélium cornéen provient de cellules mésenchymateuses issues de la crête neurale crânienne. Chez des souris transgéniques, les cellules dérivées de la crête neurale expriment une exo-protéine fluorescente EGFP. Cette EGFP verte est trouvée dans l'endothélium cornéen, le trabéculum, le stroma cornéen, et le stroma irien, mais pas dans l'épithélium cornéen. Les noyaux sont colorés en bleu par Hoechst. ***(d'après Kikuchi et al., 2011)***

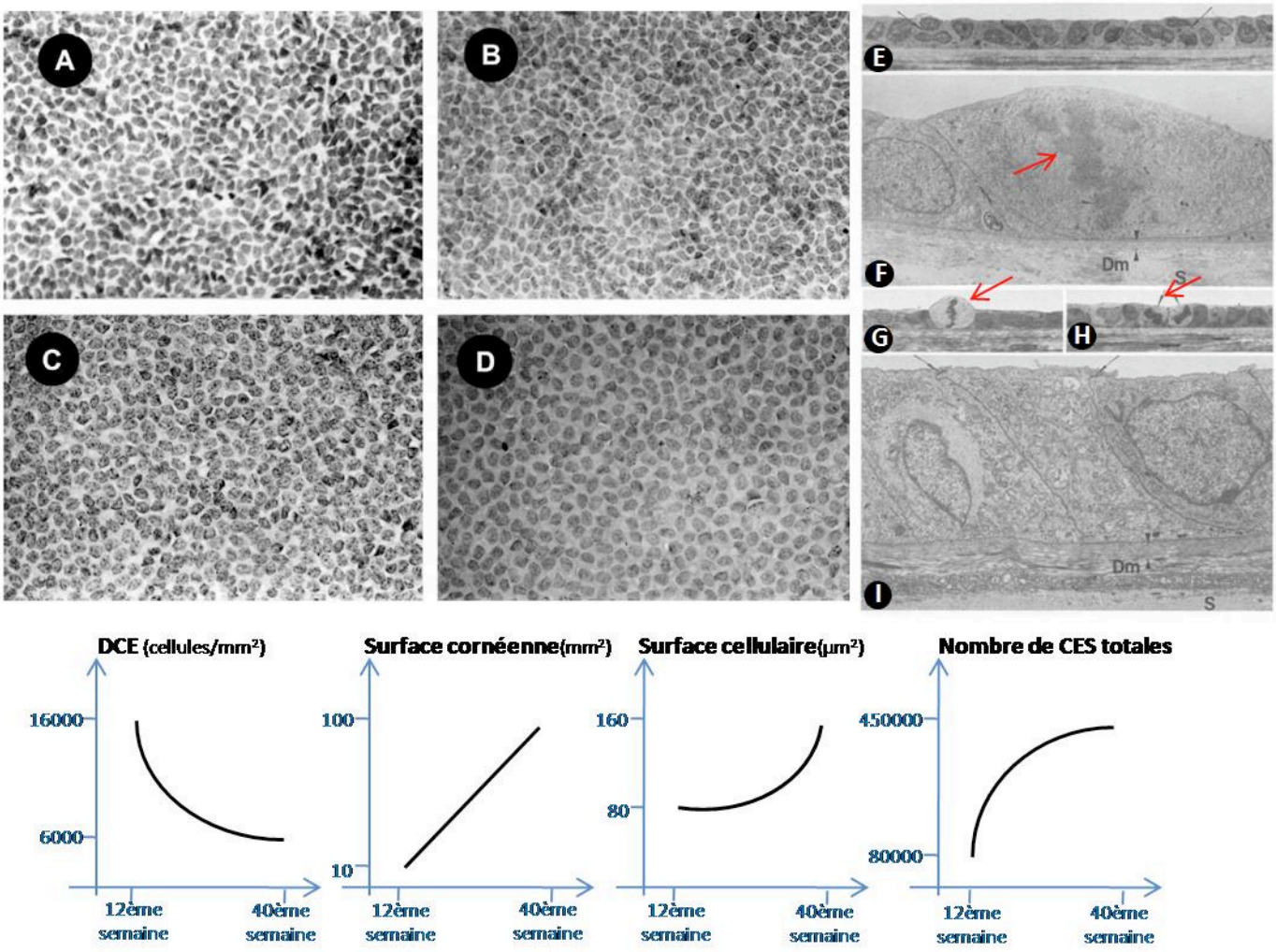

Figure 19. L'absence de corrélation entre l'augmentation du nombre de CE totales et la diminution de la DCE pendant l'embryogenèse de l'endothélium cornéen est due une croissance plus rapide de la surface postérieure de la cornée. A à D) Les noyaux sont colorés par Hematoxyline; observation en vue de face (cornée montée à plat) de l'endothélium sous microscopie optique. E à I) vue de profil (coupe transversale) de l'endothélium visualisé par MET. L'endothélium cornéen de fœtus humain à A) 15ème , B) 23ème, C) 27ème, D) 37ème, E) 16ème, F,G, H) 18ème, I) 22ème semaine de la gestation. Les flèches rouges indiquent les cellules mitotiques. Dm: Membrane de Descemet; S: stroma cornéen. ***(d'après Ko et al., 2001 ; Murphy et al., 1984)***

«endo-épithéliale» de Fuchs). Il s'agit d'une pathologie fréquente. Lorenzetti et al. retrouvent une prévalence de « gouttes » de 31,5% entre 10 et 39 ans et 70,4% au-delà de 40 ans [Lorenzetti 1967]. Au stade de l'œdème cornéen, celui-ci apparaît toujours au centre de la cornée. Sur les boutons cornéens de dystrophies de Fuchs trépanés lors de greffe de cornée, nous avons observé que la gravité des lésions endothélio-Descemétiques s'accentuait de la périphérie vers le centre **(Fig. 16A)**. Une détérioration non seulement de la morphologie, mais aussi de l'expression et de la localisation subcellulaire des protéines caractéristiques des CE ont été observées **(Fig. 16B)**.

I-3) Trabéculum

Le trabéculum (T) est un tissu de fibres collagènes situé dans l'angle irido-cornéen. Il entoure l'endothélium cornéen sur 360° et assure le drainage de l'humeur aqueuse. Le T est un maillage spongieux bordé par des trabéculocytes. Il permet à l'humeur aqueuse de se drainer dans le canal de Schlemm qui s écoule ensuite dans le système sanguin **(Fig. 17)**. Un obstacle a cette évacuation entraîne une augmentation de la pression intraoculaire jusqu'au glaucome.

Situé au dessus du stroma cornéen sans aucune barrière physique (membrane basale), le T se situe entre l'endothélium et la racine de l'iris. En raison de sa localisation anatomiquement particulière, et sa structure spongieuse, il est possible qu'il existe des échanges cellulaires entre le TM et les tissus voisins. De plus, les cellules trabéculaires, endothéliales, stromales cornéennes et stromales iridiennes dérivent de la même source embryonnaire : les cellules mésenchymateuses issues de la crête neurale crânienne [Kikuchi 2011]. McGowan et al. a d'ailleurs suggéré que le T pouvait abriter la niche des cellules souches de l'endothélium cornéen [McGowan 2007]. Les cellules trabéculaires ont cependant une morphologie très différente de celle des CE.

Il existe une zone de transition entre le T et l'endothélium cornéen **(Fig. 17)**. Cette zone étroite d'environ 100 µm entoure tout le bord extérieur de la MD et est liée directement à l'endothélium, mais comporte une monocouche de cellules qui n'ont pas les caractéristiques typiques des CE. L'anneau de *Schwalbe* est un ruban dense de collagène trouvé occasionnellement (15% des yeux) dans la zone de transition **(Fig. 17A)**. La zone de transition a également été suggérée comme zone de niche des cellules souches de l'endothélium [Whikehart 2005].

II) Embryologie et développement

L'endothélium cornéen provient de la crête neurale

L'endothélium cornéen provient des cellules mésenchymateuses issues de la crête neurale crânienne [Hayashi 1986, Gage 2005]. La différenciation cellulaire de la crête neurale se fait en trois étapes : le premier pool cellulaire forme le trabéculum et l'endothélium cornéen, le deuxième forme le stroma et le troisième forme l'iris [Jin 2002]. L'utilisation de souris doubles transgéniques porteuses du rapporteur spécifique de la crête neurale, P0-Cre/Floxed-EGFP, a permis d'identifier les cellules différenciées provenant de cette origine par l'expression d'une protéine EGFP exogène et fluorescente. On retrouve l'expression de cette protéine dans le trabéculum, l'endothélium cornéen, le stroma cornéen, et le stroma irien, mais pas dans l'épithélium cornéen **(Fig. 18)** [Kikuchi 2011].

La DCE baisse au cours de l'embryogenèse

Chez l'homme, l'embryogénèse de la cornée débute dès la 6ème semaine de développement embryonnaire et la migration des cellules mésenchymateuses de la crête neurale commence à la 7ème semaine [Hay 1980]. Au début de la genèse de l'endothélium, les CEs sont en bi ou multicouches, et forment petit à petit une monocouche à la fin de la gestation **(Fig. 19)**. Durant l'embryogénèse, l'endothélium fait l'objet de profonds remaniements : l'augmentation du nombre total de CE est rapide dans les premières semaines de l'embryogénèse de la cornée puis ralenti dans les dernières semaines de gestation. Du fait de la croissance rapide de la cornée [Denis 1998], la DCE décroît d'environ 16000 cellules/mm^2 à la 12ère semaine de gestation à approximativement 6000 cellules/mm^2 à la fin de la 40ème semaine en élargissant la surface cellulaire et formant une monocouche cellulaire **(Fig. 19)** [Ko 2000, Ko 2001].

Après la naissance, la DCE décroit rapidement pendant une période très courte, et puis la perte cellulaire se ralentie et reste constante (0,56% par an) pendant toute la vie [Murphy 1984b]. Cependant, ceci ne constitue pas un fait universel comme le témoigne l'observation de certains endothéliums cornéens de personnes âgées qui présentent une DCE à 7000 cellules/mm^2 en périphérie cornéenne **(Fig. 9)**. Ceci suggère l'existence d'un processus de régénération cellulaire chez l'adulte.

III) Capacité prolifératives de l'endothélium

Les CE humaines conservent une capacité de prolifération [Wilson 1995] dont la mise en œuvre in vitro dépend de l'âge du donneur [Mimura 2006]. Des cultures primaires de CE sont couramment obtenues in vitro [Zhu 2004, Zhu 2008]. Les oncogènes viraux, tel que l'antigène SV40-large-T et E6/E7 du papillomavirus humain, sont capables d'outrepasser les mécanismes normaux d'inhibition du cycle cellulaire et d'induire une prolifération non facteurs de croissance dépendants [Feldman 1993, Wilson 1995]. Chez les personnes âgées comme chez les jeunes, les CE n'ont pas de raccourcissement de leur télomères. L'arrêt de la prolifération ne peut donc pas être lié à un phénomène standard de sénescence réplicative où chaque mitose s'accompagne d'un raccourcissement des télomères, égrenant les tours limités de l'horloge biologique [Konomi 2007]. D'un autre côté, comme la longueur du télomère chez les CE n'est pas différente entre les jeunes et les personnes âgées, ceci indique qu'aucune duplication cellulaire importante n'a pas lieu pendant la période adulte. Malgré leurs capacités de prolifération in vitro, les CE humaines ne semblent pas se diviser in vivo et sont arrêtées en phase G1 du cycle [Joyce 1996a, Joyce 1996b]. Dans ses études, Joyce a étudié le statut proliférati f des CE par une approche immunohistochimique (IHC) sur des coupes transversales de cornée humaine, issues de donneurs d'âges différents de 6 semaines à 67 ans. Les cornées utilisées dans cette étude ont été conservées dans l'Optisol à 4°C et l'IHC a été réalisée dans les 36 heures après la mort des donneurs. La limitation de la capacité proliférative des CE in vivo ne semble pas, au moins pas complètement, due à leur incapacité intrinsèque à répondre à la stimulation des facteurs de croissance car les CE ont démontré leur capacité de prolifération en OC lorsque l'endothélium subit une lésion. Le TGF-β et l'inhibition de contact sont les deux principaux facteurs identifiés comme étant à l'origine de l'inhibition des mécanismes mitotiques au sein de l'endothélium cornéen [Joyce 1997, Joyce 2002]. Plus récemment, des phénomènes de sénescence prématurée induite par le stress ont été mis en évidence, participant à l'arrêt en phase G1 du cycle cellulaire [Konomi 2007]. D'autres mécanismes d'inhibition du cycle cellulaire des CE pourrait éventuellement être impliqués, tel que le contact des CE avec la MD, ou la séquestration au niveau de la jonction serrée par ZO-1 de ZONAB (ZO-1 associated nucleic acid binding protein) qui fonctionne comme un des promoteurs de la progression de la phase G1 quand il est localisé dans le noyau.

IMMUNOLOCALISATION

I) Principes des techniques d'immunolocalisation

I-1) Fixation des échantillons biologiques
I-2) Démasquage des sites antigéniques
I-3) Réduction du bruit de fond et du marquage non spécifique
I-4) Immunoréactions
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I-6) Observations microscopiques

II) Applications classiques

II-1) Immunohistochimie (IHC)
    A) IHC sur tissu paraffiné
    B) IHC sur tissu congelé
    C) IHC pour l'observation en MET
II-2) Immunocytochimie (ICC)

III) Notre technique hybride IHC/ICC : l'immunolocalisation sur cornée montée à plat

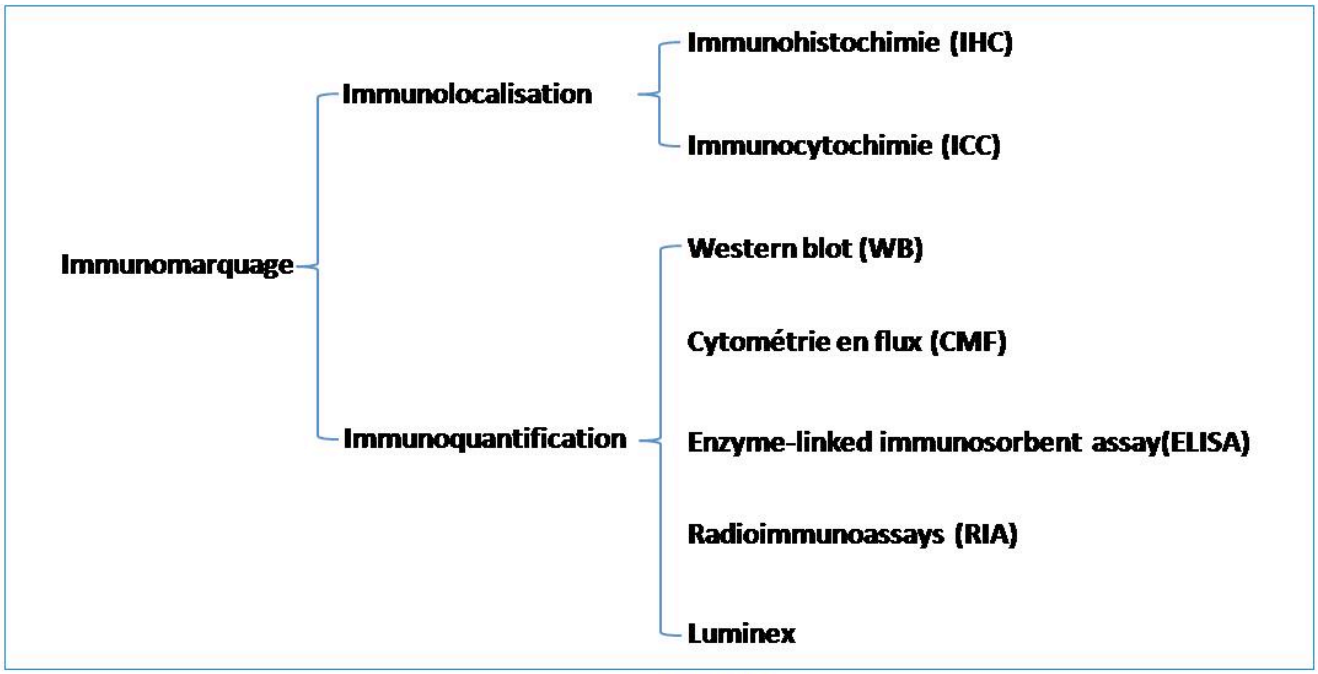


Figure 20. Classification des techniques d'immunomarquage (BiiGC/He)

	IHC (Tissus)		ICC (Cellules)
Etapes de la préparation des échantillons	-Fixation -Inclusion en paraffine ou résine -Réhydratation	-Fixation ou non -Congélation -Fixation dans le cas du tissu non fixé	-Fixation -Perméabilisation éventuelle

Tableau 2. Etapes principales de la préparation des tissus ou des cellules pour immunoréaction (BiiGC/He)

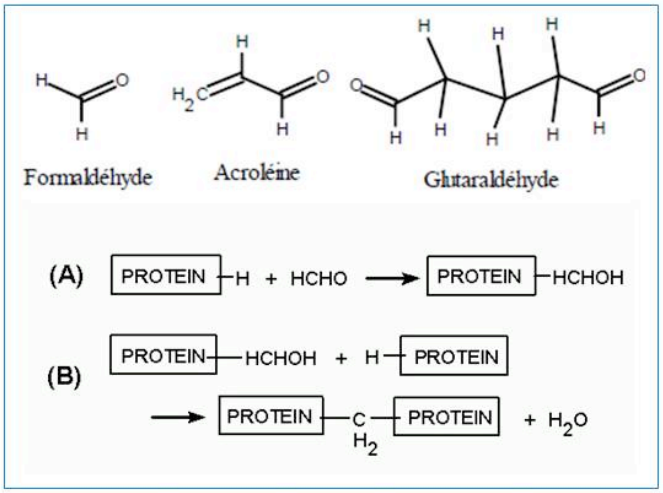


Figure 21. Différents aldéhydes et leur mécanisme de la création du pontage inter et intra-protéine (D'après « Immunohistochimie » de Garaud et Roussel)

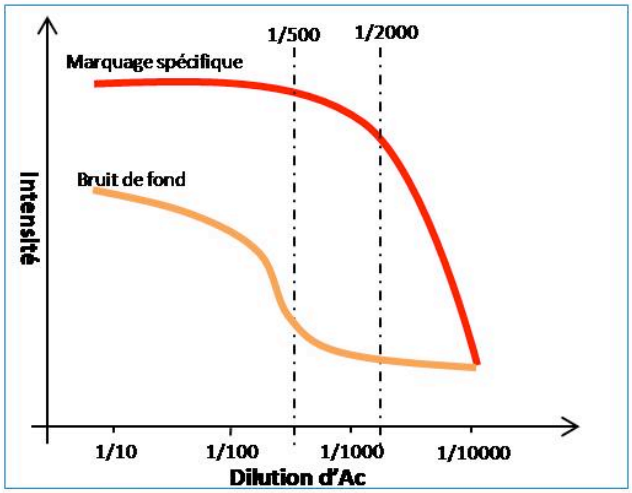


Figure 22. Réduction du bruit de fond par l'optimisation de la dilution de l'Ac (Adapté de « Immunohistochimie » de Garaud et Roussel)

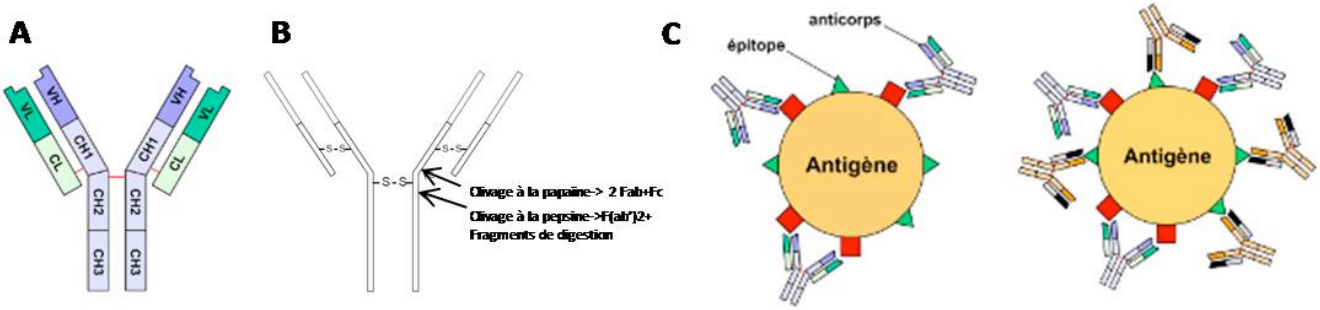


Figure 23. Anticorps (Ac). A) Structure générale de l'AC. Les ponts disulfures sont indiqués par les lignes rouges; V: variable, C: constant, H: chaîne lourde (heavy), L: chaîne légère (light). Exemple: VL: partie Variable de la chaîne légère. B) Clivage enzymatique aux sites spécifiques de l'Ac. C) Ac monoclonaux (à gauche) : liaison à un épitope spécifique et Ac polyclonaux (à droite) : liaison à des épitopes différents. **(D'après « Immunohistochimie » de Garaud JC et Roussel G)**

I) Principes des techniques d'immunolocalisation

L'immunomarquage est un terme générique désignant toutes les techniques utilisant la reconnaissance spécifique entre un anticorps (Ac) et un antigène (Ag) cible dans un échantillon biologique. Une fois fixé sur l'Ag cible, l'Ac, souvent couplé à un traceur, permet de déterminer sa localisation subcellulaire précise et/ou sa distribution sur un tissu (immunolocalisation) ou de quantifier (immunoquantification) sa présence au sein du tissu **(Fig. 20)**.

Ces techniques peuvent être effectuées soit, sur une coupe de tissu, il s'agit alors de l'immunohistochimie (IHC), soit sur des cellules étalées en monocouche sur une lame, ou issues de cultures in vitro, il s'agit alors de l'immunocytochimie (ICC). Par rapport à la simplicité relative de l'ICC, l'IHC permet d'étudier, en plus, des modèles in situ ou in vivo avec des techniques plus complexes.

Les techniques d'immunolocalisation ont en commun plusieurs étapes que nous décrivons ci-après.

I-1) Fixation des échantillons biologiques

L'étape de fixation des cellules ou des tissus permet de figer leurs structures, d'immobiliser in situ les Ag, en arrêtant le métabolisme cellulaire, notamment les processus de dégradation des protéines et des structures cellulaires, pour qu'elles ne soient pas endommagées et que les Ag ne soient pas détruits et élués par les différents bains réactionnels. Il s'agit d'une étape importante car elle conditionne la reconnaissance par l'Ac des sites antigéniques de l'Ag. Une fixation adaptée permet donc l'accès de l'Ac à l'Ag et l'exposition de l'épitope à la surface de l'Ag. A l'inverse une mauvaise fixation peut aboutir à des faux négatifs. Les différents fixateurs peuvent donner des résultats d'immunolocalisation très différents. De plus, d'autres conditions entrent en jeu telles que la durée, le pH ou la température. Il existe deux grandes catégories de fixateurs : agents de pontage chimique et agents dénaturants de protéines

Agents de pontage chimique : Aldéhydes

Les aldéhydes tels que le formaldéhyde ou le glutaraldéhyde, ont comme formule générale R-COH et réagissent essentiellement avec les groupements aminés (spécialement lysine) des protéines en créant des ponts intra- et inter-protéines **(Fig. 21)**. Cela permet d'immobiliser les protéines, de figer la structure tissulaire et cellulaire et d'empêcher l'autolyse de la cellule pendant et après la mort cellulaire.

Agents dénaturants

La dénaturation d'une protéine correspond à la désorganisation de sa structure spatiale sans aucune modification des liaisons covalentes inter- ou intra-moléculaires. Les agents dénaturants de protéines sont très nombreux, mais peu d'entre eux sont de bons fixateurs du tissu. Pour être un fixateur de tissu, il faut qu'ils possèdent deux caractéristiques :

- Inhibition de l'autolyse : le déploiement des structures protéiques entraîne des modifications au niveau des sites actifs des enzymes et leur fait perdre leurs activités. Ainsi, l'action des agents dénaturants permet de neutraliser les enzymes impliquées dans l'autolyse de la cellule et d'assurer l'intégrité de l'Ag cible au sein de la structure cellulaire et tissulaire.
- Agrégation des protéines : la modification de la structure spatiale des protéines cibles par les agents dénaturants affecte profondément leur interaction avec l'environnement aqueux et diminue leur solubilité. En particulier, des contacts entre les zones hydrophobes des protéines dépliées peuvent conduire à leur agrégation et à leur précipitation sur le tissu. Cela permet d'éviter la solubilisation des protéines cibles durant les étapes ultérieures du marquage.

Il existe deux types d'agents dénaturants dans le cas de la fixation de tissu : les agents déshydratants et les agents précipitants

- Les agents déshydratants, qui peuvent être classés en 3 groupes : 1) Les solvants miscibles à l'eau, comme le méthanol, l'éthanol, et l'acétone peuvent déshydrater très rapidement une cellule et un tissu ; 2) La chaleur utilisée pour sécher les coupes rapidement sur une plaque chauffante ou dans un four à micro-onde ; 3) La déshydratation sous vide : les coupes ou les tissus sont alors congelées et placées dans une enceinte sous vide pour obtenir une évaporation lente de la glace.
- Les agents précipitants comme l'acide picrique, l'acide acétique et les métaux comme les sels de zinc, de mercure et d'osmium. Rarement utilisés seul comme fixateur, ils sont plutôt combinés à d'autres fixateurs, comme des agents de pontage chimique, dans le but de mieux conserver la structure cellulaire et de préserver la structure spatiale des protéines afin de ne pas compromettre la reconnaissance des épitopes conformationnels par l'Ac pendant la fixation.

Adaptation à l'IHC et à l'ICC

Selon les différentes techniques d'immunolocalisation, la préparation des tissus ou des cellules avant l'immunoréaction peut comprendre une ou plusieurs étapes. **(Tableau 2)**. La fixation est une étape indispensable.

Dans la plupart des cas, le processus d'immunoréaction se fait après la préparation du tissu, mais dans certains cas particuliers, cet ordre peut être inversé :

-Cas 1 : Immunoréaction sur tissu fixé avant inclusion en IHC : en cas d'utilisation de traceurs stables pendant les processus d'inclusion ou de congélation, l'immunoréaction peut se faire sur une coupe de tissu épaisse et fixée avant l'inclusion ou la congélation du tissu. Par exemple, pour certains immunomarquages en microscopie électronique à transmission (MET), l'étape d'immunoréaction peut être effectuée éventuellement sur une coupe épaisse de tissu fixé avant l'inclusion dans la résine et la réalisation des coupes ultra-fines.

-Cas 2 : Immunoréaction sur tissu vivant: dans de rares cas, l'immunoréaction peut être réalisée sur coupe de tissu frais avant la fixation pour localiser des Ag à la surface des cellules du tissu. Cette technique, mise au point par Warrington et Pfeiffer [Warrington 1992], a permis de visualiser, sur des coupes de cerveau non fixés, des glycolipides spécifiques localisés à la surface des oligodendrocytes.

-Cas 3 : Immunoréaction sur des cellules vivantes en ICC : les protéines intégrées à la membrane cytoplasmique de cellules vivantes en monocouches in vitro ou en suspension, peuvent être directement détectées par immunoréaction sans fixer les cellules préalablement. Une fixation après immunoréaction est possible. Cette technique est souvent employée en cytométrie en flux, parfois en ICC.

I-2) Démasquage des sites antigéniques

L'étape de démasquage a pour but de restaurer l'accessibilité aux sites antigéniques de la protéine cible. Elle est très souvent utilisée en IHC, mais beaucoup moins en ICC. Les sites de fixation des Ag présents dans les tissus peuvent être peu ou non accessibles, entourés ou masqués par d'autres molécules, ou posséder une configuration non reconnue par les Ac. De plus, l'étape de fixation provoque une modification des Ag pouvant changer leur affinité pour les Ac ainsi qu'une réticulation du tissu freinant la diffusion des réactifs à l'intérieur du tissu. Le processus de démasquage est souvent nécessaire après le processus de fixation lorsque celui-ci utilise des aldéhydes, rarement après utilisation d'agents dénaturants. Cette étape est surtout importante en IHC sur des tissus inclus en paraffine ou en résine.

Trois techniques de démasquage se distinguent :

Traitement enzymatique

La digestion enzymatique peut dégager l'Ag et permettre l'exposition des épitopes. Cette technique est relativement simple et facile à contrôler, mais dépend du type et du lot d'enzymes utilisées ainsi que du temps de réaction optimal. La trypsine, la pronase et la pepsine sont les enzymes les plus employées.

Traitement dénaturant

Un pré-traitement des coupes par des agents dénaturants tels que le Dodécyl sulfate de sodium (SDS) [Robinson 2001], l'urée ou le thiocyanate de guanidine peut améliorer la qualité du marquage.

Traitement par la chaleur

Le démasquage antigénique par la chaleur ou HIER (Heat induced epitope retrieval) en anglais est la méthode la plus utilisée et la plus efficace pour l'IHC utilisant des tissus fixés avec le formaldéhyde et inclus dans la paraffine. Néanmoins, ce traitement reste inefficace lorsque le fixateur utilisé est le glutaraldéhyde ou un agent dénaturant tel que l'éthanol ou l'acétone. Les coupes, obtenues au cryostat et montées sur lame de verre, sont souvent décollées ou partiellement détruites par le traitement à la chaleur. Pour cette technique, la méthode de chauffage est sans importance (autoclave, cocotte-minute, bain-marie, micro-ondes, chauffage à la vapeur) et plus la température est haute, meilleur est le résultat. Par contre, dans les cas où les tissus sont susceptibles d'être détruits à haute température, il est possible de diminuer la température et de prolonger le temps d'incubation. Ainsi, des résultats analogues sont obtenus par des traitements à 100°C pendant 20 min, 90°C pendant 30 min, 80°C pendant 50 min ou encore 70°C pendant quelques heures. Le pH de la solution de démasquage a plus d'importance que les sels présents dans la solution. Quelques Ag bénéficient d'un traitement à pH bas (tampon glycine-HCl), mais un pH élevé donne des résultats plus constants et meilleurs que l'utilisation d'un pH 6 du tampon citrate qui est pourtant le plus fréquemment employé. La molarité quant-à elle, a peu d'effet. Différents additifs, comme les ions métalliques (Zn, plomb), l'urée ou les détergents peuvent éventuellement améliorer la réaction. Cependant, la présence d'ions calcium semble compromettre certaines immunoréactions : le chauffage avec une solution contenant un chélateur de calcium (EDTA, EGTA, tampon

citrate) peut dans ce cas être bénéfique. L'intensification du marquage après un traitement à la chaleur est généralement expliquée par le clivage des chaines polypeptidiques à pH bas ou élevé et une rupture des liaisons établies par les fixateurs permettant ainsi une "renaturation" des Ag et un accès facilité pour les Ac.

Ces techniques de démasquage peuvent donner de très bons résultats dans certains cas, mais ne sont pas universelles. Une diminution voire une inhibition de certains marquages peut parfois être observée.

I-3) Réduction du bruit de fond et du marquage non spécifique

1-3-1 Diminution du bruit de fond

Dans le cas de l'immunofluorescence, l'autofluorescence peut être intrinsèque (provoquée par les composants naturels des cellules ou des tissus, tels que nicotinamide adénine dinucléotide phosphate (NADPH), certaines matrices extracellulaires comme le collagène et l'élastine [Monici 2005, Menter 2006]) ou provoquée par la fixation. Elle peut alors provenir des doubles liaisons imines -C=N- ou encore de nouvelles molécules créées par l'étape de fixation utilisant des aldéhydes, notamment le glutaraldéhyde. L'intensité de l'autofluorescence est diminuée par l'utilisation d'une solution contenant 0,1% de borohydrure de sodium dans le PBS qui, en libérant de l'hydrogène, réduit les doubles liaisons imines et les molécules autofluorescentes. Cette étape peut se faire avant la saturation des sites non spécifiques.

En cas d'utilisation de traceurs enzymatiques, l'activité enzymatique des peroxydases endogènes et des molécules à activité péroxydasique (hémoglobine, cytochromes, catalases…) persiste après les étapes de fixation et d'inclusion du tissu dans la paraffine. Contrairement, à la peroxydase exogène qui est utilisée comme traceur, l'activité de la peroxydase intrinsèque peut introduire un bruit de fond. Le blocage de l'activité des peroxydases endogènes par un traitement à l'eau oxygénée réalisé avant la saturation des sites non spécifiques permet d'éliminer ce bruit de fond.

1-3.2 Marquage non spécifique

Causes de marquage non spécifique

Le marquage non spécifique est introduit principalement par des liaisons non spécifiques ou non désirées de l'Ac à l'échantillon. Ces réactions non spécifiques ont diverses causes :

- Fragment Fc et liaisons non spécifiques : la région Fc porte des récepteurs pour le complément. De nombreuses cellules (macrophages, mastocytes ...) expriment à leur surface des récepteurs du fragment Fc. Des marquages parasites peuvent être évités en utilisant des $F(ab')_2$ dont le fragment Fc est absent.
- Sites antigéniques de Fragment Fab et immunoréactions croisées : le motif antigénique (épitope) est constitué de 3 à 16 aminoacides [Gershoni 2007]. Il peut être séquentiel (formé d'une chaîne continue d'aminoacides) ou conformationnel (formé par le rapprochement spatial d'aminoacides). Il est possible d'avoir des réactions croisées entre un Ac et des protéines non cibles mais possédant des épitopes similaires à ceux de l'Ag recherché.
- Ac polyclonaux et reconnaissance indésirable : l'animal immunisé peut produire des Ac polyclonaux non seulement contre l'Ag recherché, mais également contre tout autre motif antigénique présent dans le mélange immunogénique. D'autre part, un mélange d'Ac polyclonaux contient également une quantité importante d'Ac non spécifiques préexistant dans le sérum. L'utilisation d'Ac monoclonaux permet d'éviter ces reconnaissances indésirables.

Diminution du marquage non spécifique

Le marquage non spécifique existe dans tous les essais d'immunolocalisation, et peut être très gênant dans les cas où la réaction immunologique est faible pour différentes raisons. Les méthodes utilisées couramment pour réduire le marquage non spécifique sont les suivantes :

- Dilution optimale des Ac : avant de déterminer la spécificité d'une immunoréaction, il est nécessaire d'éliminer les marquages non spécifiques par une dilution optimale des Ac. La dilution optimale de la plupart des Ac se situe en général entre 1:100 et 1:10 000 **(Fig. 22)**.
- Saturation des sites non spécifiques : cette méthode est utilisée juste avant la réaction immunologique pour réduire la formation des liaisons non spécifiques de l'Ac. Des solutions contenant de l'albumine de sérum bovin (BSA), du lait en poudre, ou des sérums animaux, sont incubées avec les échantillons biologiques avant et pendant l'immunoréaction.

1-3.3 Les contrôles de spécificité [Burry 2011]

Le bruit de fond et le marquage non spécifique existent universellement pour toutes les techniques d'immunolocalisation. Ils pourraient devenir négligeables si l'Ac et le protocole optimal existaient, ce qui est malheureusement rarement le cas en pratique de laboratoire.

Les contrôles de spécificité (contrôles négatifs) sont donc indispensables dans les essais d'immunolocalisation. Les méthodes utilisées pour ces contrôles sont les suivantes :

- Remplacer l'Ac primaire par des immunoglobulines non spécifiques.

Le remplacement de l'Ac primaire par des immunoglobulines de même espèce mais sans spécificité connue est très souvent utilisé comme contrôle négatif dans une réaction d'immunoréaction indirecte utilisant des Ac primaires polyclonaux. Elle peut mettre en évidence un bruit de fond, des liaisons non spécifiques avec l'Ac ou une reconnaissance non désirée par des Ac non spécifiques mais pas toujours inertes dans le sérum.

- Omettre l'Ac primaire.

L'utilisation de l'Ac secondaire seul est très souvent utilisée comme contrôle négatif dans les réactions d'immunoréaction indirecte. Cette méthode peut mettre en évidence un bruit de fond ou des liaisons non spécifiques avec les Ac secondaires.

- Omettre l'Ac secondaire.

En omettant l'Ac secondaire associé au traceur, on repère ainsi le bruit de fond intrinsèque du tissu

- Inhiber l'activité de l'antisérum à l'aide de l'Ag.

Ce contrôle consiste à pré-incuber l'Ac primaire avec des Ag préparés dans du tissu à tester. Les sites de liaison occupés par l'Ag ne pourront donc plus se fixer sur les molécules reconnues dans les tissus. Il s'agit donc d'un test d'inhibition ou de compétition qui met en évidence le marquage spécifique par comparaison des résultats du contrôle et du processus normal de réaction.

- Contrôle positif.

Pour démontrer que l'absence du marquage dans le tissu à tester est due à l'absence de l'expression d'une protéine cible, et non pas à une technique non adaptée, un autre tissu au sein duquel cette protéine cible s'exprime de façon certaine est utilisé comme contrôle positif.

I-4) Immunoréactions

Les Ac sont les réactifs essentiels en immunohistochimie. Le choix de l'Ac est primordial dans les techniques d'immunolocalisation. Il est extrêmement utile de connaître les caractéristiques des Ac (capacité (titre), avidité/affinité, spécificité, sensibilité aux agents de fixation) que l'on utilise : la réussite ou l'échec d'une immunolocalisation dépend en partie de ces paramètres.

4.1 Anticorps

4.1.1 Structure générale

Les Ac sont des glycoprotéines de la superfamille des immunoglobulines. Ils sont formés de 2 chaînes lourdes et 2 chaînes légères reliées entre elles par un nombre variable de ponts disulfures **(Fig. 23A)**. Chaque chaîne légère est constituée d'un domaine constant et d'un domaine variable; les chaînes lourdes sont composées d'un fragment variable et de 3 ou 4 fragments constants selon l'isotype. Pour un Ac donné, les deux chaînes lourdes et les deux chaines légères sont identiques.

Les domaines constants ne sont pas impliqués dans la reconnaissance de l'Ag, mais interviennent dans l'activation du système du complément. L'association entre un domaine variable porté par une chaîne lourde (V_H) et le domaine variable adjacent porté par une chaîne légère (V_L) constitue le site de reconnaissance (ou paratope) de l'Ag.

4.1.2 Fragmentation

Le clivage enzymatique des sites spécifiques de l'Ac permet d'isoler différents fragments **(Fig. 23B)** qui peuvent être utiles dans l'application des techniques d'immunolocalisation.

-La digestion à la papaïne produit 2 fragments Fab et un fragment Fc [Porter 1959].

-La digestion à la pepsine fournit un fragment $F(ab')_2$ divalent et qui a la même affinité Ac-Ag et des produits de digestion du fragment Fc [Dolby 1964, Nisonoff 1964].

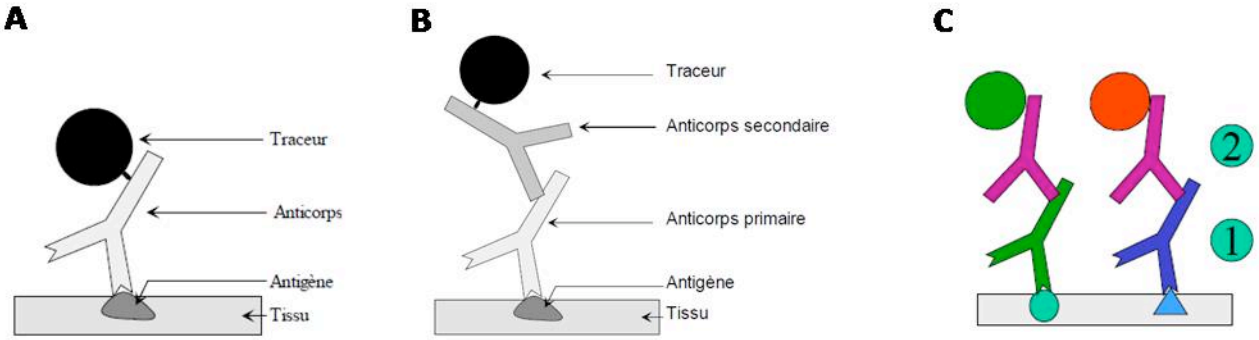


Figure 24. Trois principaux types d'immunoréaction utilisé dans l'immunolocalisation. A) Immunoréaction directe: traceur-anticorps-antigène-tissu; B) Immunoréaction indirecte: traceur-anticorps secondaire-anticorps primaire-antigène-Tissu; C) Marquages multiples **(D'après « Immunohistochimie » de Garaud et Roussel)**

	IHC			ICC
Echantillons biologiques	Tissus paraffinées	Tissus congelés	Observation à MET	Culture cellulaire in vitro ou cellules en suspension
Fixation	Oui	A: Oui B: Non	Oui	Oui
Durcissement de tissu	Oui, inclusion en paraffine	Oui, congélation	Oui, inclusion en résine ou congélation	Non
Découpe de tissu	Oui (3 à 20µm)	Oui (5 à 20µm)	Oui (50 à 100nm)	Non
Réhydratation du tissu	Oui	Non	Oui, dans le cas de l'inclusion en résine	Oui, pour l'utilisation des fixateurs déshydratants ou de le précipitation
Perméabilisation membranaire	Non	Oui, pour l'utilisation des fixateurs de l'aldéhyde	Non	Oui, pour l'utilisation des fixateurs de l'aldéhyde
Fixation	Non	A: non B: Oui	Non	Non
Démasquage	Souvent	Peu	Peu	Peu
Réduction de 1) l'autofluorescence* 2) l'activité enzymatique Intrinsèque** 3) l'accrochage non spécifique de l'Ac	1) Optionnel* 2) Optionnel** 3) Oui	1) Optionnel* 2) Optionnel** 3) Oui	1) Non 2) Optionnel** 3) Oui	1) Non 2) Non 3) Oui
Immunoréaction	Oui	Oui	Oui	Oui
Révélation du marquage	Non* Oui**	Non* Oui**	Non*** Oui**	Non
Contre coloration	Oui	Oui	Rare	Oui
observation	Microscope optique** ou à fluorescence*	Microscope optique** ou à fluorescence*	MET	Microscope à Fluorescence

Tableau 3. Etapes principales des protocole d'IHC et d'ICC. * Dans le cas d'utilisation des traceurs fluorescents; ** Dans le cas de d'utilisation de certains traceurs enzymatiques, tel que la peroxydase; *** dan le cas d'utilisation de traceurs métaux lourds. **(BiiGC/He)**

4.1.3 Ac monoclonaux et polyclonaux

Un Ag possède généralement plusieurs épitopes différents qui représentent autant de sites de liaison aux Ac. On peut classer une population d'Ac selon sa capacité à reconnaître un seul ou plusieurs épitopes, On parle alors respectivement d'Ac monoclonaux et polyclonaux **(Fig. 23C)**.

- Les Ac monoclonaux sont des Ac ne reconnaissant qu'un seul type d'épitope sur un Ag donné. Ils sont par définition tous identiques et produits par un seul clone de plasmocyte.
- Les Ac polyclonaux sont un mélange d'Ac reconnaissant différents épitopes sur un Ag donné, chaque idiotype étant sécrété par un clone de plasmocyte différent.

Au cours de la réponse immunitaire, un organisme synthétise des Ac dirigés contre plusieurs épitopes d'un Ag : la réponse est dite polyclonale. La purification de l'Ac à partir du sérum est alors nécessaire influençant la qualité de l'Ac. Les Ac polyclonaux contiennent souvent des Ac non spécifiques qui peuvent donner un bruit de fond plus important par rapport des Ac monoclonaux.

4.2 Ag et épitope.

Un Ag est une substance biologique, reconnue par des Ac. Les Ag sont généralement des protéines, des polysaccharides, des lipides associés à des protéines, ou des acides nucléiques. La notion d'épitope est primordiale dans la compréhension du mécanisme de reconnaissance de l'Ag par l'Ac. Dans le cas d'Ag protéique, on nomme épitope ou déterminant antigénique, la partie de l'Ag reconnue par l'Ac. Un même Ag peut comporter plusieurs épitopes (identiques ou différents). Il existe des épitopes séquentiels, correspondant à une séquence d'acides aminés, et des épitopes conformationnels, liés à la structure tertiaire de la protéine et donc sensibles à la dénaturation.

La valence antigénique est le nombre d'Ac capables de se lier simultanément à un Ag. La valence est donc proportionnelle à la surface de cet Ag mais ne reflète pas le nombre d'épitopes en raison de l'encombrement spatial que peuvent occasionner les anticorps.

4.3 Interaction spécifique Ac-Ag

La réaction entre un Ac et un Ag donne un complexe (Ac-Ag). C'est une réaction d'équilibre entre les vitesses d'association et de dissociation de l'Ac à l'Ag.

Les termes affinité et avidité sont souvent employés l'un pour l'autre. Ces concepts sont en effet très proches. L'affinité reflète la force de liaison entre un site de liaison de l'Ac (Fab) et un site d'un Ag monovalent. L'avidité reflète quant à elle la force de liaison d'un Ac avec un Ag monovalent ou multivalent. Les Ac sont souvent divalents : la présence de deux liaisons qui ne se rompent pas de façon synchrone augmente considérablement la stabilité de la liaison d'un Ac avec un Ag multivalent (ou un réseau d'Ag, ce qui est souvent le cas de marquages de tissus fixés). L'avidité peut ainsi être 1000 fois plus élevée que l'affinité intrinsèque. Pour cette raison il est préférable d'utiliser pour les marquages d'immunolocalisation des Ac complets ou des F(ab')2 plutôt que des Fab.

On peut accroitre l'intensité de la réaction en augmentant la concentration en Ag et en Ac : une bonne fixation empêchant l'élution de l'Ag est souhaitable; mais l'augmentation de la concentration en Ac est très souvent associée à une augmentation du bruit de fond et des marquages non spécifiques. Le test d'un Ac à différentes dilutions permet de déterminer le bon compromis entre marquage spécifique et bruit de fond (voir partie 4.2).

Il existe principalement trois types d'immunoréactions :

- Immunoréaction directe **(Fig. 24A)** : L'Ac reconnaissant l'Ag est directement couplé au traceur. Cette technique est la plus simple et la plus rapide. Par contre, la sensibilité de cette technique est inférieure à celle de l'immunoréaction indirecte.
- Immunoréaction indirecte **(Fig. 24B)** : L'Ac primaire reconnaissant l'Ag, est révélé par un Ac secondaire qui porte le traceur. Cette technique est beaucoup plus souvent employée par rapport à l'immunoréaction directe, en raison de sa meilleure sensibilité, car une amplification de l'intensité du marquage peut se faire par la fixation de plusieurs Ac secondaires à un Ac primaire. Néanmoins, elle est plus longue que l'immunoréaction directe.
- Marquages multiples : co-localisation de différents marqueurs **(Fig. 24C)** : La détection simultanée de deux Ag (rarement plus de 3 Ag) par des Ac couplés à des traceurs différents permet de décrire leurs relations spatiales et de réduire des échantillons précieux. Le traitement du même échantillon biologique par différents Ac couplés à des révélateurs de couleurs (microscopie optique) ou d'aspects (microscopie électronique) facilite la distinction des différents Ag.

L'immunoréaction peut se faire à 4°C, à température ambiante (TA), ou 37°C. Le temps de fixation diminue avec l'augmentation de la température.

Les combinaisons les plus souvent utilisées sont : 4°C pendant une nuit (12h), TA durant 1 à 2h et 37°C pendant 30 minutes à 1h.

I-5) Révélation

La reconnaissance de l'Ag par l'Ac ne peut être observée qu'après la mise en œuvre d'un système révélateur qui la rend visible. Le traceur conjugué à l'Ac a souvent une taille plus petite que celle de l'Ac afin de ne pas masquer le site de fixation de l'Ac à l'Ag et de ne pas constituer un obstacle dans l'accès de l'Ac aux tissus et aux différentes structures subcellulaires.

Dans la plupart des cas, l'Ac est conjugué à une enzyme qui peut catalyser une réaction de production de couleur (ex : immunoperoxydase). La peroxydase est utilisée préférentiellement pour le marquage des tissus animaux. Ceci nécessite une étape de révélation et éventuellement d'intensification ainsi que des lavages intermédiaires. Parfois, une étape préliminaire de suppression de l'activité enzymatique endogène peut s'avérer nécessaire. Les traceurs enzymatiques sont souvent utilisés au cours des réactions d'IHC et permettent une observation par microscopie optique ordinaire. La contre coloration peut se faire avec des colorants chimiques comme l'hématoxyline. La coupe de tissu peut être conservée longtemps après un processus de déshydratation et un montage en milieu hydrophobe du tissu.

Les Ac peuvent aussi être conjugués avec un fluorochrome (ex : FITC, Rhodamine, Texas Red ou Alexa Fluor). Les techniques d'immunolocalisation utilisant des traceurs fluorescents ont un nom spécifique : il s'agit de l'immunofluorescence (IF). La sensibilité, le contraste obtenu, ainsi que la précision de la localisation de ce marquage sont généralement supérieurs aux traceurs enzymatiques. L'IF est une technique rapide et très reproductible : pas de bains de révélation ou d'intensification nécessaires. La contre coloration nucléaire se fait grâce à des agents intercalants de l'ADN, comme Hoechst et iodure de propidium. Le tissu peut être conservé par congélation avant observation.

Dans le cas où il est nécessaire d'obtenir une localisation très précise de l'ultrastructure en microscopie électronique, l'immunolocalisation peut aussi se faire sur des coupes de tissu ultrafines en utilisant des Ac couplés à des micro-billes de métaux lourds comme l'or ou l'argent.

I-6) Observations microscopiques

Selon le type de traceurs, différentes techniques microscopies sont disponibles.

-Microscopie optique ordinaire : Pour les traceurs enzymatiques, les dépôts colorés par des réactions immunoenzymatiques s'observent à l'aide d'un microscope optique ordinaire. Il s'agit principalement de l'IHC.

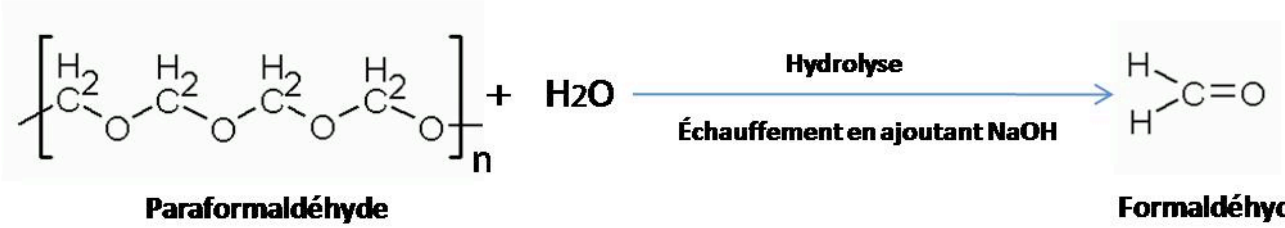
-Microscope à fluorescence : Ce sont des microscopes équipés d'une ou de plusieurs sources de lumière (lampe à arc, laser, etc.) et de filtres permettant de sélectionner les longueurs d'onde d'excitation et d'émission des traceurs fluorescents utilisés. L'IHC et l'ICC utilisent ce type de microscopie.

-Microscope confocal et microscope à balayage laser : Le microscope confocal représente une évolution du microscope à fluorescence. Dans un microscope à fluorescence classique, l'échantillon est illuminé uniformément de telle sorte que les éléments situés en dessous et au dessus du plan focal contribuent à la formation de l'image, ce qui diminue la résolution et le contraste de l'image dans le plan focal. Dans un microscope confocal, l'image des éléments situés hors du plan focal est arrêtée par le diaphragme du détecteur. Le contraste et la définition des images sont donc bien meilleurs. De plus, le balayage du faisceau permet un "découpage" de l'image dans des plans horizontaux et verticaux : les sections optiques ainsi obtenues peuvent être combinées par informatique pour reconstituer une image en trois dimensions.

-Microscopes électroniques : MET et MEB : En microscopie électronique, le marquage est visible grâce à des traceurs de masse moléculaire élevée : les atomes lourds (majoritairement l'or, l'argent ou l'osmium) dévient le faisceau d'électrons. On observe le plus souvent des coupes de tissu par microscope électronique à transmission (MET), et plus rarement la surface des cellules par microscope électronique à balayage (MEB). Cet outil permet d'établir une localisation ultra-précise des protéines.

	Composition	Remarques
Bouin	-la solution aqueuse saturée (1,4%) d'acide picrique (75ml) -formol (25ml) -acide acétique glacial (5ml)	L'avantage de cette méthode est de mieux conserver la structure cellulaire du tissu, et l'inconvénient est de donner la difficulté pour exposer les sites antigéniques de la protéine cherchée.
Carnoy I	-éthanol (3 volumes) -acide acétique glacial (1 volume)	
Carnoy II	-éthanol à 95% (6 volumes) -chloroforme (3 volumes) -acide acétique glacial (1 volume)	La méthode "Carnoy II" permet de mieux fixer les tissus gras et cireux.
Methacarn	-méthanol (6 volumes) -chloroforme (3 volumes) -acide acétique glacial (1 volume)	Ce fixateur est proposé par Puchtler et al. (1970).
Formaldéhyde-sublimé	-$HgCl_2$ (6 g) -acide acétique glacial (5 ml) -formol (10 ml) -eau distillée (85 ml)	Ce fixateur contenant du mercure peut donner de bons résultats pour la révélation des immunoglobulines (Curran et Gregory, 1980) .
Formol-zinc	-$ZnSO_4$ (0,3 à 1 g) ou $ZnCl_2$ (0,16 g) -formaldéhyde (4 % en final) -NaCl (5 g) -eau distillée q.s.p.100 ml	Le zinc présent dans les fixateurs peut donner de bons résultats en IHC en stabilisant la structure native des protéines.
TRIS-zinc	-acétate de calcium (0,5 g) -acétate de zinc (5 g) -chlorure de zinc (5 g) -tampon TRIS 0,1M pH 7,41 (1 l)	Ce fixateur est proposé par Beckstead (1994) .
Fixation à la chaleur	Fixations à 60, 80, 100 ou 120°C dans des tampons seuls ou des solutions de fixateurs classiques en concentration diminuée	La fixation à la chaleur de tissus en tampon seul semble provoquer d'importants artefacts en particulier au niveau des noyaux .

Tableau 4. Les fixations « secondaires » pour l'IHC (Adapté de « Immunohistochimie » de Garaud et Roussel)



Paraformaldéhyde **Formaldéhyde**

Figure 25. Préparation de « methanol free formaldehyde » à partir de la paraformaldéhyde en poudre (D'après Kiernan, 2000)

II) Applications classiques

Dans tout protocole utilisant une immunoréaction, plusieurs d'étapes conditionnent la qualité des résultats, notamment celles qui influencent la reconnaissance spécifique de l'Ag par l'Ac. Le choix de l'Ac est ainsi le premier élément déterminant : non seulement par sa spécificité, mais aussi par la qualité de la reconnaissance des épitopes stables et constamment exposés à la surface de l'Ag. Pour cela, l'Ac est souvent choisi en fonction de la technique utilisée (Western Blot, ICC, IHC, ELISA, etc...). Cependant, cette reconnaissance ne peut avoir lieu, même en présence d'Ac de bonne qualité, si les sites antigéniques du tissu ne sont pas pleinement accessibles. Ainsi, la maîtrise du traitement de l'échantillon biologique, permettant d'améliorer l'exposition des épitopes de l'Ag à l'Ac avant la réaction immunologique, est primordiale dans la réussite de la technique d'immunolocalisation.

Dans cette partie de notre exposé, nous présentons des protocoles expérimentaux correspondant à différentes techniques d'immunolocalisation. Nous insisterons en particulier sur les étapes de fixation et de démasquage antigénique.

L'immunolocalisation regroupe les techniques d'ICC et d'IHC. Les étapes principales du protocole des différentes techniques d'IHC et celles d'ICC sont synthétisées dans le **Tableau 3**.

II-1) Immunohistochimie (IHC)

L'IHC est la méthode principale utilisée pour localiser des protéines dans des cellules d'une coupe de tissu massif. Le tissu est coupé soit en tranches fines de 3 à 15 de micromètre pour observation en microscopie optique ordinaire ou à fluorescence, soit en en tranches ultra-fines de 50 à 200 nanomètres d'épaisseur pour observation en MET. On distingue l'IHC sur tissu paraffiné ou congelé pour l'observation par microscope ordinaire ou/et à fluorescence, et l'IHC pour l'observation en MET.

A) IHC sur tissu paraffiné

Il s'agit de technique la plus utilisée de l'immunolocalisation tant pour le diagnostic en clinique que pour la recherche biologique. Les tissus fixés et inclus dans la paraffine peuvent se conserver très longtemps. L'accès facile à des Ac commerciaux adaptés est un avantage indéniable de cette technique.

Les principales étapes du protocole de cette technique sont décrites dans le **Tableau 3**. Les deux étapes critiques de cette technique sont la fixation et le démasquage antigénique, du fait de l'existence de multiples possibilités.

Etape critique 1 : la fixation

Les fixateurs :

Le formaldéhyde est le fixateur le plus utilisé. Il peut être employé seul ou de façon combinée avec d'autres fixateurs ou agents chimiques. Une concentration à 35 ou à 40% de formaldéhyde dans le formol commercial est souvent utilisée pour l'histologie simple, mais une concentration plus basse (4% de formaldéhyde) donne généralement un meilleur résultat en IHC. Il est possible de préparer du formaldéhyde à 4% à partir de formol commercial qui contient également près de 10 à 15% de méthanol dont le but est de stabiliser le formaldéhyde concentré dans la solution aqueuse mais qui peut néanmoins perturber les mécanismes de fixations ainsi que certaines immunoréactions. De ce fait, il est conseillé de préparer les solutions de formaldéhyde à partir de paraformaldéhyde qui est un polymère du formaldéhyde et qui se présente sous forme d'une poudre blanche et insoluble dans l'eau. En le chauffant dans un milieu alcalin (NaOH), le paraformaldéhyde est hydrolysable en méthylène glycol qui est la forme active du formaldéhyde dans l'eau **(Fig. 25)**. Cette préparation est également appelée "methanol free formaldehyde" en anglais.

Le paraformaldéhyde et le formol sont souvent dissouts ou dilués dans du tampon phosphate comme le PBS. Le pH final ciblé est à 7,45. L'utilisation d'un tampon TRIS est déconseillée car il contient des amines qui réagissent avec les différents aldéhydes. Par contre, il peut être utilisé après l'étape de fixation pour bloquer les groupements de fixateurs restés libres.

De très nombreux travaux ont démontré que l'utilisation seule du formaldéhyde est suffisante pour fournir une préservation optimale du tissu et donner d'excellents résultats en IHC pour la plupart de cas. C'est donc un fixateur de choix. Il existe d'autres fixateurs qui ne sont pas utilisés de façon courante, mais qui peuvent

A) Tampons utilisés pendant le traitement de chaleur/Heat induced Epitope retrieval (HIER)

Tampons	Composition
Tampon Tris-HCl 100 mM, pH 10	-Tris (12 g) -eau distillée (800 ml) -ajuster le pH à 10 avec de l'HCl -eau distillée q.s.p. :1 litre
EDTA 1 mM, pH 8.0	-EDTA (0,37 g) -eau distillée (800 ml) -pH ajusté à 8.0 avec de le NaOH -eau distillée q.s.p.1 litre
Tampon citrate 10 mM, pH 6.0	-solution A : acide citrique ($C_6H_8O_7$, H_2O) 0,1 M (2,1 g dans 100 ml H_2O) -solution B :citrate de sodium ($C_6H_5O_7Na_3$, $2H_2O$) 0,1 M (2,94 g/100 ml H_2O) -mélanger 9 ml de solution A et 41 ml de solution B -compléter à 500 ml avec H_2O.
Tampon glycine-HCl 0,05M, pH 3,6	-glycine3,8 g -eau distillée (800 ml) -ajuster le pH avec de l'HCl -eau distillée q.s.p.1 litre

B) Démasquage enzymatique

Enzymes	Préparation	Traitement
Trypsine	Dissoudre la Trypsine à 0,1% dans du tampon TBS à pH 7,8. Ajouter 0,1% de chlorure de calcium	Incuber pendant 5 à 30 min à 37°C avec agitation vigoureuse (Curran et Gregory , 1978)
Pepsine	Dissoudre la Pespsine à 0,4% dans une solution d'acide chlorhydrique à 0,01N	Laisser agir pendant 20 à 30 minutes à 37°C (Reading, 1977)
Pronase	Dissoudre la Pronase à 0,1 mg/ml dans du tampon Tris-HCl 50 mM pH 7,6	Traiter les coupes pendant 5 min à 37°C (Finley et al., 1978)
protéase VI	Dissoudre la Protéase VI à 0,06 % dans du tampon Tris 0,5 M pH 9,6	Laisser agir pendant 10 minutes à température ambiante

C) Démasquage par des agents dénaturants

Agents dénaturants	Préparation	Traitement de la coupe
Urée	3 à 8 M (soit 180 à 480 g/L) dans de l'eau	Plusieurs heures à température ambiante ou une dizaine de minutes à chaud (four à micro-ondes, bain-marie) (Hausen et Dreyer, 1982, Shi et al., 1996)
Sodium dodécyl sulfate (SDS)	0,1-1% dans de l'eau distillée ou du tampon (PBS, Tris, etc.)	5-10 min à température ambiante
Guanidine thiocyanate (GdSCN)	4M (soit 473 g/L) dans de l'eau distillée.	2 h à 4°C

Tableau 5. Différentes méthodes de démasquage des sites antigéniques pour l'IHC (Adapté de « Immunohistochimie » de Garaud et Roussel)

également donner des très bons résultats. Ce sont donc des fixateurs de secours au cas où la fixation avec le formaldéhyde ne serait pas satisfaisante. Ces fixateurs « secondaires » sont décrits dans le **Tableau 4** [Puchtler 1970, Curran 1980, Beckstead 1994].

Conditions de fixation par formaldéhyde (temps, température etc.)

La fixation est souvent réalisée à 0°C (dans un bac à glace) ou à 4°C (au frigo) pendant 24 heures, et le temps de la fixation peut dépasser plus de 6 jours. Généralement, le temps de fixation est calculé en fonction de l'épaisseur des blocs de tissus, (en général 1h d'incubation pour chaque millimètre d'épaisseur de tissu). La température élevée et le pH neutre peut accélérer le processus de la fixation [Helander 1994]. La fixation à température ambiante est aussi courante avec un temps de fixation plus court par rapport à 4°C.

Etape critique 2 : le démasquage des sites antigéniques

Traitement par la chaleur/HIER

L'IHC sur tissu paraffiné nécessite fréquemment une étape de démasquage des sites antigéniques. Le démasquage par la chaleur est la méthode la plus utilisée. Les coupes sont chauffées au bain-marie, au four à micro-ondes ou dans une cocotte-minute, dans l'un des tampons cités **Tableau 5A**). Le tampon citrate pH 6 est le plus couramment utilisé.

Exemple de protocole:

- Déparaffinage ou suppression de la résine, réhydratation des coupes
- Immerger les lames dans du tampon citrate de sodium 10 mM pH 6. Chauffer dans un four à micro-ondes à 750 W pendant 5 min, 3 fois de suite. L'ébullition est normale. Entre chaque cycle, réajuster si nécessaire le niveau de tampon avec de l'eau distillée pour éviter le séchage des coupes au cours de l'ébullition.
- Laisser les lames refroidir dans le bain de tampon pendant 20 min à température ambiante.
- Rincer les lames dans du PBS.

Le four à micro-ondes soit le système de chauffage le plus couramment utilisé. L'utilisation d'un bain-marie thermostaté permettant d'éviter l'ébullition conserve mieux les coupes tissulaires L'utilisation d'une cocotte-minute permettant d'atteindre une température plus élevée (120°C) est encore plus efficace. Le tampon citrate peut être remplacé par une solution d'EDTA qui semble donner de meilleurs résultats [Pileri 1997].

Traitements enzymatiques

En donnant des ruptures des sites spécifiques au sein des protéines, la digestion enzymatique du tissu permet de dégager un espace autour de l'Ag, où un réseau de pontage entre les différentes protéines a été établit par les aldéhydes, afin d'exposer l'épitope de l'Ag. Les différents traitements enzymatiques sont détaillés dans le **Tableau 5B** [Reading 1977, Curran 1978, Finley 1978].

Traitements par agents dénaturants

Contrairement aux autres méthodes de démasquage, les agents dénaturants ne coupent pas de liaisons covalentes naturelles au sein d'une protéine ni celles créées par des fixateurs aldehydiques entre les différentes protéines. Ces traitements libèrent des espaces en détachant des liaisons non covalentes entre les molécules et restaurent ainsi certains épitopes à la surface de l'Ag en modifiant la structure spatiale (la conformation tertiaire et quaternaire) de la protéine. Les différents traitements utilisant des agents dénaturants sont détaillés dans le **Tableau 5C** [Hausen 1982, Shi 1996].

B) IHC sur tissu congelé

Cette technique, moins utilisée que l'IHC en coupe paraffinée, est utile pour le diagnostic en anatomo pathologie et surtout dans le domaine de la recherche biologique. L'avantage de cette technique est double : d'une part, les Ag moins altérés sont plus facilement reconnus par l'Ac et d'autre part, la mise en oeuvre de cette technique est plus rapide. Les différentes étapes de cette technique sont récapitulées dans la **Figure 26**.

La fixation du tissu peut se faire, soit avant la congélation et la réalisation de la coupe, soit, et c'est le cas le plus fréquent, après ces deux étapes. Pour une fixation préalable à la congélation, le fixateur le plus utilisé est le PFA 4% à 4°C pendant une nuit. Pour réaliser une fixation après découpe du tissu, le protocole utilise du PFA 4% à 4°C pendant 10 à 15 minute, ou du méthanol ou encore de l'acétone à -20°C pendant 30 minutes.

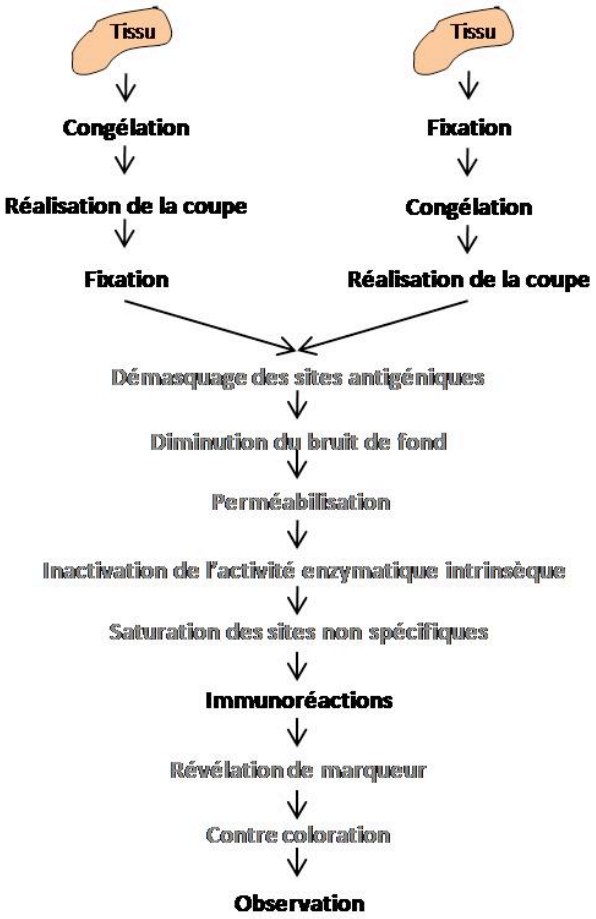


Figure 26. Différentes étapes de l'IHC sur coupes de tissus congelés. Les étapes en écriture grise optionnelles selon le cas précis. **(BiiGC/He)**

Figure 27. Méthode d'IHC pour l'observation en MET : immunoréaction avant la découpe ultrafine. Les étapes en écriture grise sont optionnelles. **(BiiGC/He)**

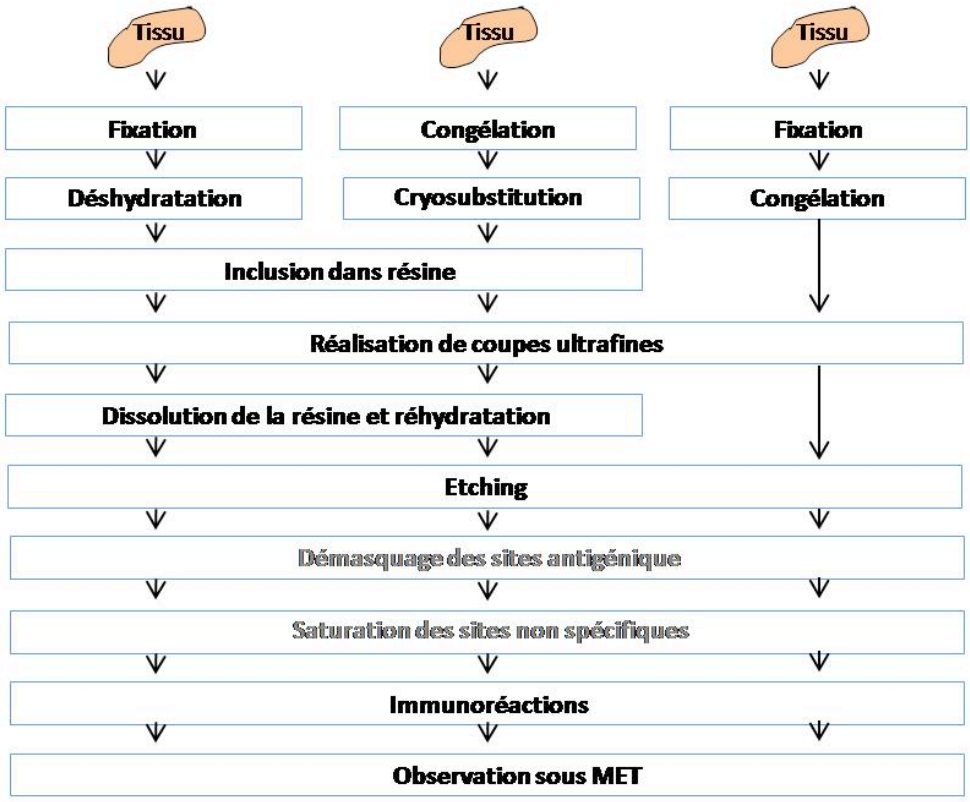


Figure 28. Méthodes d'IHC pour l'observation à la MET: immunoréaction après la découpe ultrafine. Trois différents protocoles sont distincts; Les étapes en écriture grise optionnelles. **(BiiGC/He)**

L'étape de démasquage des sites antigéniques bien que rarement réalisée, se fait préférentiellement par un traitement par la chaleur en raison de la fragilité de la coupe tissulaire congelée.

En immunofluorescence, le traitement de la coupe tissulaire avec 0,1 à 1% de borohydrure de sodium dans du PBS pendant 15 à 30 minutes à température ambiante permet le démasquage des sites antigéniques et entraine une diminution de l'autofluorescence. Ceci est dû à des phénomènes de suppression de liaisons covalentes intrinsèques ou créés par le formaldéhyde qui sont responsables du masquage des épitopes de l'Ag et de l'autofluorescence. Un démasquage par traitements enzymatiques ou dénaturants peut être réalisé dans des cas difficiles pour obtenir une immunoréaction spécifique.

C) IHC pour l'observation en MET

Afin de visualiser la localisation précise de l'ultrastructure de l'Ag, l'IHC peut être effectuée sur des coupes de tissu ultrafines suivi d'une observation par MET.

Deux protocoles se distinguent en fonction de l'ordre dans lequel sont réalisées la découpe du tissu et l'immunoréaction.

-Méthode 1 : immunoréaction réalisée avant la découpe ultrafine **(Figure 27**)

L'avantage principal de cette technique réside dans la facilité de l'immunoréaction spécifique de l'Ac aux sites antigéniques de l'Ag qui ne sont alors pas masqués ou perturbés par la fixation « forte » du glutaraldéhyde ou par le processus d'inclusion du tissu dans la résine. Le fixateur le plus couramment utilisé est le PFA à 2 à 4% dans du PBS à pH 7,4. D'autres solvants tels que l'éthanol, le méthanol, ou l'acétone peuvent être aussi employés. L'épaisseur de la coupe de tissu allant de quelques µm à 100 µm conditionne la facilité ou non de la pénétration des Ac mais aussi des techniques de démasquage. En cas d'utilisation d'agents de pontage comme fixateurs, deux étapes supplémentaires sont alors nécessaires : une saturation des groupements aldéhydiques par un traitement à la glycine 100mM, ou au chlorure d'ammonium 50mM dans du PBS pendant 15 à 20 minutes à TA, et une perméabilisation par des détergents comme le Triton X100, la saponine 0,01% à 0,1% dans du PBS pendant 10 à 15 minutes à TA. Les traceurs conjugués à l'Ac sont souvent des billes de métaux lourds, mais peuvent aussi être des enzymes comme la peroxydase qui est alors associée à une étape de révélation des marqueurs par la diaminobenzidine (DAB).

-Méthode 2 : immunoréaction réalisée après la découpe du tissu **(Figure 28**)

L'étape de fixation reste ici identique : préfixation avec des mélanges de formaldéhyde contenant au moins 1% de glutaraldéhyde, puis post-fixation au tétroxyde d'osmium. La cryosubstitution pouvant éventuellement remplacer la fixation classique par glutaraldéhyde, a pour but de ne pas introduire de liaisons covalentes masquant les sites antigéniques de l'Ag.

Le processus est long : les tissus frais congelés sont laissés plusieurs jours à environ -80°C dans plusieurs bains de solvant (acétone, éthanol, etc.) qui ont pour rôle d'extraire progressivement la glace présente dans les tissus. On peut éventuellement ajouter dans le solvant un fixateur comme le tétroxyde d'osmium [Moreira 1996] ou le glutaraldéhyde par exemple afin de mieux conserver l'ultrastructure du tissu.

Trois différents protocoles sont distincts au sein de cette méthode (Figure 28). Le premier conserve mieux l'ultrastructure cellulaire, le deuxième en évitant l'utilisation d'une fixation « forte » par glutaraldéhyde permet un meilleur accès de l'Ac aux sites antigéniques en diminuant l'effet pontage et le troisième permet également un accès facilité aux sites antigéniques dans les cas où ces derniers seraient masqués par le processus d'inclusion du tissu dans la résine.

La plupart des immunohistologistes utilisent la méthode 1 en raison d'une plus grande facilité de mise en oeuvre du processus d'immunoréaction grâce à une fixation utilisant du formaldehyde mais aussi par l'obtention d'une présélection du tissu avant la réalisation des coupes ultrafines. La méthode 2 peut également donner des bons résultats [Garaud 1980, Bendayan 1983].

II-2) Immunocytochimie (ICC)

A la différence de l'IHC, l'ICC est souvent utilisée en recherche biologique comme méthode d'immunolocalisation sur des cellules monocouches en boîte de culture in vitro ou des cellules isolées et étalées en monocouche sur une lame. La technique d'ICC est beaucoup plus simple et rapide à réaliser par

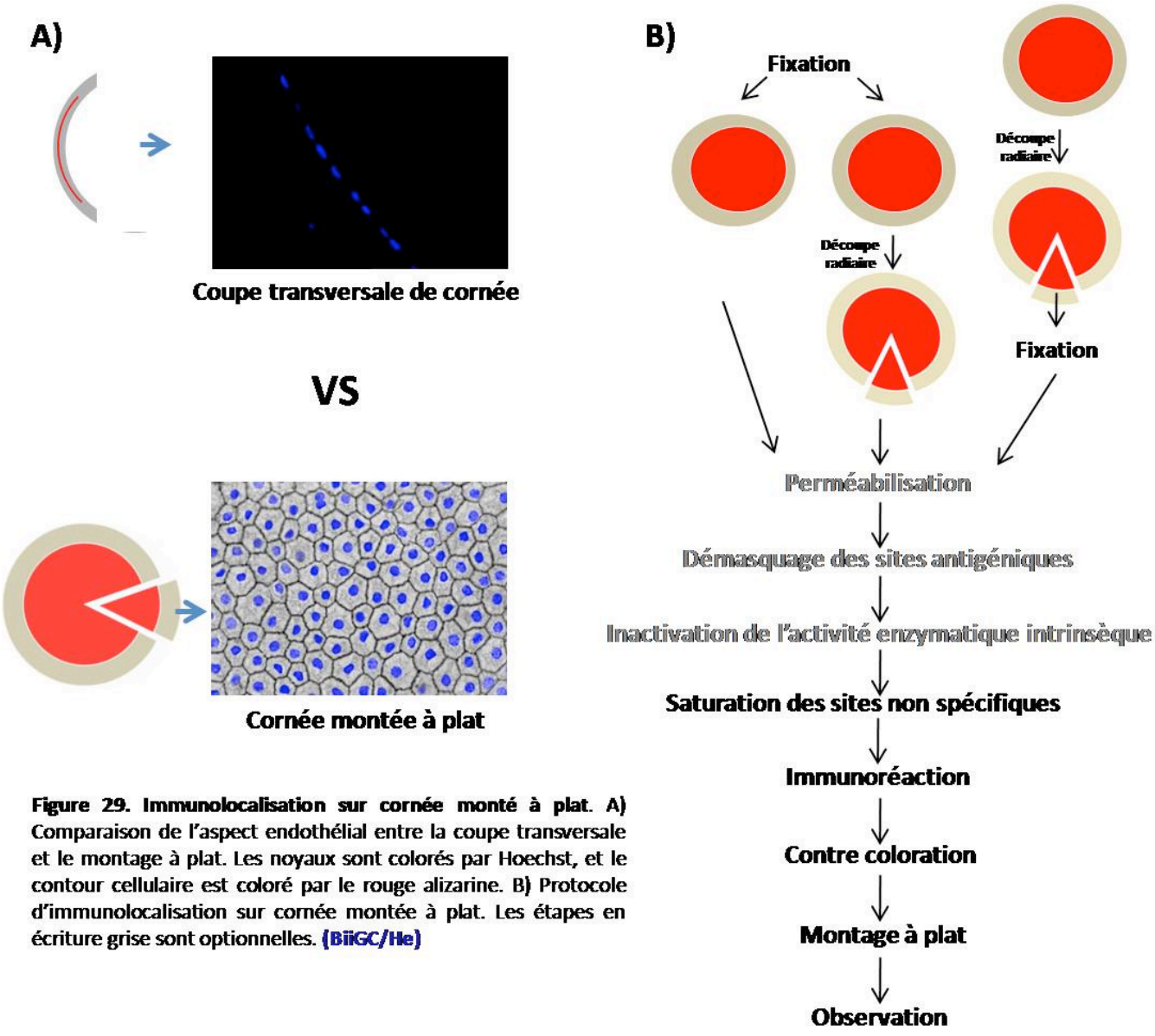


Figure 29. Immunolocalisation sur cornée monté à plat. A) Comparaison de l'aspect endothélial entre la coupe transversale et le montage à plat. Les noyaux sont colorés par Hoechst, et le contour cellulaire est coloré par le rouge alizarine. B) Protocole d'immunolocalisation sur cornée montée à plat. Les étapes en écriture grise sont optionnelles. **(BiiGC/He)**

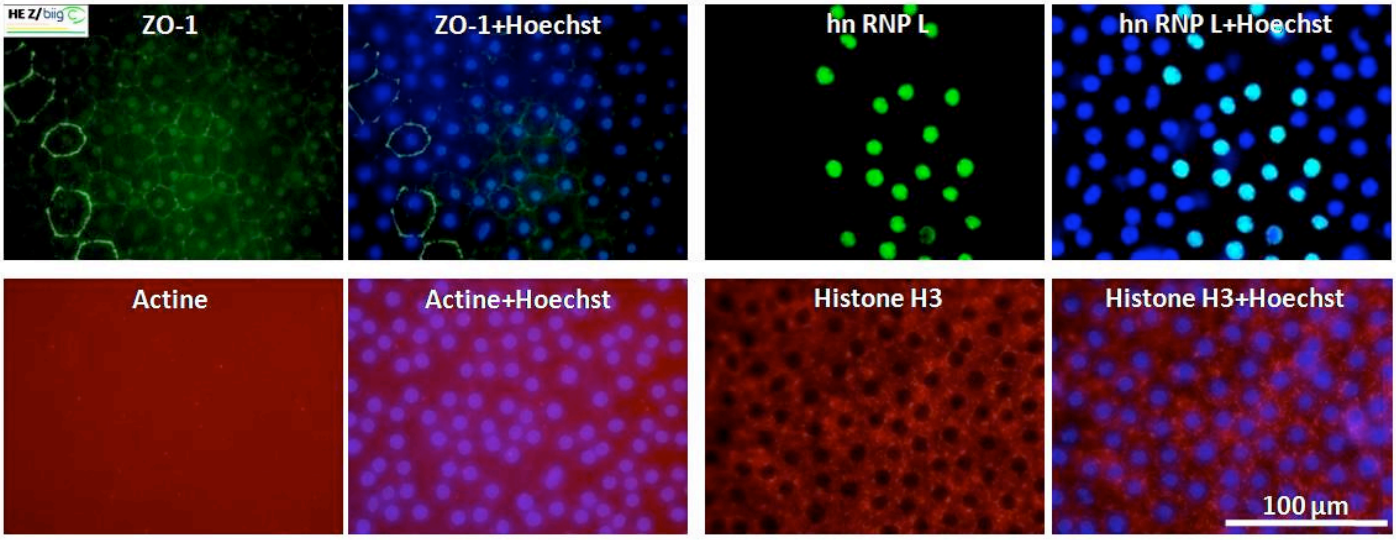


Figure 30. Echec de l'utilisation du PFA 4% en immunolocalisation sur l'endothélium cornéen montée à plat. Analyse comparative en immunolocalisation de 4 protéines exprimées de façon constante par les CE sur la cornée humaine fixée par PFA 4%. ZO-1 devrait marquer le contour de toutes les CE ; l'actine devrait marquer la face apicale des CE ; hn RNP L devrait marquer le noyau de toutes les CE et ainsi qu'histone H3. Cependant, avec la fixation standard de PFA 4% (pour IHC et ICC), l'immunomarquage de tous ces 4 protéines est faussé. **(observations de BiiGC/HE)**

rapport aux techniques d'IHC en raison de l'absence d'inclusion, de congélation et de la réalisation de coupes tissulaires.

Les étapes principales du protocole sont illustrées dans le **Tableau 3** :

La fixation se fait par du PFA 4% dans du PBS à pH 7,4 pendant 15 minutes à température ambiante, ou par du méthanol, éthanol ou acétone pendant 10 minutes à -20°C. Les méthodes de démasquage sont très peu utilisées. La perméabilisation du tissu utilisant 1% de Triton X100 pendant 5 minutes à température ambiante est requise seulement après fixation par du PFA. Les traceurs utilisés dans l'ICC sont des fluorochromes permettant d'omettre l'étape de révélation du marqueur. Une réaction d'ICC peut ainsi être effectuée en 3 à 4 heures.

III) Notre technique hybride IHC/ICC : l'immunolocalisation sur cornée montée à plat

L'étude de la biologie fondamentale de la CE est primordiale tant pour la compréhension de certaines pathologies primitives (dystrophie de Fuchs en premier lieu), que de la perte cellulaire accrue pendant la conservation cornéenne, ou encore de la reconstitution in vitro de l'endothélium cornéen à partir de cellules souches en espérant un jour pouvoir pratiquer une thérapie cellulaire directement in situ chez un patient, ou en utilisant un greffon de la qualité améliorée pendant la conservation en OC, ou encore un endothélium reconstitué in vitro par un processus de bioengineering.

L'étude des protéines de structures et des protéines fonctionnelles est un des outils majeurs de la biologie cellulaire car ce sont les principaux effecteurs de réactions biologiques. Parmi les techniques d'étude, le Western Blot permet une analyse quantitative de l'expression des protéines, mais nécessite une quantité protéique importante qu'en seul endothélium cornéen ne pourrait fournir. L'IHC avec son approche qualitative apparait donc comme la technique de choix pour analyser en première instance l'expression des protéines au sein de l'endothélium cornéen. Le blocage en phase G1 du cycle cellulaire des CE humaines in vivo est une notion bien démontrée, principalement par l'équipe de Joyce du Schepens Eye Research Institute, en utilisant initialement une technique d'IHC sur des coupes transversales de cornée [Joyce 1996a, Joyce 1996b]. Avec cette méthode, seules quelques CE sont visualisées « de profil » **(Fig. 29A)**

Il nous semblait pourtant plus judicieux tant pour la localisation que la quantification d'utiliser au sein de l'endothélium cornéen, une nouvelle technique d'immunolocalisation sur montage à plat. Pour cela, l'immunoréaction est effectuée directement sur l'endothélium cornéen fixé, la cornée est ensuite montée à plat entre lame et lamelle afin d'avoir une vue globale de l'ensemble de la surface endothéliale. Notre technique originale est ainsi une technique « hybride » : la réaction d'immunolocalisation sur tissu solide s'apparente à l'IHC, alors qu'au niveau de sa mise en œuvre, elle s'apparente plus à de l'ICC. Ses principales étapes en sont illustrées dans la **Figure 29B**.

Ses difficultés n'en demeurent pas moins nombreuses :

1) La fixation au PFA 4%, pourtant la plus utilisée en IHC et ICC ne nous semble pas être adaptée **(Fig. 30)**.

2) Les techniques de démasquage antigénique sont délicates à mettre en oeuvre. La cornée se rétracte et se déforme sous les traitements par la chaleur, rendant l'observation impossible sous microscope. Les traitements enzymatique ou par agents dénaturants décollent souvent des CEs de la MD.

L'un des objectifs était de pallier à ces difficulté afin d'aboutir à une technique désormais robuste. **Ce travail est présenté dans la partie expérimentale.**

CELLULES SOUCHES (CS)

I) Définition et classification

I-1) Classification des CS selon leur potentiel de différenciation
I-2) Classification des CS selon leurs origines
I-3) Progéniteurs, précurseurs et cellules d'amplification transitoire
I-4) CS cancéreuses

II) Cellules souches adultes (CSA)

II-1) Diversité des CSA et controverses
II-2) Auto renouvellement et niches des CSA
II-3) Identification des CSA
II-4) CSA et thérapie cellulaire
II-5) Questions essentielles sur les CSA

III) Modèle épithélial cornéen : de l'identification des CS à l'utilisation en clinique humaine

III-1) Rappel anatomique de la surface occulaire
III-2) Concepts de régénération de l'épithélium cornéen par les CS limbiques
III-3) Insuffisance en CS limbiques (ICSL) : causes, conséquences et diagnostic
III-4) Traitement de l'ICSL par greffe de CS/ progénitrices limbiques
III-5) Reconstitution in vitro du microenvironnement spécifique du limbe et thérapies cellulaires dérivées
    A) Microenvironnement spécifique du limbe
    A-1) Microenvironnements favorisant le maintien du caractère de CS (stemcellness)
    A-2) Microenvironnements assurant la différenciation spécifique
    B) Thérapies cellulaires dérivées
    B-1) A partir des CS limbiques isolées
    B-2) A partir d'autres types de CSA autologues
III-6) Reconstitution in vivo du microenvironnement spécifique du limbe et traitements dérivés

IV) Etat actuel des connaissances et perspectives de recherche sur les CS souches endothéliales cornéennes

IV-1) Arguments suggérant le potentiel régénératif de l'endothélium cornéen chez l'homme
    A) Hétérogénéité entre CE du centre et de la périphérie d'une cornée saine
    B) Observation de phénomènes pathologiques touchant les CE
    C) Découverte in vitro de cellules progénitrices en nombre supérieur en périphérie
IV-2) Nécessité de preuves directes des capacités régénératives des CE
    A) Analyse critique des travaux existants
    B) Questions restant en suspens
IV-3) Thérapies du futur : la reconstitution de l'endothélium in vitro
    A) Reconstitution de l'endothélium in vitro
    B) Nécessité d'études du microenvironnement

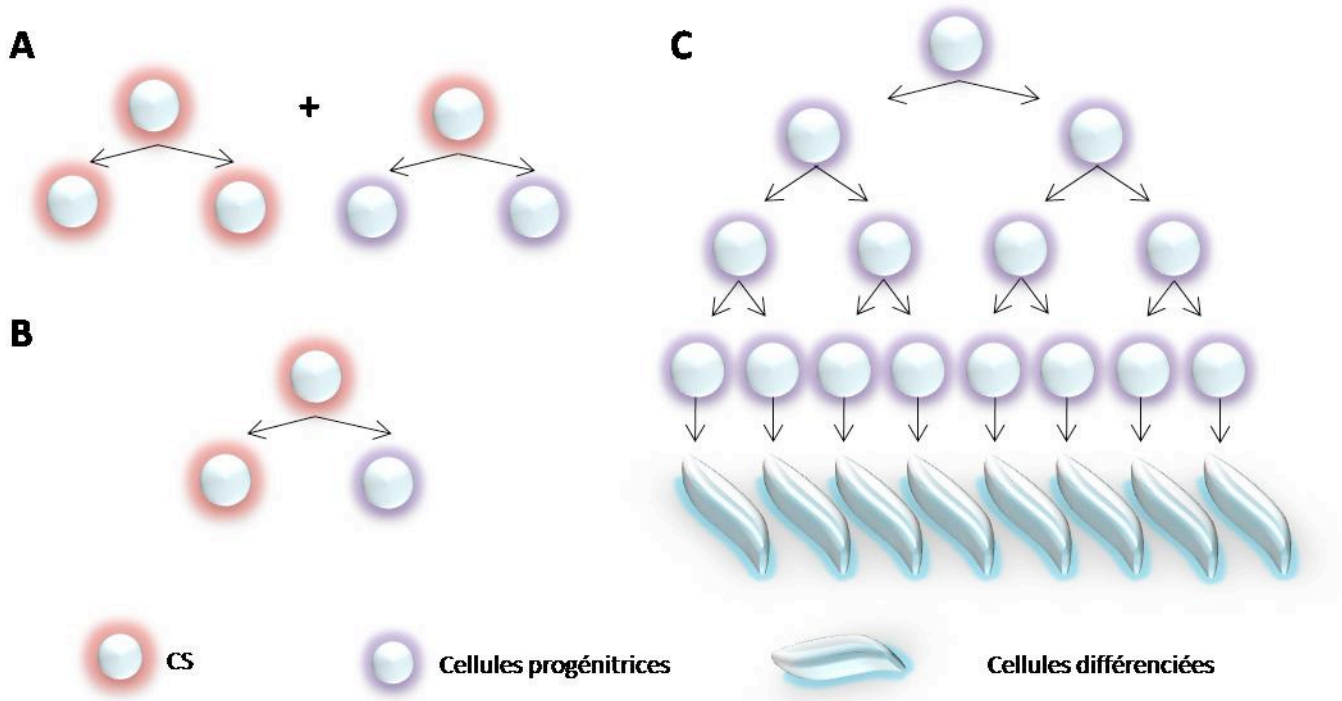


Figure 31. Auto-renouvellement et la différenciation des cellules souches (CS). A) Autorenouvellement de CS par deux divisions symétriques de CS; B) Autorenouvellement de CS par une seule division asymétrique; C) Différenciation terminale après l'expansion des cellules progénitrices. **(BiiGC/He)**

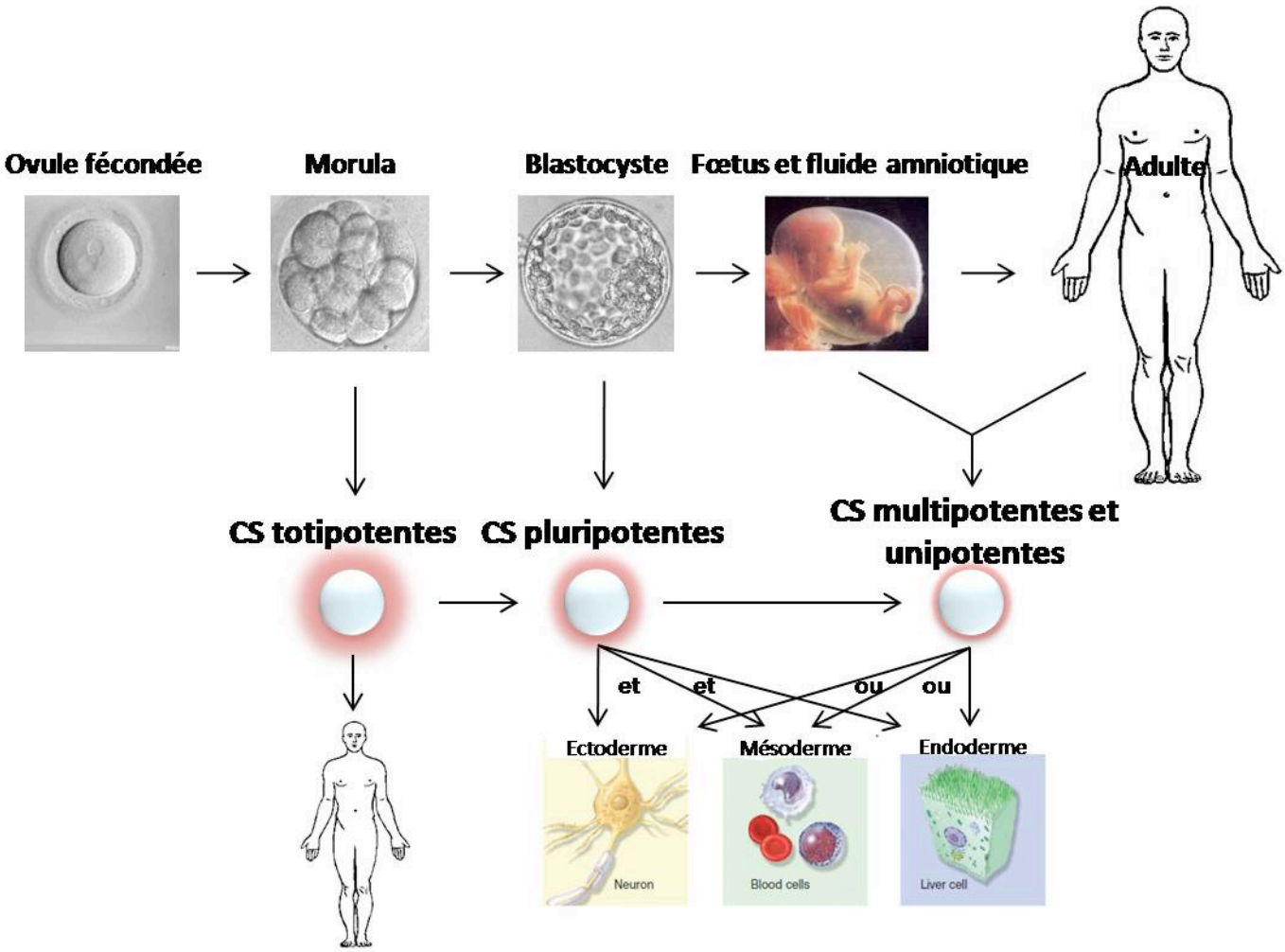


Figure 32. Classification de CS selon leur potentiel de plasticité **(BiiGC/He)**

I) Définition et classification

Les cellules souches (CS) sont indispensables au développement des tissus et des organes pendant l'embryogenèse et la croissance après la naissance. Elles permettent aussi l'homéostasie des tissus et organes en assurant le renouvellement de leurs cellules et la régénération tissulaire **(Fig. 31)**.

- Cellule souche (CS) :

Il s'agit d'une cellule indifférenciée qui se caractérise par des capacités de différenciation en cellules spécialisées et de multiplication quasi infinies permettant de maintenir en permanence un pool de CS (autorenouvellement) [Lewis 1964, Lajtha 1967].

Pendant le développement ou en cas d'atteintes tissulaires (régénération physiologique ou pathologique), la CS se divise de façon asymétrique en deux cellules filles qui présentent des propriétés différentes de prolifération et de différenciation. Cette asymétrie s'explique soit par la ségrégation de facteurs intrinsèques différents après la séparation cellulaire, soit par la position de ces cellules dans des microenvironnements distincts appelés niches. La cellule fille qui garde le même profil que la CS mère reste dans la niche et contribue au maintien d'un pool stable de CS (autorenouvellement). L'autre cellule fille, nommée cellule progénitrice, s'engage dans une voie de différenciation aboutissant à un seul type cellulaire mature dans la plupart de cas [Lajtha 1979].

- Etat indifférencié :

Une des propriétés fondamentales des CS est de ne pas avoir de structure tissulaire spécifique, qui leur permettrait d'accomplir des fonctions cellulaires spécifiques. Leur caractère non spécialisé leur permet de donner naissance à n'importe quel type de cellules spécialisées.

- Différenciation :

La différenciation cellulaire est un concept de biologie du développement décrivant le processus par lequel les CS se spécialisent en un « type » cellulaire. La morphologie d'une cellule peut changer radicalement durant la différenciation. Le matériel génétique reste le même, mais le matériel épigénétique qui décrit l'ensemble de l'expression génique sans altération des séquences nucléotidiques est modifié.

Au cours de sa différenciation, une cellule passe par plusieurs stades, en devenant de plus en plus spécialisée. Les différents signaux intra et extracellulaires impliqués dans le processus de différenciation cellulaire commencent à être démembrés. Les signaux externes comprennent les secrétions chimiques d'autres cellules, les contacts inter-cellulaires et certaines molécules du microenvironnement autour de la CS. L'interaction de ces signaux au cours de la différenciation permet à l'ADN des cellules d'acquérir des marqueurs épigénétiques qui limitent l'expression de l'ADN dans la cellule et peut être transmis par la division cellulaire.

I-1) Classification des CS selon leur potentiel de différenciation

On distingue 4 types de CS selon leur potentiel de différenciation. **(Fig. 32)**

- La CS totipotente

Ovule fécondé ou cellule issue des premières divisions de cet œuf jusqu'au quatrième jour (morula de 2 à 8 cellules), cette cellule est la seule à permettre le développement complet d'un individu, y compris des tissus extra-embryonnaires comme le placenta, à condition d'être placé *in vivo* pour permettre une orientation de l'embryon qui reste impossible *in vitro*. C'est seulement à ce stade que peut s'opérer un clonage reproductif (vrais jumeaux) par scission embryonnaire. Étymologiquement totipotence signifie "qui peut tout" indiquant que théoriquement ces cellules peuvent se différencier en tout type cellulaire de l'organisme (cellules épithéliales, neuronales, hépatiques...). Quatre jours après fécondation et de division continue, des cellules totipotentes commencent à se spécialiser en cellules pluripotentes et multipotentes.

- La CS pluripotente

Elle fait partie des cellules souches embryonnaires (CSE). Les CSE ne peuvent pas produire un organisme entier car elles ne peuvent pas générer de tissus extra-embryonnaires comme le trophoblaste et le placenta, mais peuvent se différencier en cellules issues de n'importe lequel des 3 feuillets embryonnaires, y compris les cellules germinales. Elles ne peuvent pas, à elles seules, aboutir à la création d'un individu complet

puisqu'elles proviennent de la masse cellulaire interne du blastocyste (au stade de 40 cellules) alors que le placenta qui nourrit l'embryon et le protège de tout rejet par le système immunitaire est produit par la couche cellulaire externe (ou trophectoderme). Elles ont vocation de former tous les tissus de l'organisme. Le clonage reproductif à partir des CSE n'est pas possible.

- La CS multipotente

Les CS multipotentes regroupent les CS tissulaires (ou CS somatiques) du foetus et de l'adulte. Elles peuvent aussi être retrouvées dans le liquide amniotique. Elles sont à l'origine de plusieurs types de cellules différenciées mais conservent leur capacité d'auto renouvellement. Les CS multipotentes peuvent donner naissance à plusieurs types cellulaires, mais elles sont déjà engagées dans une certaine voie de différenciation. On dit que ce sont des cellules déterminées. Leurs potentialités sont donc plus restreintes que celles des CSE. Les cellules hématopoïétiques des mammifères, par exemple, donnent des globules rouges, des plaquettes, des lymphocytes T ou B, des macrophages, mais elles ne peuvent pas donner des cellules musculaires. Un autre exemple de CS multipotentes est apporté par les cellules de la crête neurale qui migrent à partir du tube neural au cours de l'embryogénèse et qui donnent notamment naissance aux mélanocytes, aux neurones et aux cellules gliales du système nerveux périphérique.

- La CS unipotente

Elle ne peut produire qu'un seul type cellulaire (tout en s'auto renouvelant) comme la peau, foie, muqueuse intestinale, testicule.

Note 1 : Stricto sensu les cellules totipotentes (ovules fécondés) ne peuvent pas être considérées comme étant des CS, mais seulement comme des cellules indifférenciées, car il leur manque la capacité d'auto-renouvellement.
Note 2: Beaucoup d'auteurs considèrent qu'il n'existe pas de CS multi- ou unipotentes. Il s'agirait plutôt selon eux soit de cellules progénitrices, soit de CS dont les capacités de différenciation ont été sous-estimées.
Note 3 : Certains organes, tels que le cœur ou le pancréas, ne renferment pas de CS et n'ont donc aucune possibilité de régénération en cas de lésion.

I-2) Classification des CS selon leurs origines

Quatre types de CS se distinguent par leur origine : CS embryonnaires, CS adultes, CS fœtales et amniotiques et CS induites.

- Cellules souches embryonnaires (CSE)

Ce sont des CS pluripotentes présentes dans l'embryon peu de temps après la fécondation jusqu'au stade de développement dit de blastocyste ou blastomère, où elles constituent encore la masse cellulaire interne (les autres cellules les plus externes du blastocyste constituent le trophectoderme).

Les CSE humaines dérivent d'embryons préimplantatoires surnuméraires au stade de blastocyste, issus de programmes de fécondation *in vitro* Trounson 2001. Des CSE humaines pluripotentes ont été obtenues en culture pour la première fois en 1998 Thomson 1998. Ces cellules montrent une capacité de prolifération stable (auto renouvellement) sur une période importante impliquant la voie de signalisation Wnt Sato 2004, et ont une activité télomérase Thomson 1998. Les CSE humaines sont caractérisées par l'expression de marqueurs impliqués dans la pluripotence, tels que les facteurs de transcription Oct4, Nanog, Sox2, Foxd3, Rex1 Yeo 2007, le récepteur du TDGF1 ou le facteur de croissance GDF3 par exemple Pera 2004. Les CSE humaines peuvent également être caractérisées par l'expression de la phosphatase alkaline, ou par l'activité télomérase, en plus de marqueurs spécifiques tels que SSEA-3 et 4 (stage-specific embryonic antigens 3 and 4), Tra-1–60, Tra-1–81 ou le complexe majeur d'histocompatibilité de classe 1 (MHC-1) Stojkovic 2004. Les CSE humaines présentent un grand intérêt dans le cadre de leur utilisation potentielle en médecine régénérative, de par leur capacité de différenciation pluripotente. Cependant, leur utilisation thérapeutique pose plusieurs problèmes : 1) d'ordre éthique (utilisation d'embryons humains) ; 2) de par leur fort potentiel mitogène et tumorigène, le risque de générer des tumeurs après leur greffe est important ; 3) la possibilité d'un rejet de ces CSE après transplantation n'est pas écartée.

- CS fœtales et CS du liquide amniotique et du sang de cordon

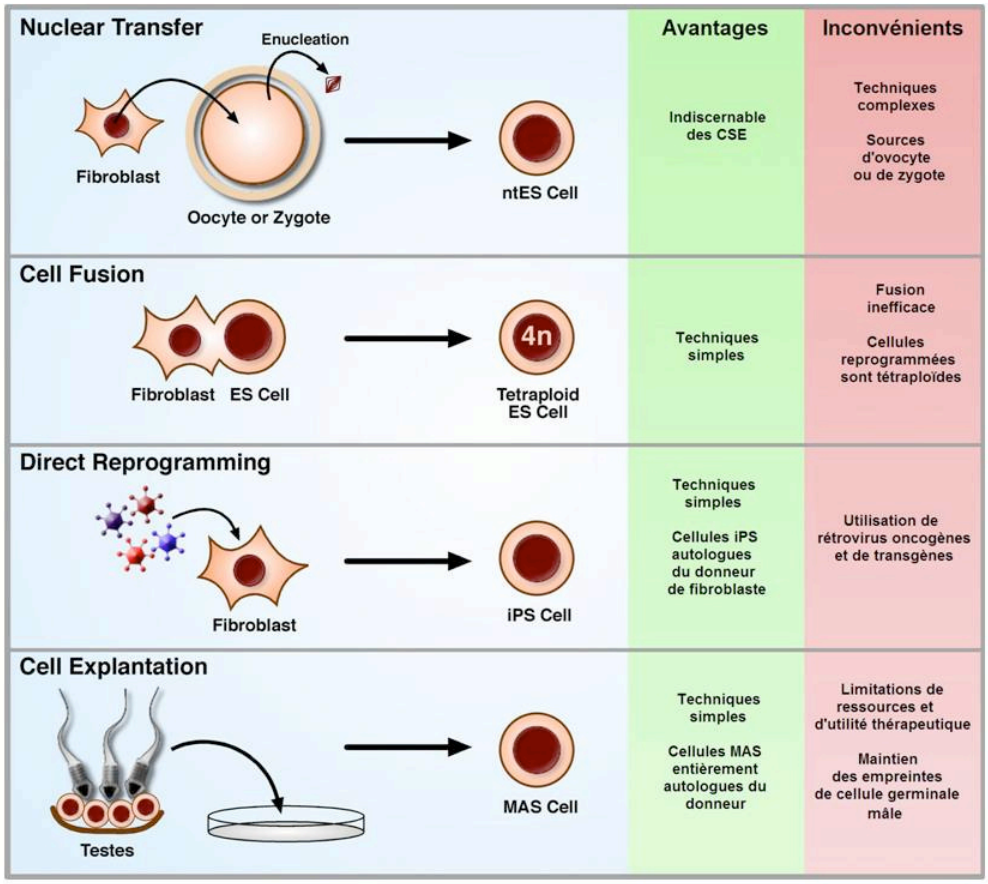


Figure 33. Différentes SC pluripotentes régénérées à partir des cellules somatiques (D'après Rodolfa, 2008)

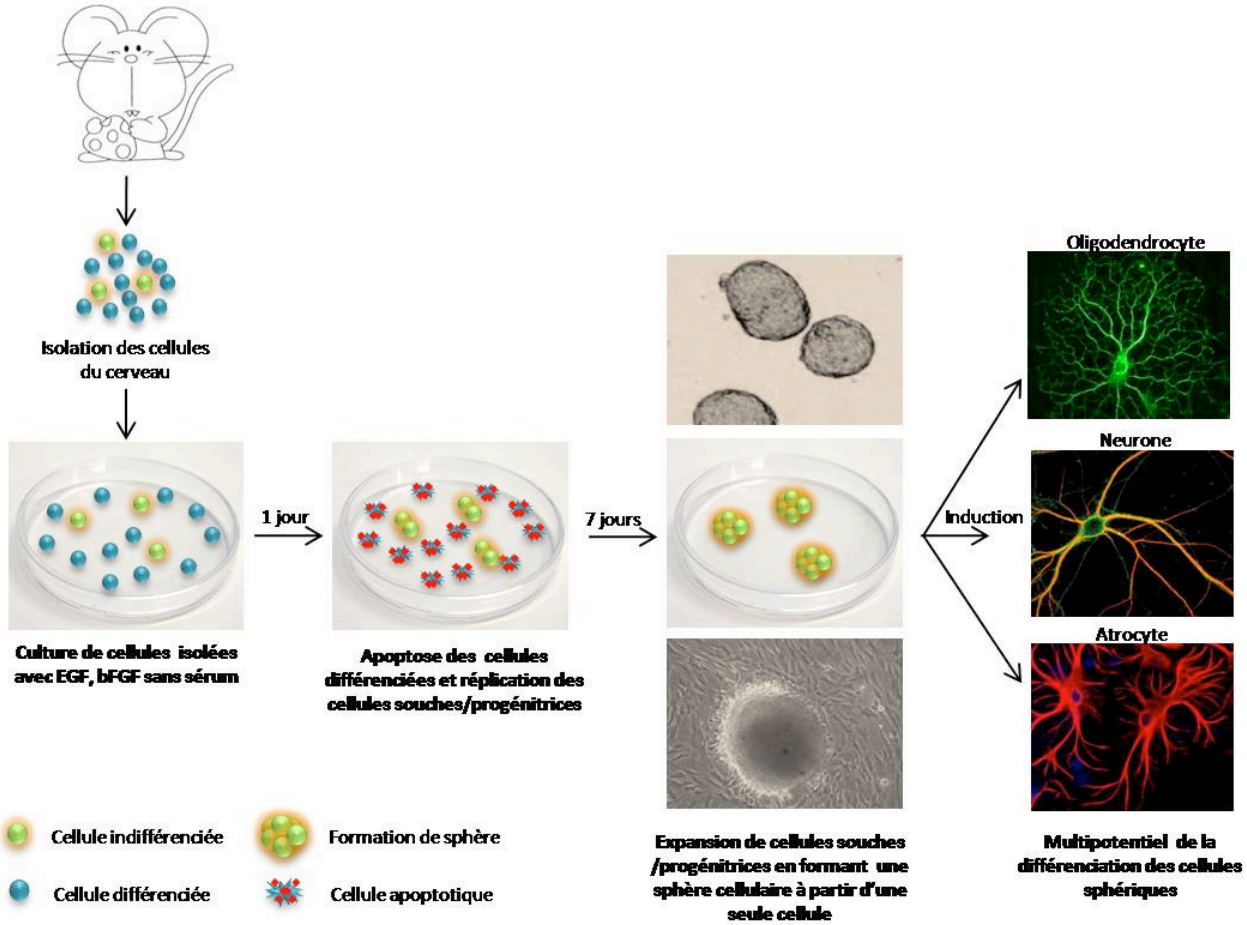


Figure 34. Identification et isolation des cellules souches neurales par l'essai de formation sphérique (Adapté de Reynolds et Weiss, 1992)

Ce sont des CS multipotentes d'origine fœtale ou trouvées dans le liquide amniotique et dans le sang du cordon ombilical (ou dans le sang plancentaire). A la différence des CSE, les CS fœtales issues du tissu fœtal à un stade tardif (>5 semaines) ont perdu la pluripotence et sont déjà engagées vers des types cellulaires particuliers. Elles sont plus difficiles à cultiver et à contrôler in vitro par rapport aux CSE. Les CS amniotiques sont très actives, et peuvent se différencier vers les lignées cellulaires adipogéniques, ostéogéniques, myogéniques, endothéliales vasculaires, hépatiques et aussi neuronales [De Coppi 2007]. Des banques de CS amniotiques existent aux Etats-Unis et collectent des CS multipotentes dans du liquide amniotique pour un usage autologue. Le sang du cordon contient des CS hématopoïétiques et mésenchymateuses et offre des possibilités de traitement de certaines leucémies. Mais ces cellules ne se divisent pas spontanément, sont de culture difficile et, une fois le processus de division engagé, se différencient et meurent très rapidement [Rocha 2005].

- CS adultes (CSA)

Les CSA, aussi appelées CS somatiques, sont des cellules indifférenciées que l'on trouve au sein d'un tissu spécifique composé en majorité de cellules différenciées chez adulte. Ce sont généralement des cellules multipotentes et unipotentes. Elles sont capables de donner naissance à un ou plusieurs types cellulaires. Elles sont à la base du renouvellement naturel d'un tissu et de sa réparation à la suite d'une lésion. Les CS représentent un petit pourcentage du pool cellulaire total. Les CS adultes sont à l'heure actuelle très étudiées. Leur utilisation ne pose pas de problème d'éthique et l'espoir est important de pouvoir les utiliser à des fins thérapeutiques à la place des CSE ou fœtales. De plus, la possibilité d'autogreffe des CS adultes pourrait rendre la thérapie encore plus sécurisée. Cependant, les difficultés sont encore importantes pour pouvoir les maintenir en culture.

- CS pluripotente régénérée à partir des cellules somatique **(Fig. 33)** [Rodolfa 2008]

4 méthodes d'induction de la pluripotence existent à partie des cellules matures ou CS somatiques.

Transfert nucléaire : le matériel génétique (chromatine) d'un ovocyte ou d'une cellule œuf (zygote) est remplacé par celui d'une cellule différentiée comme le fibroblaste. Suivant le développement jusqu'au stade blastocyste, les cellules pluripotentes ntES (nuclear transfer embryonic stem cell) peuvent être générées comme dans un embryon dérivé de l'ovule fécondé. Le succès du clonage de Dolly utilisant cette technique en 1997 a été la première preuve du maintien de la capacité de développement complète du noyau de certaines cellules somatiques [Wilmut 1997].

Fusion de cellule : l'hybridation entre les CSE et les cellules somatiques donne des lignées de CSE tétraploïde où le phénotype pluripotent est dominant.

Reprogrammation directe: l'induction de certains facteurs de transcription utilisant des vecteurs rétroviraux peut être suffisante pour régénérer le phénotype pluripotent. La première étude réalisée en 2006 [Takahashi 2006], a démontré que l'introduction dans des cellules somatiques des quatre facteurs de transcription Oct3/4 (Pou5f1) et Sox2, déjà identifiés dans le maintien de la pluripotence des CSE [Avilion 2003], ainsi que c-Myc et Klf4, gènes surexprimés dans de nombreux cas de tumeurs et contribuant à l'auto renouvellement et à la capacité de prolifération rapide des CSE [Cartwright 2005], permettait d'induire de la pluripotence dans ces cellules différentiées. Ces cellules, générées à partir de cellules embryonnaires murines ou fibroblastiques adultes, ont été nommées **iPS** (induced pluripotent stem cells), et présentent les propriétés morphologiques, de prolifération, et d'expression de gènes des CSE [Takahashi 2006].

Explantation de cellules : Les cellules pluripotentes peuvent être obtenues directement à partir du tissu testiculaire de souris néonatales [Kanatsu-Shinohara 2004] et adultes [Guan 2006] après l'explantation des cellules testiculaire et leur culture in vitro en présence des facteurs de croissance appropriés.

I-3) Progéniteurs, précurseurs et cellules d'amplification transitoire

Il s'agit de populations cellulaires transitoires entre CS et cellules matures différenciées [Emerson 1996, Emsley 2005]. Elles participent au processus de différenciation et possèdent une capacité de prolifération supérieure à celle des CS. La différence entre les CS et ces cellules transitoires est que les CS peuvent s'auto-renouveler indéfiniment, alors que ces cellules transitoires n'ont pas de capacité d'auto-renouvellement et ne peuvent se diviser qu'un nombre limité de fois.

- Progéniteurs ou cellules progénitrices

Après la division d'une CS, les progéniteurs, aussi appelés cellules progénitrices, quittent la niche où les CS résident et migrent vers les tissus [Ogawa 1983, Schofield 1983]. Les CS ont un cycle cellulaire très long, l'accélération de la division des CS pouvant induire une erreur pendant la réplication de l'ADN. Au contraire, les cellules progénitrices peuvent se diviser beaucoup plus facilement et rapidement et jouent donc un rôle primordial dans la réparation du tissu. Les cellules progénitrices sont destinées de se différencier en un seul type cellulaire. Exception faite dans de rares cas où elles peuvent se différencier en plusieurs types cellulaires appartenant à la même lignée cellulaire : elles sont alors uni ou mutipotentes [Kawamoto 1997]. La différence entre les cellules progénitrices et les CS réside dans la capacité du renouvellement du soi : les CS peuvent s'auto-renouveler indéfiniment, alors que l'auto-renouvellement des cellules progénitrices est restreint dans le temps. La majorité des cellules progénitrices sont quiescentes et ne possèdent qu'une petite activité dans le tissu où elles résident. Au cours de processus de régénération physiologique ou de réparation, les cellules progénitrices sont activées. Elles vont alors proliférer en se différenciant et en migrant vers l'endroit qui leur est suggéré pour remplacer les cellules mortes et réparer le tissu. Les CS et les cellules progénitrices se situent souvent près ou au sein du tissu qu'elles régénèrent, mais pas toujours, tel que les CS et progénitrices hématopoïtiques résidant dans la moelle osseuse. Les facteurs de croissance et les cytokines libérés par le tissu endommagé jouent le rôle très important de messager et de stimulateur des cellules progénitrices quiescentes.

-Note: Les termes de CS adulte et de cellule progénitrice sont parfois confondus ou égaux. En théorie, ces deux populations se différencient par un auto-renouvellement infini pour les CS et limité pour les progéniteurs. Mais en pratique, ce n'est pas toujours évident de les distinguer. Beaucoup de propriétés sont partagées entre les CS et les cellules progénitrices, et leurs définitions exactes restent controversées. D'ailleurs, ce concept est toujours en train d'évoluer. Le terme CS/progénitrice est souvent utilisé au cours de la découverte des cellules immatures adultes dont le statut n'est pas clairement défini.

- Précurseurs

C'est un type de cellules partiellement différenciées par rapport aux cellules souches/progénitrices multipotentes. Elles possèdent une grande capacité de prolifération en se différenciant vers un seul type cellulaire spécifique. On utilise souvent ce terme dans la culture primaire des cellules souches/progénitrices, où un seul type de cellules matures est obtenu à partir de ces cellules sans leur donner de microenvironnements spécifiques.

- Cellules d'amplification transitoire

Les cellules d'amplication transitoire (CAT), sont aussi nommées cellules progénitrices d'amplification transitoire, progéniteurs proliférants, progéniteurs activés ou committed progenitors en anglais. Ces termes sont utilisés pour les progéniteurs activés in vivo en insistant sur leur caractère proliférant.

I-4) CS cancéreuses

Durant plusieurs décennies, les chercheurs en oncologie ont été confrontés à deux visions concurrentes des tumeurs : d'une part, toutes les cellules d'une tumeur sont identiques et ont la même capacité à se diviser et à former de nouvelles tumeurs ; d'autre part, seules les cellules spécifiques sont capables de former de nouvelles tumeurs : les CS cancéreuses.

La notion de CS cancéreuse date du 19ème siècle, mais pendant longtemps les scientifiques n'ont pas été en mesure de mener à bien les expériences nécessaires pour prouver leur existence en séparant les CS cancéreuses des autres cellules d'une tumeur [Sell 2010]. La première démonstration a été réalisée en 1997 par une équipe de chercheurs de l'Université de Toronto [Bonnet 1997]. Ils ont implanté dans des souris immunodéficientes des cellules cancéreuses prélevées chez des patients atteints de leucémie, et ont ainsi pu démontrer qu'une très petite partie de ces cellules leucémiques étaient capables de reproduire la maladie chez la souris. Par la suite, les CS cancéreuses ont aussi été identifiées dans différents tissus solides tels que le cerveau [Singh 2003], le sein [Al-Hajj 2003], le colon [O'Brien 2007], le mélanome [Schatton 2008] etc. Les chercheurs ont conclu que ces cellules cancéreuses rares pouvaient être considérées comme des CS cancéreuses.

Les CS cancéreuses présentent des caractéristiques communes avec des CS normales : elles se renouvellent indéfiniment et peuvent se différencier en différents types cellulaires. L'origine des CS cancéreuses est encore très discutée. Elles pourraient être l'aboutissement d'une mutation d'un gène impliqué

dans les mécanismes d'auto-renouvellement et de différenciation des CS, progénitrices, matures ou bien les cellules tumorales [Martinez-Climent 2006, Wu 2008].

Bien qu'elles ne constituent que moins de 1% des cellules tumorales, elles jouent un rôle essentiel dans l'évolution de la maladie puisqu'elles seraient à l'origine des métastases et des récidives. La découverte des CS cancéreuses a des implications importantes dans les futures thérapies anticancéreuses.

II) Cellules souches adultes (CSA)

Malgré leur importante plasticité, les CSE, ainsi que les induced pluripotent stem cells ont une utilisation qui reste délicate : d'une part, d'un point de vue éthique, et d'autre part, du fait de leur pouvoir tumorigène et immunogène [Boyd 2005]. Les études cliniques utilisant la greffe de CSA sont de plus en plus fréquentes. Bien que leur capacité de différenciation soit plus restreinte que celle des CSE, les CSA présentent l'avantage de pouvoir être isolées chez un patient (greffe autologue) dans le cadre de la thérapie cellulaire. De plus, la faisabilité de la reprogrammation des CSA d'un tissu à l'autre pourrait contre balancer l'inconvénient du manque de plasticité et aboutir à la thérapie cellulaire destinée à certains tissus chez lesquels des CS spécifiques n'ont pas encore été trouvées, tel que le cœur [Wagers 2004, Takeuchi 2009].

Les CSA sont capables de s'auto-renouveler tout au long de la vie mais également de se différencier pour permettre le maintien de l'intégrité physiologique des tissus. Des CSA ont été identifiées dans de nombreux tissus et organes, tel que le cerveau, la moelle osseuse, le sang, les vaisseaux, les muscles squelettiques, la peau, les dents, le cœur, l'intestin, le foie, les épithéliums ovarien et cornéen, les testicules… Elles résident dans un endroit spécifique dans chaque tissu, appelé « niche de CS ». Dans de nombreux tissus, des preuves récentes suggèrent que certaines CSA sont en fait des péricytes, cellules qui composent la couche externe des petits vaisseaux sanguins. Les CSA peuvent être quiescentes pendant de longues périodes jusqu'à ce qu'elles soient activées par un besoin cellulaire, un processus pathologique, ou une lésion tissulaire.

Les CSA adultes sont rares : dans l'intestin grêle, il y a moins de 10 CSA par crypte pour une population cellulaire totale de 300 cellules [Marshman 2002]. Dans le muscle squelettique, les cellules satellites occupent 5% des noyaux et dans la moelle osseuse, la CS hématopoïétique (multipotente) représente une proportion de 0,01% parmi toutes les cellules.

L'identification des CS au sein des différents tissus et organes est encore en cours. L'absence de marqueurs moléculaires bien définis et caractéristiques constitue un frein aux études actuelles mais reste une condition requise pour leur isolement et leur caractérisation phénotypique et moléculaire.

De plus, il est difficile de mettre en place des méthodes de culture pour étudier le comportement des cellules souches isolées, surtout les CSA. Les CSE peuvent être cultivées in vitro pendant plus d'un an sans se différencier, mais ce n'est pas le cas des CSA. Ceci s'explique probablement par une forte dépendance du « stemcellness » des CSA à un microenvironnement spécifique (niche de CS).

La réussite de certaines thérapies cellulaires à partir de CSA est très encourageante : ainsi l'identification des CS spécifiques à l'épithélium cornéen ainsi que leur isolement et culture in vitro jusqu'à leur application à la thérapie cellulaire. Ce modèle thérapeutique est un bel exemple en ophtalmologie. A la différence de l'épithélium cornéen, l'endothélium cornéen humain est considéré à ce jour comme un tissu non régénératif. Cependant, les besoins actuellement croissants et la nécessité de proposer aux patients de nouvelles thérapeutiques nous poussent à rechercher et à identifier des CS spécifiques endothéliales.

II-1) Diversité des CSA et controverses

Diversité des CSA

Les CSA sont multi ou unipotentes. Contrairement aux CSE, elles sont souvent spécifiques à un seul ou à plusieurs tissus. Il existe donc une grande variété de CSA capables de répondre aux besoins des différents tissus [Coulombel 2007].

Dans le système hématopoïétique, l'existence de CS dans la moelle osseuse a été suggérée avant même leur identification formelle, pour expliquer la production quotidienne de millions de cellules sanguines. C'est sur ce postulat que les greffes de moelle osseuse ont été proposées dans les années 1975, alors que les CS hématopoïétiques n'ont été identifiées à l'échelon clonal que dix ans plus tard [Kondo 2003].

Pour la peau, les greffes ne permettent pas de restaurer un revêtement cutané fonctionnel : il n'y a pas de régénération des follicules pileux, des glandes sébacées, et des glandes sudoripares, car seule la population des CS épidermiques est greffée [Levy 2005]. Par contre, la coopération de plusieurs types de CS est requise pour

le maintien d'un épiderme fonctionnel. Or les CS du bulbe, les plus immatures, sont à l'origine des CS épidermiques et des cellules du follicule pileux mais sont difficilement transplantables dans les conditions actuelles. Pour l'intestin, les cellules épithéliales des villosités se renouvellent aussi tous les 30 jours à partir de CS localisées au fond des cryptes. Si celles-ci sont clairement identifiées, elles ne peuvent être ni cultivées, ni amplifiées ex vivo.

Pour les autres tissus, l'identification de CS est beaucoup plus récente et reste encore incertaine : le cerveau et le muscle constituent les exemples les plus emblématiques, mais on pourrait également citer la glande mammaire, les bronches, la prostate, ainsi que nous l'avons vu précédemment l'épithélium cornéen etc... Chacune de ces populations a des caractéristiques spécifiques et il n'y a pas de stratégie univoque pour leur identification.

Controverse sur les CSA

Les CSA sont définies comme des CS multi ou unipotentes. Le renouvellement cellulaire pendant un processus de réparation d'un tissu endommagé provient de cellules progénitrices et souches spécifiques. Le fonctionnement du système de régénération et de réparation des tissus adultes semble pourtant plus compliqué.

Le foie constitue une situation unique, puisque ce sont les hépatocytes eux-mêmes, cellules différenciées, qui sont en première ligne pour la régénération tissulaire, et que le rôle des cellules dites ovales, CS de secours localisées dans les canaux d'Hering à la jonction des canalicules biliaires, est encore bien hypothétique.

Dans le cœur et le rein, la notion de CS est plus difficile à concevoir, puisque ces organes sont constitués d'une architecture complexe faisant appel à des constituants d'origine différente (épithéliale, endothéliale, cardiomyocytaire, etc).

On connaît depuis longtemps les CS dites mésenchymateuses, isolées au sein de la moelle osseuse et présentant des capacités d'adhérence au plastique bien reconnues sur le plan phénotypique. Elles ont un potentiel avéré, in vitro comme in vivo, de progéniteurs ostéoblastiques, chondrocytaires, adipocytaires etc., utilisé d'ailleurs en thérapeutique Caplan 2005, 2007. Cette population ne mérite probablement pas l'appellation de souche et son rôle physiologique est inconnu.

Indépendamment, une controverse s'est récemment développée à propos de la possible existence, dans les tissus adultes de CS très rares, qui auraient un potentiel non plus multipotent, mais pluripotent (proche de celui des CSE) Serafini 2006. Ces cellules, généralement isolées à partir de la moelle osseuse chez l'adulte possèdent également des propriétés d'adhérence et sont désignées par des termes variés permettant de les distinguer des CS mésenchymateuses décrites ci-dessus. Elles expriment deux propriétés potentiellement intéressantes d'un point de vue thérapeutique : elles peuvent être amplifiées ex vivo sous forme indifférenciée, et peuvent, dans des milieux de culture adaptés, adopter de multiples destins cellulaires, endodermiques (foie, cellules sécrétrices d'insuline), ectodermiques (neurones) ou mésodermiques (muscle, cellules endothéliales vasculaires).

Le consensus actuel mentionne que les CSA sont peu capables de se dédifférencier et de prendre un autre chemin de différenciation que celui du tissu dans lequel elles résident, comme le font par exemple les cellules du membre sectionné des salamandres Coulombel 2003. Cependant, plusieurs articles suggèrent que les CS adultes, placées dans un environnement différent de leur tissu d'origine, sont capables de suivre une autre voie de différenciation, ce qui est désigné par le terme de transdifférenciation. La transdifférenciation pourrait être due, soit à une reprogrammation intrinsèque des CS afin d'acquérir de nouvelles capacités d'adaptation au nouveau microenvironnement Takeuchi 2009, soit à une simple fusion cellulaire avec les cellules du tissu où les CS sont transplantées Alvarez-Dolado 2003.

II-2) Auto-renouvellement et niche des CSA

Auto-renouvellement

Une CS est définie par sa capacité de production d'autres CS identiques (auto-renouvellement) et de cellules engagées dans le processus de différenciation (cellules progénitrices). Par une seule division asymétrique, une CS peut accomplir ces deux tâches à la fois **(Fig. 31A)**. Par une division symétrique, une CS peut donner deux cellules filles identiques: deux CS, ou deux cellules progénitrices **(Fig. 31B)**.

La division asymétrique est le mécanisme permettant aux CS de maintenir leur nombre constant dans la niche [Alison 2009], alors que la division symétrique permet l'amplification des CS au cours du développement de l'individu mais aussi de rétablir le nombre de CS après une perte cellulaire causée par une lésion tissulaire [Morrison 2006]. Toutefois, ces mécanismes peuvent être responsables d'évènements délétères : la disparition de la division asymétrique des neuroblastes chez Drosophila melanogaster est létale et ce phénomène expansif aboutit à des lésions cancéreuses similaires à celles retrouvées quand on transplante des neuroblastes à un adulte [Caussinus 2005]. Paradoxalement, le manque de signalisation adéquate peut induire l'épuisement du capital de CS. Ceci a déjà été constaté en cas d'absence de la signalisation Wnt dans l'intestin des souris où l'expression de Tcf-4 a été supprimée [Korinek 1998].

L'auto-renouvellement des CS est maintenu non seulement par des mécanismes intrinsèques, mais aussi par le microenvironnement extrinsèque.

Niche de cellules souches

Les CSA in vivo se maintiennent dans leur état indifférencié pendant toute la vie. Cependant, elles rentrent en sénescence quand elles sont mises en culture in vitro : la morphologie change, l'auto-renouvellement se perd et la capacité de prolifération diminue. Ce phénomène courant met en évidence l'importance du microenvironnement dans le maintien de l'état indifférencié des CS adultes.
Le concept de niche de CS a été proposé en 1978 par Schofield comme une explication hypothétique devant le paradoxe concernant l'immortalité des CS in vivo et leur sénescence in vitro [Schofield 1978]. Schofield a proposé le postulat suivant : un microenvironnement spécifique in vivo, nommé « niche de CS », maintient les propriétés des CS [Schofield 1983]. La localisation précise des structures anatomiques où les CS s'accumulent a été identifiée dans différents tissus adultes et nommée définitivement niche de CS [Arai 2008].

Les CSA résident dans leur niche qui maintient leur état indifférencié, limite leur prolifération exubérante et régule leur participation à la régénération et la réparation d'un tissu [Scadden 2006].

Les mécanismes de maintien de l'état indifférencié des CS par la niche sont très complexes. Ils font intervenir différents éléments : l'interaction directe entre les CS, ou entre les CS et les cellules matures qui fonctionnent comme des cellules nourricières, ou encore entre les CS et des composants de matrice extracellulaire, mais aussi la teneur en oxygène, le pH, la concentration des ions tel que le Ca2+, la présence de certains facteurs de croissance comme les cytokines. La reproduction in vitro des conditions d'une niche est un élément déterminant pour les futures thérapies cellulaires

II-3) Identification des CSA

Les différents tissus ont souvent leurs propres CS situées au sein du tissu ou dans des tissus voisins (sauf le sang et la lymphe). Ces CS ainsi que leur niche peuvent être identifiées directement sur le tissu en utilisant des marqueurs moléculaires. Après l'identification, les CS sont isolées et marquées en culture et puis transplantées dans le tissu à régénérer afin de vérifier leur capacité de repeuplement sans altération. En cas de réponse positive, leur statut de CS spécifiques au tissu est confirmé.

A ce jour, il n'existe pas de marqueur universel pour toutes les CSA. Chaque type de CS possède ses propres marqueurs spécifiques. Selon les caractéristiques de chaque CS, des marqueurs non spécifiques peuvent être utilisés pour identifier ou présélectionner éventuellement des CS sur certains tissus.

Marqueurs spécifiques

Les protéines sont des acteurs principaux dans les différentes activités cellulaires. Leur expression au sein de différents types cellulaires et au cours des différents stades de différenciation nous permet d'identifier des populations cellulaires spécifiques. Les CS et progénitrices expriment des protéines spécifiques qui conditionnent ainsi leurs caractéristiques. Ainsi ces protéines peuvent servir comme marqueurs permettant d'identifier des CS au sein d'une large population cellulaire.

Une centaines de marqueurs plus ou moins spécifiques ont déjà été identifiés chez différents types de CS. Pour information, voici les adresses de quelques sites internet où comprenant des listes très complètes de marqueurs :

http://en.wikipedia.org/wiki/stem_cell_marker ou http://stemcells.nih.gov/info/scireport/appendixE.asp

L'utilisation de marqueurs spécifiques par immunolocalisation est actuellement l'un des principaux outils dans l'identification des CS de différents tissus. Alors que certains marqueurs très spécifiques ont été bien identifiés, il reste encore des CS spécifiques de nombreux tissus à découvrir. L'identification de ces CS spécifiques peut utiliser dans un premier temps des marqueurs identifiés correspondant à des tissus de même origine embryonnaire. De plus, un seul marqueur positif ne peut souvent pas identifier précisément une population de CS spécifiques. Il faut souvent combiner plusieurs marqueurs pour pouvoir distinguer les CS parmi de nombreuses cellules matures et progénitrices.

Les CS les plus étudiées sont les CS hématopoïétiques et CD34 est considéré comme leur marqueur spécifique. Cependant, il est présent non seulement dans les CS primitives isolées de la moelle osseuse, mais aussi dans les différentes progénitrices dérivées. Pour bien distinguer ces cellules souches primitives, il faut examiner également l'expression de CD38, CD33 et HLA-DR qui ne sont exprimées que chez les cellules progénitrices.

Marqueurs ou méthodes non spécifiques

- Bromodésoxyuridine (BrdU), ou H3-Thymidine

L'incorporation à l'ADN des analogues de thymidine repérables (bromodésoxyuridine (BrdU), ou H3-Thymidine) durant la réplication en phase S du cycle cellulaire, peut permettre de situer les CS dans leurs niches respectives, et de les distinguer de leur descendance [Cegielski 2004, Chan 2006]. Après une exposition courte du tissu à ces traceurs de prolifération, toutes les cellules possédant une capacité de prolifération ont incorporé les traceurs dans leur ADN. L'observation se fait après une période longue de re-exposition du tissu dans un milieu normal. Les cellules progénitrices, dérivant des CS et présentant un fort pouvoir prolifératif, élimineront très rapidement le BrdU ou la H3-Thymidine par plusieurs cycles effectués. Seules les CS en se divisant lentement resteront marquées fortement. Cette technique met ainsi en évidence les LRC (Label Retaining Cells) correspondant aux CSA, au sein de leur microenvironnement spécifique *in vivo*.

- Hoechst 33342

Le Hoechst 33342 peut se fixer sur l'ADN et ainsi le rendre fluorescent. Il est couramment utilisé pour colorer les noyaux des cellules vivantes ou fixées. Une petite proportion de cellules vivantes colorées plus légèrement par Hoechst et nommée « side population (SP) », peut être isolée par cytométrie en flux grâce à la capacité de l'efflux de Hoechst via la glycoprotéine transmembranaire BCRP/ABCG2 [Scharenberg 2002]. Cette SP possède des caractéristiques communes avec les CS. La propriété d'efflux du Hoechst 33342 est ainsi utilisée conjointement pour l'identification et la purification des CS potentielles de différents tissus, en absence de véritables marqueurs de surface cellulaire. La première SP isolée provenait des cellules d'une moelle osseuse murine et la proportion de CS hématopoïétiques dans la SP était 1000 fois grande qu'au sein de la population cellulaire de moelle osseuse [Goodell 1996]. La SP a ensuite été mise en évidence chez certains tissus solides. Dans la zone sub-épendymale située dans le prosencéphale des souris adultes, la proportion des CS neurales est 7,5 fois plus importante dans la SP que dans la population cellulaire totale. Ce rapport de proportion de CS neurales augmente à 278 fois dans l'embryon de souris à 14ème jour [Kim 2003]. Ce phénomène de population de CS très concentrée dans la SP a aussi été démontré chez la souris dans les glandes mammaires, la peau et les testicules, et l'épithélium pulmonaire [Summer 2003, Challen 2006]. Cette capacité à effluer le Hoechst, ainsi que l'expression de la BCRP représente un marqueur commun à certaines CS tissulaires [Zhou 2001].

- Essai de formation sphérique en culture

A l'origine, il s'agit une technique pour isoler et augmenter le nombre de CS in vitro. Le succès de cette méthode est un argument supplémentaire en faveur de l'existence de cellules souches/progénitrices parmi les cellules isolées d'un tissu [Sukach 2007].

En 1992, Reynolds et Weiss ont employé un système de culture sans sérum, nommé test de formation de neuro-sphères, afin d'isoler et d'amplifier in vitro des CS neurales provenant du cerveau d'une souris adulte [Reynolds 1992]. Dans cet essai, les cellules isolées du cerveau ont été séparées et dissociées les unes des autres de façon chimique (Trypsine-EDTA) et mécanique (pipettage). Elles ont été ensuite cultivées dans un milieu de culture contenant des facteurs de croissance (EGF (epidermal growth factor) et bFGF (basic fibroblastic growth factor)). La plupart des cellules (les cellules différenciées) sont mortes après quelques jours de culture sans l'ajout de sérum. Cependant une petite population cellulaire a survécue et a proliférée en formant des amas cellulaires sphériques. La subculture de ces cellules de neuro-sphères a donné un pool cellulaire expansif et

indifférencié capable de donner naissance aux trois types majeurs de cellules matures du système nerveux central **(Fig. 34)** [Reynolds 1992].

Cette méthode a été ensuite généralisée pour identifier et isoler non seulement des cellules indifférenciées dans différentes tissus sains [Toma 2001, Yamagami 2006, Oshima 2009, Mimura 2010], mais aussi des CS cancéreuses de différentes origines [Ghods 2007, Pan 2010, Rajasekhar 2011, Zou 2011].

Cette méthode identifie non seulement les CSA, mais aussi les progéniteurs qui possèdent une importante capacité de multiplication.

La maintenance de l'état indifférencié de CSA nécessite la présence de leur microenvironnent spécifique (« leur niche »). Après les processus d'isolation des CSA, de leur niche et d'amplification cellulaire in vitro, les « CS » obtenue ne sont plus de véritables « CS », mais plutôt des progéniteurs qui ont déjà perdu une partie de leur capacité d'auto-renouvellement et sont engagés partiellement dans la voie de la différenciation.

Confirmation de l'identité des cellules souches

La difficulté est d'ordre expérimentale : il faut, en effet, garder présent à l'esprit qu'une CSA, à l'exception des cellules germinales, n'est pas reconnaissable sur des critères phénotypiques et qu'aucune combinaison de marqueurs n'est suffisamment spécifique pour identifier une population pure de CS. L'identité des CS ne peut être confirmée donc que rétrospectivement et indirectement, par leur fonction, qui est de produire des cellules différenciées in vivo pour repeupler et reformer un tissu endommagé. Idéalement, cette démonstration doit être effectuée in vivo après transplantation d'une seule CS. Cette preuve expérimentale a été obtenue pour quatre tissus : le système hématopoïétique, la glande mammaire, la peau, et, à un moindre degré le muscle [Coulombel 2007].

II-4) CSA et thérapie cellulaire

Aujourd'hui, face aux cas où l'organisme ne peut pas restaurer un tissu ou un organe endommagé, la greffe de tissu ou la transplantation d'organe est presque le seul moyen thérapeutique. Mais le besoin en greffons est bien supérieur à l'offre à travers le monde. De plus, les problèmes d'immuno-compatibilité ainsi que de nombreuses difficultés et complications des techniques chirurgicales représentent un frein important au processus de greffe d'organe ou de tissu.

L'application potentielle la plus importante de la thérapie cellulaire utilisant des CSA est de réparer ou de reformer le tissu ou l'organe endommagé. Par rapport à la greffe de l'organe, les avantages de la thérapie cellulaire seraient multiples: simplicité de la technique chirurgicale, accès facile aux matériels (cellules, organes), accroissement des possibilités d'autogreffes évitant tous les problèmes immunitaires de l'allogreffe, accessibilité au traitement de maladies beaucoup plus diverses ou fréquentes telles que la maladie d'Alzheimer, lésion de la moelle épinière, maladies cardio-vasculaires, diabète, ostéoarthrose, brûlure ou encore maladies de la cornée parmi elles la dystrophie endothéliale (une des principales causes de greffe) …

Il existe deux stratégies possibles d'utilisation des CS en thérapeutique : soit la stimulation de l'activité de la réparation des CS endogènes résiduelles au sein du tissu endommagé, soit l'introduction des CS ou de progéniteurs exogènes dans le tissu endommagé. Les deux stratégies peuvent être envisageables en fonction du tissu concerné [Korbling 2003].

Stimulation de l'activité de réparation endogène

De nombreux essais cliniques récents dans le domaine cardiovasculaire, notamment en période post-infarctus, ont consisté à greffer par voie intramyocardique, des cellules de moelle osseuse [Minami 2005, Fazel 2006]. L'idée initiale était de remplacer des cardiomyocytes défaillants, mais on sait aujourd'hui que le principal effet de ces cellules médullaires est trophique, réduisant l'apoptose, stimulant l'angiogenèse et probablement l'activation de précurseurs myocytaires locaux. Une autre possibilité de stimulation des CS endogènes est d'agir sur les cellules nourricières constituant leur niche tissulaire, et de leur faire délivrer un message de prolifération aux CS avoisinantes. Le bien-fondé de cette stratégie a récemment été démontré dans le système hématopoïétique [Adams 2007, Napoli 2008] et pourrait être exploré pour d'autres, notamment dans le cerveau.

Essais en cours utilisant des CS exogènes

Le terme CS exogène désigne tout type de CS hors du tissu en traitement. A l'heure actuelle, hormis les CS hématopoïétiques et les CS épidermiques, il n'existe encore que peu de protocoles thérapeutiques utilisant les CS adultes au sens strict du terme. En effet, par manque du microenvironnement in vitro bien adapté à la maintenance de l'état initial de CS, les essais publiés ces dernières années utilisent des cellules appelées progéniteurs, certes descendants immédiats des CS, mais ayant déjà perdu les attributs cardinaux que sont

l'auto renouvellement et la multipotence [Coulombel 2007]. C'est le cas de la transplantation des îlots béta de Langherans dans le diabète insulinodépendant, ou des neurones fœtaux dans la maladie de Hungtington, peut-être aussi de certains progéniteurs ostéogéniques dans certaines pathologies osseuses. Précisons que, si le tissu est à renouvellement lent, ce qui est le cas dans ces exemples, ces progéniteurs seront très efficaces. Mais, dans le cas des pathologies musculaires, hépatiques, ou encore neurologiques (maladies neurodégénératives) il n'y a pas actuellement de possibilités de réparation efficace par des CS tissulaires [Martino 2006, Chun 2010]. La limitation principale est le nombre de cellules, leur source, les difficultés de prélèvement, des écueils qui n'existent pas dans le cas des CSE disponibles en nombre illimité et à différents stades de leur différenciation. Il faut y ajouter notre difficulté à les isoler et les amplifier in vitro en nombre suffisant, mais également l'altération de ces cellules elles-mêmes, notamment chez des patients âgés, car les CSA vieillissent également, ce qui s'accompagne d'une perte de potentiel [Janzen 2006], ou peuvent être atteintes par le processus pathologique que l'on cherche à réparer. A contrario, les CSA présentent peu de risques, et l'on peut les utiliser en situation autologue (le patient étant son propre pourvoyeur de cellules), alors que la tolérance immunologique est un problème majeur posé par les CSE. Voici l'intérêt que suscitent les études des CSA dites généralistes comme les CS mésenchymateuses, ou les cellules multipotentes si contestées. Ces cellules possédant de la multipotence peuvent être prélevées d'un autre tissu saint du patient et appliquées chez le même patient au tissu endommagé.

II-5) Questions essentielles sur les CSA

La connaissance portant sur les CSA est encore très loin d'être achevée. Quelles sont les attentes dans ce domaine? Les sept questions posées par l'institut national américain de la recherche des CS peuvent nous montrer l'orientation de la recherche dans le domaine des CSA. (site web : http://stemcells.nih.gov/info/basics/basics4.asp)

1) Combien de types de CSA existe-il et dans quels tissus ?
2) Comment évoluent les CSA pendant le développement et comment maintiennent-elles leur l'état indifférencié chez adulte ? Sont-elles le reste de la puissance de CSE ou dérivées d'autres mécanismes ?
3) Comment les CS maintiennent-elles leur état indifférencié au sein d'une population cellulaire différenciée? Quelles sont les caractéristiques de la niche qui contrôlent le comportement des CSA ?
4) Est-ce que toutes les CS ont la capacité de transdifférenciation? Est-il possible de contrôler efficacement ce processus toute en sécurité ?
5) Si les bénéfices de la transplantation de CS ne sont que trophiques, quelles en sont les mécanismes ? Est-ce le contact seul entre les cellules donneuses et les cellules receveuses qui est nécessaire, ou est-ce seulement les substances biologiques secrétées par les cellules donneuses, ou bien les deux ?
6) Quels sont les facteurs qui contrôlent la prolifération et la différenciation des CS?
7) Quels sont les facteurs qui stimulent la repopulation par les CS d'un tissu endommagé, et comment intervenir pour améliorer ce processus ?

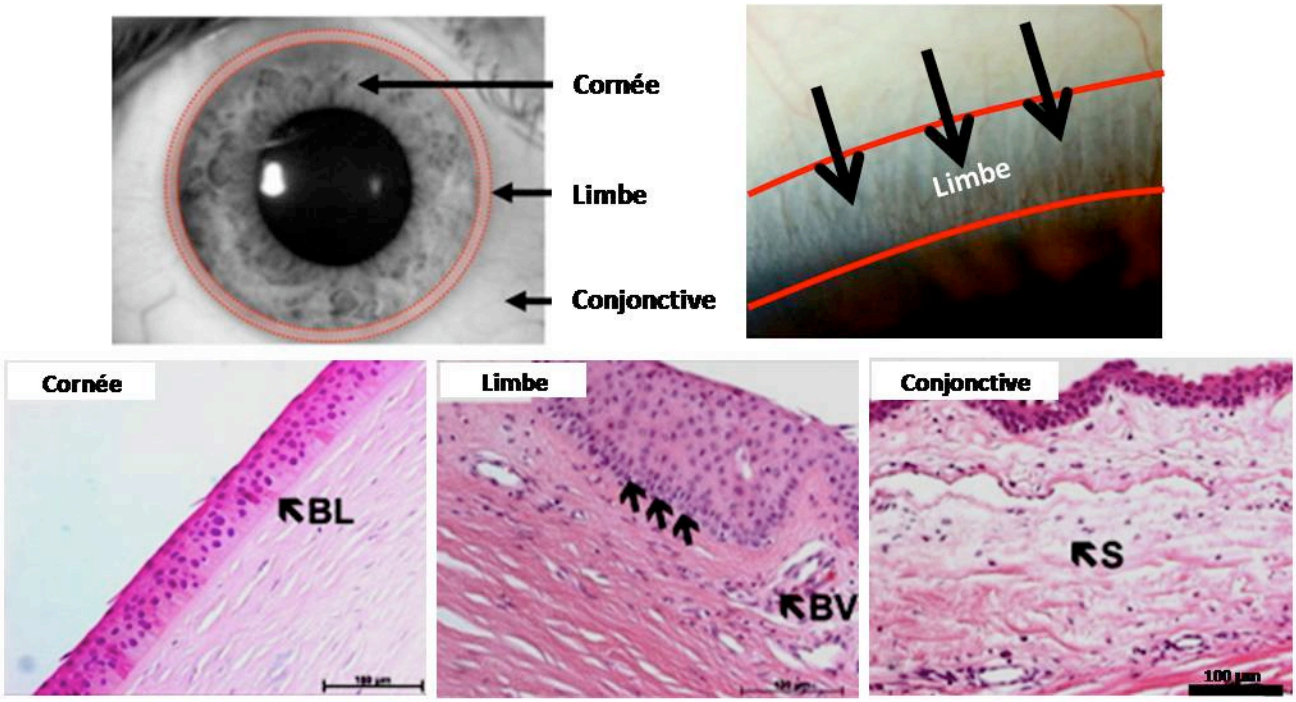


Figure 35. Organisation anatomique et histologique de l'épithélium cornéen, limbique et conjonctival de la surface oculaire (D'après Ahmad et al., 2010)

	Epithélium limbique		Epithélium cornéne	
	Basal	Supra-basal	Basal	Supra-basal
Marqueurs de CS limbiques				
P63α	++	-	-	-
ABCG2	+++	+/-	-	-
Intégrine α9	+++	+/-	-	-
Vimentine	+++	+/-	-	-
CK 19	++	-	-	-
N-cadhérine	++	-	-	-
Marqueurs des cellules différenciées				
CK3/CK12	-	+++	+++	+++
Connexine 43	-	+++	+	+++
Involucrine	-	+++	+	+++
NGFR ($p75^{NTR}$)	-	+++	+++	+++
E-cadhérine	+/-	+++	+++	+++

Tableau 6. Distinction de CS limbiques par la présence de certains marqueurs de CS et l'absence de certains marqueurs de cellules différenciées (D'après Secker et Daniels, 2008)

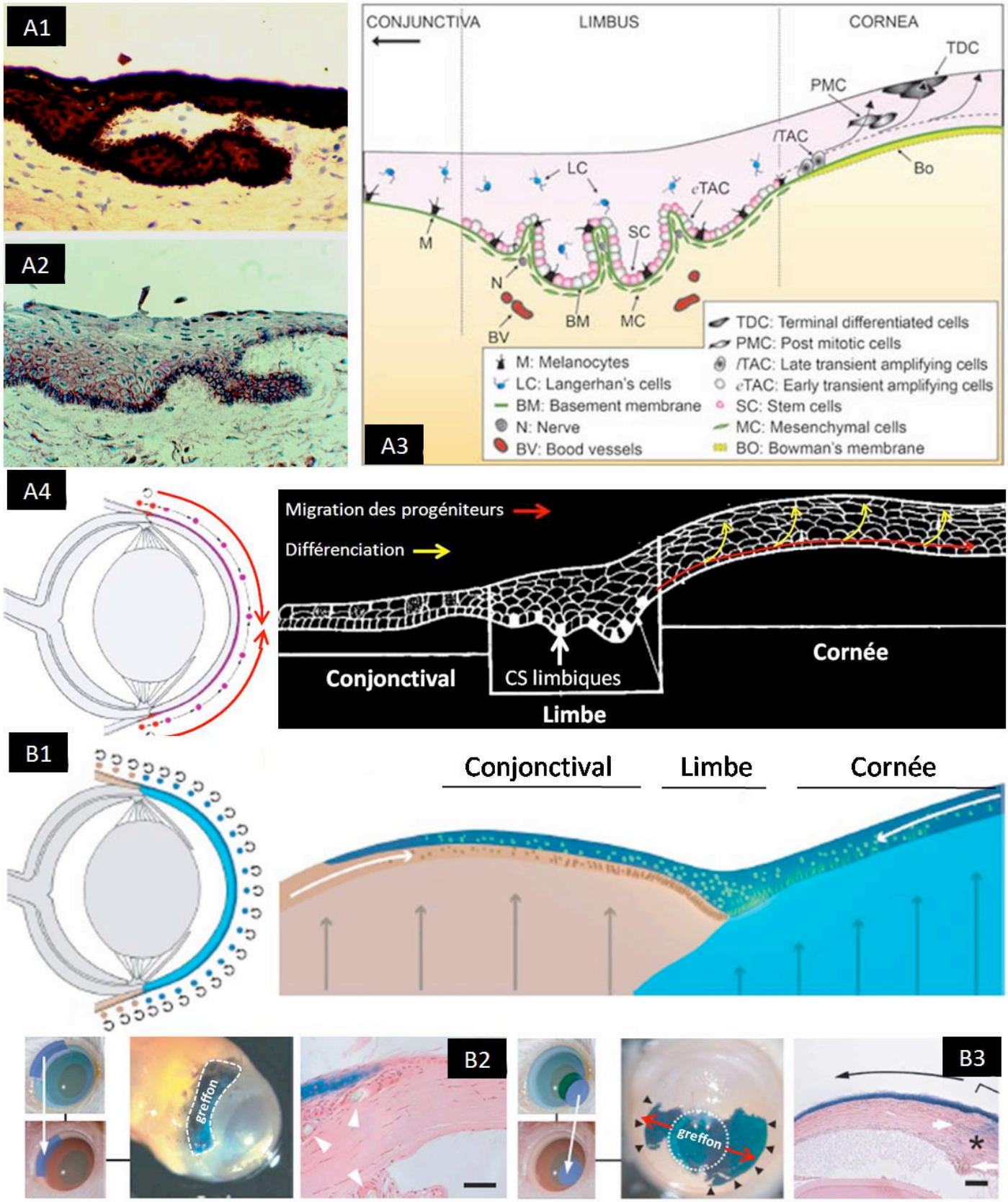


Figure 36. Deux modèles complètement différents de la régénération de l'épithélium cornéen. A1 et A2 montrent que la niche de CS limbiques se trouvent dans les cryptes limbiques **(D'après Dua et al., 2005)**: A1: l'expression ubiquitaire de CK14 indique la nature des cellules dans les cryptes sont épithéliale cornéenne; A2: seules une partie de cellules basales dans les cryptes montrent ABCG2 positif. A3: la figure explicative de la niche de CS limbique et les microenvironnements à l'entourage. **(D'après Li et al., 2007)** A4: le modèle classique de la régénération de l'épithélium cornéen par la niche de CS limbiques. B1: le modèle de défi contredit à celui classique évoque l'existence de CS à toute la surface de l'œil et la direction de la migration des CS est du centre de l'épithélium vers limbe. Les deux petites photos à gauche de B2 et B3 montre que les greffons (cornées bleues) chez lesquels les cellules expriment toutes exogène β-galactosidase est transplanté sur des cornées normales (cornées rouges). 4 mois après la greffe, les cellules limbiques ne migrent pas du tout vers le centre de la cornée (B2), au contraire, les cellules du centre cornéen migrent (indiqué par flèches rouges) vers limbe (B3). **(D'après Majo et al.,2008)**

III) Modèle épithélial cornéen : de l'identification des CS à l'utilisation en clinique humaine

III-1) Rappel de l'anatomie de la surface occulaire

La surface occulaire est couverte par l'épithélium cornéen, limbique et conjonctival. L'épithélium cornéen recouvre entièrement la surface de la cornée, tissu non vascularisé, jusqu'à la limite du limbe. Le limbe mesurant de 1,5 à 2 mm de largeur est situé aux limites de la cornée et forme ainsi une barrière entre l'épithélium cornéen et conjonctival **(Fig. 35)**[Ahmad 2010].

L'épithélium cornéen, transparent, est formé de 5 à 7 couches de cellules. Il constitue une barrière étanche par ses jonctions intercellulaires et sa surface parfaitement lisse. La couche basale formée de cellules cubiques repose sur la membrane de Bowman attachée au stroma cornéen avasculaire. L'épithélium cornéen se renouvelle rapidement, son cycle de vie est compris entre 19 et 35 jours. Ce renouvellement prouve l'existence de CS spécifiques. L'épithélium cornéen joue un rôle de protection et facilite la dispersion du film lacrymal à la surface de la cornée.

L'épithélium conjonctival repose sur un chorion vascularisé, et contient des cellules caliciformes sécrétant un mucus qui constitue la couche profonde du film lacrymal. Il est en continuité avec l'épithélium cornéen par l'intermédiaire du limbe.

Le limbe constitue une zone de transition entre l'épithélium cornéen et conjonctival. L'épithélium limbique est composé de presque 10 couches de cellules dont la couche basale contient des CS. Le limbe remplit de multiples fonctions dont deux essentielles dans le maintien de l'intégrité de la surface cornéenne : il est le siège des CS spécifique qui régénèrent l'épithélium cornéen et représente une frontière non seulement anatomique mais également fonctionnelle entre la conjonctive et la cornée. La perte de son intégrité favorise alors l'envahissement cornéen par une prolifération d'origine conjonctivale et peut entraîner une baisse d'acuité visuelle s'expliquant par l'absence de transparence de l'épithélium conjonctival [Chen 1991].

III-2) Concepts de la régénération de l'épithélium cornéen par les CS limbiques

Les premières découvertes sur la régénération de l'épithélium cornéen par CS limbiques

En 1971, Davanger et Evensen ont été les premiers à suggérer que la régénération de l'épithélium cornéen est assurée par une population de cellules situées dans la zone limbique. Ils ont observé la migration centripète de cellules pimentées situées au limbe après lésion de l'épithélium central [Davanger 1971].

En 1986, Schermer et al. ont démontré que les cellules basales limbiques n'expriment pas de cytokératine K3, marqueur de la différenciation et exprimé universellement par toutes les cellules épithéliales cornéennes et les couches non basales des cellules limbiques [Schermer 1986]. Ceci suggère que les cellules basales situées au limbe seraient des CS.

En 1989, Cotsarelis et al ont découvert l'incorporation durable de H3-thymidine dans les cellules basales de l'épithélium limbique, suggérant leur long cycle cellulaire et ainsi leur statut de CS [Cotsarelis 1989]. Au cours de la même année, Kenyon et Tseng ont présenté des travaux portant sur le succès de plusieurs autogreffes limbiques réalisées en cas de troubles de la vision secondaires à diverses lésions à la surface oculaire [Kenyon 1989], confirmant à leur tour l'existence de CS limbique [Tseng 1989].

La niche des CS limbiques

Dans les années 80, la localisation des CS de l'épithélium était supposée dans la couche basale du limbe [Schermer 1986, Cotsarelis 1989, Kenyon 1989]. En 1991 leur localisation a été précisée dans les palissades de Vogt du limbe cornéo-scléral qui sont hautement pigmentées en raison de la présence de mélanocytes [Townsend 1991]. Mais la localisation précise de la niche dans le limbe n'a pas pu être mise en évidence à cause du manque de marqueurs spécifiques de CS limbiques jusqu'à 2005 où Dua et al. ont précisé pour la première fois la localisation anatomique de la niche et l'ont nommée « cryptes épithéliales limbiques » en utilisant un marqueur spécifique à toutes les cellules épithéliales cornéennes : CK14 **(Fig. 36A1)** [Dua 2005] et un marqueur de CS : ABCG2/BCRP **(Fig. 36A2)**[Dua 2005]. Dans la même année, une équipe américaine a non seulement confirmé la

localisation de la niche de CS limbiques avec le marqueur ABCG2, mais aussi caractérisé in vitro les cellules situées dans la niche [de Paiva 2005].

L'état indifférencié de CS nécessite le support de microenvironnements spécifiques de la niche qui sont définis par la nutrition abondante (vaisseaux sanguins), les matrices extracellulaire spécifiques et aussi des cellules spécifiques pour maintenir les caractéristiques des CS [Walker 2009]. A la différence de l'épithélium cornéen, la membrane basale des cryptes épithéliales limbiques est fenêtrée et ondulée avec des papilles du stroma s'étendant vers le haut [Shanmuganathan 2007]. Cette structure anatomique fournit non seulement une grande surface dans un petite espace, mais permet aussi aux cellules basales des cryptes une communication directe avec les cellules et les structures stromales au dessous du limbe, notamment les petits vaisseaux sanguins qui fournissent les différents facteurs de croissance et les nutriments. De plus, la membrane basale des cryptes limbiques peut séquestrer et ainsi moduler certains facteurs de croissance tels que les cytokines qui sont impliquées dans le contrôle de la fonction de CS limbiques [Klenkler 2004]. Les CS au sein de leur niche expriment l'intégrine α-9 et la N-cadhérine mais pas la connexine 43 qui est un marqueur de différenciation [Stepp 1995, Matic 1997, Hayashi 2007]. Par définition, les cellules participant au maintien de la niche doivent fournir un abri pour protéger les CS des différentes stimulations et stress cellulaire, tel que la prolifération, la différenciation et l'apoptose [Moore 2006]. Ainsi, les mélanocytes dendritiques situés dans la couche basale du limbe pourraient être les premières cellules de maintien de part leur capacité de protection contre l'irradiation ultraviolette [Narisawa 1997, Higa 2005]. Les fibroblastes limbiques et les cellules épithéliales limbiques supra-basales peuvent exprimer IL-6, composant effectif dans le maintien de l'état indifférencié des CS limbiques [Notara 2010].

Les études de la niche de CS limbiques sont encore loin d'être achevées. L'identification précise des CS limbiques sans les confondre avec les cellules progénitrices et les éléments du microenvironnement de la niche pourtant cruciaux dans le maintien de l'état indifférencié des CS, ne sont pas encore découverts. La **Figure 36A3** [Li 2007] schématise la niche des CS limbiques.

Les marqueurs des CS limbiques

Plusieurs marqueurs ont été proposés pour les CS la niche de CS limbiques **(Tableau 6)** [Secker 2008] notamment p63α qui a une meilleure spécificité par rapport à l'ABCG2 [de Paiva 2005, Krishnan 2010]. Cependant, l'activation de l'expression de p63α au sein des cellules basales de l'épithélium cornéen après stimulation du système de réparation dans les suites d'une lésion épithéliale a été démontrée [Di Iorio 2005]. Ceci prouve que ce marqueur bien connu pour sa spécificité aux CS limbiques est non seulement spécifique aux CS, mais aussi aux progéniteurs dérivés de CS limbiques, car seuls les progéniteurs peuvent quitter la niche et migrer vers le tissu endommagé. Le concept de CS limbiques nécessite encore des preuves définitives, car, à l'heure actuelle, il n'existe aucun marqueur moléculaire bien défini et limité exclusivement aux CS [Schlotzer-Schrehardt 2005].

La controverse

Le modèle de régénération par la migration centripète de cellules d'amplification transitoire sur la couche basale de l'épithélium cornéen et par la différenciation progressive de ces cellules immatures situées aux couches basales en se déplaçant vers la couche supérieure a été hypothétiquement posé en 1983 [Thoft 1983]. Dans les années suivantes, la découverte de la niche des CS limbiques et ses applications cliniques ont presque confirmées cette hypothèse **(Fig. 36A4).**

Cependant, deux études ont contribué à rendre le modèle plus complexe. En 2008, Majo et al. ont publié Nature que chez la souris des CS oligopotentes existent de façon diffuse dans la couche basale de l'épithélium de la surface de l'œil, mais aussi au niveau du limbe. La régénération de l'épithélium cornéen dans les conditions physiologiques est due au renouvellement et à la division asymétrique des CS au sein de l'épithélium cornéen. L'accumulation des CS au niveau du limbe est due à la migration des CS cornéennes et conjonctivales en sens opposés **(Fig. 36B1, -B2 et -B3)** [Majo 2008]. Dans une autre étude concernant l'épithélium cornéen humain ex vivo, une équipe de Nouvelle Zélande a pu montrer que l'épithélium cornéen central est capable de régénérer de façon autonome et initiale une lésion dans la zone intermédiaire (3 à 7 mm de diamètre) à la surface de la cornée. Les CS limbiques ne participent au processus de réparation que 12h après la lésion [Chang 2008].

En 2010, une équipe américaine a défendu dans « Nature » le modèle « classique » de régénération de l'épithélium cornéen [Sun 2010] en citant des preuves cliniques et biologiques contredisant l'hypothèse de Majo. Ce débat sur le modèle de régénération de l'épithélium cornéen et de la localisation des CS à la surface oculaire n'est pas encore conclu définitivement. Ceci montre que les connaissances actuelles sur la CS et sur les éléments qui maintiennent l'état indifférenciée des CS restent encore très limitées.

III-3) Insuffisance en CS limbiques (ICSL) : causes, conséquences et diagnostic

Une destruction sévère du limbe et donc de ses CS peut se rencontrer au cours de diverses situations pathologiques [Puangsricharern 1995, Tsubota 1995]. L'étiologie la plus fréquente de l'ICSL est représentée par les brûlures chimiques de la surface oculaire. Le syndrome de Steven-Johnson, des traumatismes chirurgicaux répétés (en particulier l'ablation itérative d'une néoplasie intra-épithéliale cornéenne étendue…) sont des causes iatrogènes possibles. La pemphigoide cicatricielle est une cause auto-immune non exceptionnelle. Les causes congénitales (aniridie, kératodermie congénitale…) sont rares.

L'absence ou le dysfonctionnement des CS limbiques entraîne une incapacité de régénération de l'épithélium cornéen. Il en résulte un envahissement progressif de la surface cornéenne par le tissu conjonctival accompagné d'une néo-vascularisation et d'une baisse d'acuité visuelle allant jusqu'à la cécité.

Les signes objectifs d'une ICSL ne sont pas spécifiques. Le signe le plus évocateur est la conjonctivalisation de la surface cornéenne dont le diagnostic de certitude repose sur les examens cytologiques et d'IHC. Le premier signe d'ICSL serait la disparition des palissades de Vogt [Friedman 1981]. Une néo-vascularisation cornéenne est généralement visible. Un marquage diffus de l'épithélium cornéen par la fluorescéine en dehors des zones de kératite est aussi un signe précoce de dysplasie épithéliale [Huang 1989]. L'absence de marqueurs spécifiques de l'épithélium cornéen tels que l'AE5, la K3, la K12 et la présence du marqueur spécifique de l'épithélium conjonctival (K19) sur une biopsie de la surface cornéenne ou sur une empreinte cornéenne permettent un diagnostic de certitude [Pellegrini 1999].

III-4) Traitement de l'ICSL par greffe de CS/ progénitrices limbiques

La réalisation d'une kératoplastie transfixiante a été longtemps la seule possibilité pour rendre transitoirement aux malades atteints d'ICSL une acuité visuelle. Cependant, les risques de rejet sont considérablement majorés par l'inflammation de la surface oculaire et le tissu fibrovasculaire de nature conjonctival recouvre rapidement le greffon. Ces greffes sont vouées à l'échec.

La restauration de la transparence cornéenne peut se faire de manière moins agressive et durable par la greffe d'une partie du limbe apportant des CS limbiques, avec de bons résultats [Kenyon 1989]. On distingue 2 types de greffes :

-**Autogreffe :** Dans le cas de l'ICSL unilatéral, l'autogreffe peut apporter du tissu limbique de l'œil sain à l'œil malade [Kenyon 1989, Copeland 1990, Holland 1996]. Cependant, si la surface du limbe prélevé sur l'œil sain est trop importante, il existe un risque de défaillance en CS sur l'œil sain [Jenkins 1993].

-**Allogreffe :** Dans les cas d'ICSL bilatérale, une allogreffe peut être réalisée à partir d'un œil d'un donneur [Coster 1995]. De bons résultats ont été rapportés [Henderson 2001] mais les risques de rejet constituent un frein à cette technique [Turgeon 1990]. Le limbe est une région vascularisée, riche en cellules immunocompétentes. La confrontation des cellules Langerhans de l'œil du receveur avec celles du greffon favorise le rejet [Thoft 1993]. Le patient doit donc recevoir des immunosuppresseurs à long terme et le taux de succès à long terme (3 à 5 ans) est d'environ 50% [Tseng 2006].

III-5) Reconstitution in vitro du microenvironnement spécifique du limbe et thérapies cellulaires dérivées

A) Microenvironnements spécifique du limbe

La plupart des CSA sont multipotentes. La voie de différenciation ou de transdifférenciation d'une CS en cellule mature dépend de deux éléments : de la génétique intrinsèque de la cellule et du microenvironnement au sein duquel elle se situe. Une intervention au niveau génétique reste actuellement délicate car de nombreux effets délétères pourraient être envisagés, notamment l'apparition de mutations cancérigènes. Le microenvironnement cellulaire influence indirectement la programmation génétique des cellules. De

nombreuses expériences ont montré qu'une action à ce niveau est plus sécurisée et plus facile à mettre en œuvre de façon contrôlée. Des applications cliniques ont d'ailleurs déjà été décrites.

Cependant, le terme de microenvironnement reste vaste et tous les éléments situés en périphérie cellulaire font partir de ce microenvironnement : le contact direct entre la matrice extracellulaire et les cellules avoisinantes, les différentes substances biologiques telles que les facteurs de croissance, cytokines, hormones etc., les éléments nutritifs, ainsi que les différents éléments physiques tels que la teneur en oxygène, le pH, la température, la concentration des ions etc.

La notion de microenvironnement est primordiale dans la maintenance du caractère souche des CS, mais aussi dans la différenciation d'une CS en cellule mature. La reconstitution in vitro d'un épithélium cornéen à partir de CSA nécessite la présence d'un microenvironnement adapté afin de garantir aux CS leur caractère souche et la différenciation des progéniteurs en cellules épithéliales cornéennes.

En s'appuyant sur l'organisation cellulaire en couches des différents stades de différenciation des cellules de l'épithélium cornéen in vivo, il est envisagé de reconstituer in vitro et in vivo un nouvel épithélium durable et fonctionnel à partir de CS de différentes origines.

A-1) Microenvironnements favorisant le maintien du caractère de CS (stemcellness)

A-1.1) Co-culture avec des cellules embryonnaires ou amniotiques inactivées

-Co-culture avec des fibroblastes 3T3 : La co-culture de CS utilisant des fibroblastes 3T3 inactivés est souvent employée dans la reconstitution d'un épithélium cornéen in vitro. Les fibroblastes 3T3 sont des cellules primitives isolées au sein d'un embryon de souris. Ils ont une très grande capacité de prolifération. Leur prolifération doit être inhibée soit par un traitement à la mitomycine C, soit par irradiation afin que l'épithélium cornéen puisse avoir une prolifération modérée pendant la co-culture [Rheinwald 1975]. Les fibroblastes 3T3 inactivés secrètent des facteurs de croissance et des constituants de la matrice extracellulaire qui favorisent le maintien à l'état indifférencié et la croissance des CS in vitro [Wolf 2011]. La co-culture avec ces cellules inactivées dans un milieu sans sérum donne non seulement un effet prolifératif, mais aussi un effet anti-apoptotique aux CS limbiques [Tseng 1996]. De nombreuses études utilisent cette méthode de co-culture, notamment pour pouvoir garder des cellules indifférenciées dans l'épithélium reconstitué afin d'obtenir un greffon épithélial efficace à long terme après greffe [Koizumi 2001b, a, Nakamura 2003b, Nakamura 2004b, Nakamura 2006].

-Co-culture avec des cellules épithéliales amniotiques : Les cellules épithéliales amniotiques favorisent in vitro l'expansion des CS limbiques. La co-culture avec ces cellules inactivées par mitomycine C est plus efficace en terme de croissance clonale de CS limbiques par rapport aux fibroblastes 3T3 [Chen 2007].

A-1.2) Utilisation de membranes basales de la matrice extracellulaire

-Membrane amniotique : la membrane amniotique comporte une monocouche de cellules adhérentes à une membrane basale sur une matrice stromale avasculaire. Elle mesure de 20 à 500µm d'épaisseur. Cette membrane sans cellules amniotiques est utilisée comme support et substrat à la reconstitution de l'épithélium cornéen in vitro à partir de CS. Cette membrane favorise la croissance de l'épithélium et aide les CS à maintenir leur caractère souche in vitro [Grueterich 2003, Shortt 2009]. De plus, elle possède des propriétés anti-inflammatoire et anti-angiogénique [Tseng 1999, Hao 2000] qui sont très avantageuses pour la transplantation en raison de leurs effets sur la diminution de la réaction immunitaire pourvoyeuse de rejet de greffe. La méthode de transplantation d'un épithélium cornéen reconstitué utilisant la membrane a effectivement montré une amélioration significative de la transparence cornéenne et de la stabilité de la surface oculaire dans les cas d'auto et d'allogreffe avec des résultats meilleurs que la transplantation utilisant des membranes synthétiques comme la fibrine [Tseng 1998, Tsai 2000, Shortt 2008]. L'utilisation d'une membrane amniotique intacte montre un meilleur résultat dans le maintien du caractère souche des CS limbiques par rapport à l'utilisation d'une membrane dénudée [Sudha 2008, Krishnan 2010], mais les cellules amniotiques sont immunogènes et favorisent éventuellement un rejet.

-Membrane de fibrine : cette membrane est également utilisée comme support à l'épithélium reconstitué in vitro. Son utilisation a donné de bons résultats cliniques dans le domaine des greffes car elle possède une capacité dans le maintien du caractère souche des CS limbiques et est dégradable in vivo [Rama 2001, Marchini 2011, Nassiri 2011, Sonmez 2011].

-Membrane de laminine-5 : le collagène IV, la laminine-1, la laminie-5 et la fibronectine sont des composants majeurs de la membrane basale de l'épithélium cornéen et du limbe [Schlotzer-Schrehardt 2007, Blazejewska 2009] ont comparé les effets de ces 4 matrices extracellulaires sur la transdifférenciation des CS des follicules pileux en progéniteurs épithéliaux cornéens. Seule la membrane de laminine-5 a montré son efficacité dans l'adhésion et la prolifération de ces CS. De plus, combiné avec la co-culture en présence de des fibroblastes limbiques, les CS des follicules pileux ont réussi à se transdifférencier en progéniteurs épithéliaux cornéens [Blazejewska 2009]. La laminie-5 est aussi un composant de la membrane amniotique. Quand les CS limbiques sont transférées sur la membrane amniotique, elles libèrent de la métalloprotéase-9 (MMP-9) activée qui va alors dégrader la laminine-5. La dégradation de laminine-5 produit des fragments de la chaine-2 de laminine 5 qui favorisent la mobilité des CS somatiques et la prolifération de l'épithélium [Cheng 2009].

A-1.3) Milieux de culture

-Milieu de culture sans sérum : un milieu de culture sans sérum supplémenté en EGF et/ou en bFGF favorise l'expansion des CS/progénitrices en inhibant la prolifération et la survie des cellules différenciées. Cette méthode est utilisée dans les cultures de microsphères dont le but est d'isoler des CS/progénitrices in vitro [Reynolds 1992, Sukach 2007]. Elle constitue aussi un moyen de maintien le caractère souche des CS limbiques et de favoriser la croissance des cellules progénitrices épithéliales [Kruse 1991b, Yang 2008, Yokoo 2008, Lekhanont 2009]. Certaines substances biologiques du sérum, telles l'acide rétinoïque (vitamine A) et le TGF-beta stimulent la conversion des CS limbiques en cellules d'amplification transitoires, augmente légèrement le nombre et la taille des colonies clonales formées au sein de la culture des CS limbiques et favorise la perte du caractère souche. Par contre, ces substances inhibent la prolifération rapide des cellules progénitrices situées au sein de l'épithélium cornéen et diminue de façon importante le nombre et la taille des colonies clonales formées pendant la culture des progéniteurs épithéliaux [Kruse 1993b, a, Kruse 1994a, Kruse 1994b]. L'utilisation de sérum semble donc plus favorable à l'expansion des progéniteurs épithéliaux cornéens que des CS limbiques. La combinaison milieu sans sérum et co-culture avec des fibroblastes 3T3 inactivés donne non seulement un effet expansif, mais aussi un effet anti-apoptotique aux CS limbiques [Kruse 1991a, 1993a, Tseng 1996]. La combinaison milieu sans sérum et co-culture avec des fibroblastes limbiques constitue in vitro un microenvironnement plus proche de celui de la niche des CS limbiques et favorise in vitro le maintien du caractère souche des CS limbiques [Notara 2010]. Un milieu sans sérum est aussi utilisé pour la préparation du greffon épithélial à partir de CS d'autres origines [Yang 2008].

-Milieu de culture à faible concentration en Ca2+: une concentration extracellulaire basse en Ca2+ (≤0,4mM) favorise le maintien à l'état indifférencié des CS/progénitrices limbiques [Kawakita 2008, Meyer-Blazejewska 2010].

A-2) Microenvironnements assurant la différenciation spécifique

A-2.1) Co-culture avec les cellules impliquées dans le microenvironnement limbique ou épithélial cornéen

Divers types cellulaires situés au sein du limbe et du stroma cornéen sont utilisés pour la co-culture avec des CS autres que les CS limbiques afin de favoriser leur transdifférenciation en progéniteurs épithéliaux cornéens.

-Fibroblastes stromaux du limbe : afin, d'obtenir des microenvironnements optimaux pour la transdifférenciation des CS des follicules pileux en progéniteurs épithéliaux cornéens, Blazejewska et al. ont essayé la co-culture de ces CS avec différentes fibroblastes susceptibles de participer au microenvironnement favorisant la différenciation des CS limbiques vers des cellules épithéliales cornéennes [Blazejewska 2009]. Dans cette étude de l'induction de la transdifférenciation, les fibroblastes situés dans la partie centrale et périphérique du stroma cornéen sur le versant épithélial et les fibroblastes stromaux du limbe ont été isolés et étudiés en comparaison avec les fibroblastes 3T3. Parmi ces 4 types de fibroblastes, seuls ceux du limbe ont induit une transdifférenciation efficace. Cette étude a été effectuée chez la souris. L'induction d'une transdifférenciation en cellules épithéliales cornéennes à partir de CS mésenchymateuses de la moelle osseuse chez le lapin a été réussie en co-culture avec des fibroblastes du limbe [Gu 2009]. L'utilisation de ce milieu de culture pré-incubé avec ces cellules à la surface oculaire de l'œil après grattage de l'épithélium cornéen et limbique, favorise la repopulation de l'épithélium cornéen et inhibe l'envahissement par l'épithélium conjonctival [Amirjamshidi 2011]. Les

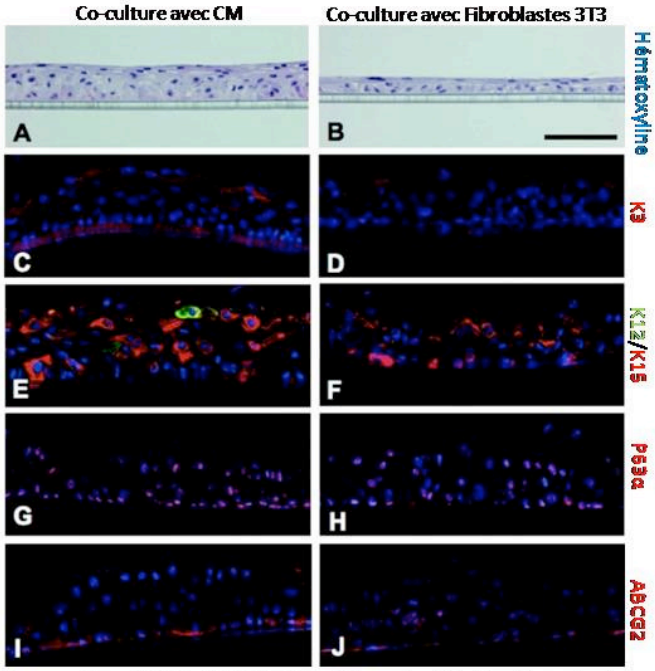


Figure 37. PMID 19136703. Effet de l'induction de la différenciation des CS limbiques en cellules épithéliales cornéennes par la co-culture avec les cellules mésenchymateuse de la moelle osseuse, mais non avec les fibroblastes 3T3. A et B: l'histologie de l'épithélium cornéen reconstitué in vitro . C à J: expression des différents marqueurs examinée par l'IHC. K3, K12, K15 sont des marqueurs des cellules épithéliales cornéennes; P63 α et ABCG2 sont les marqueurs de cellules souches limbiques. **(D'après Omoto et al., 2009)**

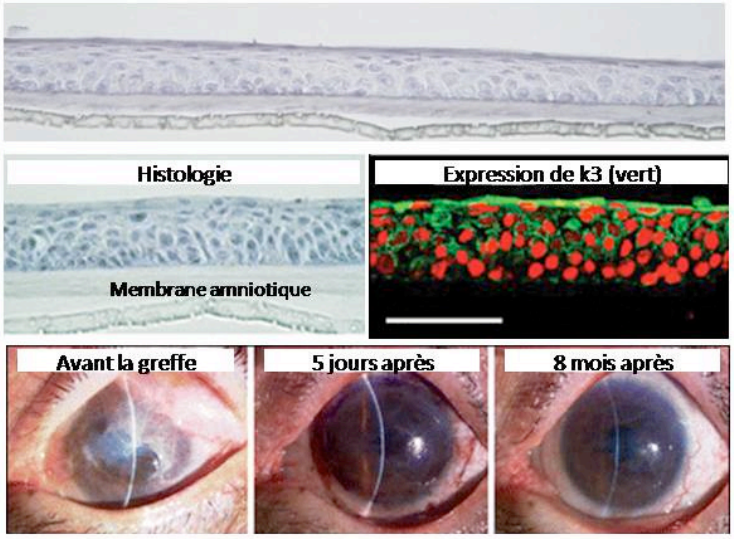


Figure 38. Thérapie à partir des cellules souches de l'épithélium de la muqueuse buccale. Les trois photos en haut montrent que l'épithélium reconstitué in vitro sur la membrane amniotique à partir d'un prélèvement de cellules épithéliales de la muqueuse buccale est morphologiquement similaire à l'épithélium cornéen normal et exprime le marqueur de cellules épithéliales cornéennes : K3. Les trois photos en bas montrent les résultats cliniques avant et après la transplantation d'une diamètre de 19mm de cet épithélium similaire avec la membrane amniotique chez un patient atteints de l'ICSL. **(D'après Nalamura et al., 2004 et 2006)**

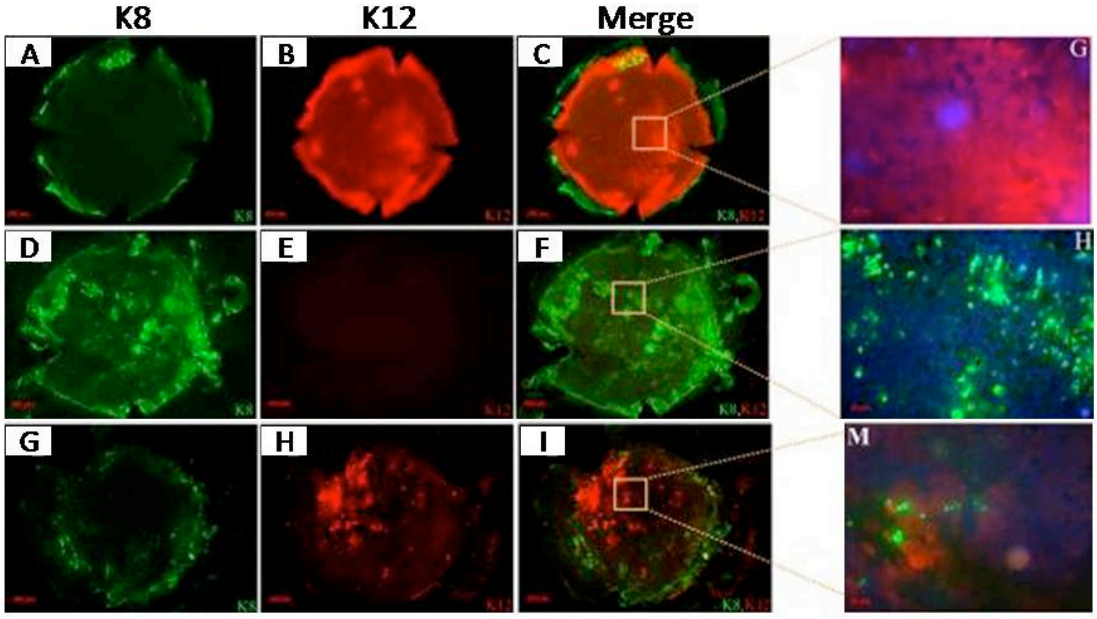


Figure 39. Induction in situ de la transdifférenciation des cellules souches de l'épithélium conjonctival en épithélium cornéen en cas d'ICSL. Epithélium cornéen normal (A,B,C). Envahissement de l'épithélium conjonctival en cas d'ICSL sans aucun traitement (D,E,F). Transdifférenciation partielle en épithélium cornéen en cas d'ICSL après l'application locale du surnageant de la culture des fibroblastes limbiques humains (G,H,I) : mise en évidence en utilisant le marqueur spécifique de l'épithélium cornéen K12 et celui de l'épithélium conjonctival : K8. **(D'après Amiriamshidi et al., 2011)**

auteurs suggèrent que les fibroblastes limbiques sont des acteurs essentiels dans le microenvironnement de la niche des CS limbiques et assurent la différenciation spécifique de l'épithélium cornéen.

-Cellules mésenchymateuses : les CS mésenchymateuse de la moelle osseuse expriment in vitro les facteurs de croissance des kératocytes et des hépatocytes, facteurs indispensables à la prolifération de cellules épithéliales, et aussi la N-cadhérine qui joue un rôle important dans le maintien du caractère souche. L'épithélium reconstitué à partir des CS limbiques en co-culture avec ces cellules souches mésenchymateuses est de meilleure qualité que celui obtenu par co-culture avec des fibroblastes 3T3 inactivés. Les cellules apicales sont mieux différenciées et les cellules basales gardent leur état indifférencié (**Fig. 37)** [Omoto 2009]. La co-culture avec des cellules mésenchymateuse provenant du limbe est aussi efficace dans le maintien du caractère souche des CS limbiques que la co-culture avec des fibroblastes 3T3 inactivés. Les cellules basales de l'épithélium reconstitué in vitro sur une membrane amniotique dénudée sont indifférenciées comme le prouve l'absence d'expression de CK3 et l'expression forte de p63 [Zhang 2010]. Par ailleurs, la co-culture avec des cellules mésenchymateuses du limbe paraît plus acceptable par les autorités de santé que celle utilisant des fibroblastes murins 3T3 inactivés.

-Fibroblastes cornéens: En co-culture avec ces fibroblastes, les cellules mésenchymateuses de la moelle osseuse ont réussi à se transdifférencier in vitro en cellules similaires aux cellules épithéliales cornéennes distinguées par leur morphologie cellulaire et l'expression du marqueur spécifique CK12 [Jiang 2010].

A-2.2) « Airlifting »

L'épithélium cornéen reconstitué est posé sur un insert de culture. En contrôlant le volume du milieu de culture, la surface de l'épithélium est maintenue à l'interface entre le liquide et l'air pendant 5 à 7 jours. Cette méthode simple induit efficacement une maturation des cellules des couches superficielles et une stratification de l'épithélium reconstitué in vitro à partir de CS de différentes origines [Nakamura 2004a, Omoto 2009, Jiang 2010, Levis 2010, Meyer-Blazejewska 2010].

A-2.3) Concentration extracellulaire élevée en Ca^{2+}

Le Ca^{2+} joue un rôle important dans l'initiation de la différenciation des CS limbiques en cellules épithéliales cornéennes [Sun 2000]. Contrairement à une concentration basse en Ca^{2+} (≤0,4mM), une concentration élevée (1,2mM) en Ca^{2+} favorise la différenciation des CS/progénitrices limbiques et d'autre origines en cellules épithéliales cornéennes matures [Ang 2010, Meyer-Blazejewska 2010]. L'augmentation de l'expression des canaux chlorure calcium-dépendant pendant la stratification épithéliale montre le rôle du Ca^{2+}. Les canaux chlorure calcium-dépendant donc joue un rôle dans la différenciation des CS limbiques en cellules épithéliales cornéennes [Connon 2006]. Cette méthode est aussi utilisée dans l'induction de la différenciation.

B) Thérapies cellulaires dérivées

B-1) A partir des CS limbiques isolées

Seule une petite biopsie de limbe d'environ 1 à 2 mm^2 est prélevée et cultivée in vitro dans un microenvironnement limbique spécifique afin de reconstituer un épithélium cornéen in vitro. Cet épithélium reconstitué déposé sur une membrane servant de support est ensuite transplanté à la surface de la cornée et du limbe (diamètre de 15 à 19 mm) après avoir retiré chirurgicalement le tissu conjonctival.

-**CS limbiques autologues :** en cas d'ICSL unilatérale, afin de ne pas prendre le risque d'introduire une ICSL sur l'œil sain secondaire à l'exérèse d'un greffon limbique trop important, l'autogreffe limbique peut être remplacée par un processus de thérapie cellulaire utilisant des CS limbiques ne nécessitant qu'un petite biopsie de tissu limbique de 1 mm^2 [Pellegrini 1997]. Les CS limbiques sont alors cultivées au sein de microenvironnements afin de favoriser leur croissance (co-culture avec des fibroblastes 3T3 sur une membrane amniotique ou de fibrine). Ces cellules cultivées sont ensuite transférées directement ou après induction du processus de différenciation par un processus de stratification in vitro en l'épithélium cornéen (processus d' « airlifting »

combiné à une concentration élevée en calcium dans le milieu) sur la surface cornéenne et limbique du patient [Rama 2001, Daniels 2007, Shortt 2007, Shortt 2010].

- CS limbiques hétérologues : dans le cas d'ICSL bilatérale, la culture des CS à partir d'un limbe donneur peut aussi donner de bons résultats à long terme, résultats similaires à ceux de la greffe autologue [Shortt 2007]. Mais l'observation de la disparition des CS du donneur après 9 mois chez le receveur rend l'utilisation de cette pratique controversée [Daya 2005]. Ce mécanisme reste encore mal élucidé. L'inconvénient de cette thérapie cellulaire est le risque de rejet et la nécessité d'un traitement immunodépresseur systémique.

B-2) A partir d'autres types de CSA autologues

Dans le cas d'ICSL bilatérale, la transdifférenciation d'autres types de CS autologues en cellules épithéliales cornéennes au sein d'un microenvironnement spécifique peut être envisagée afin d'éviter l'allogreffe de limbe ou de CS limbiques. Il a été démontré que différentes sources de CSA faciles à prélever possèdaient la capacité ou le potentiel de se transdifférencier en cellules épithéliales cornéennes.

-CS de l'épithélium de la muqueuse buccale (Fig. 38) [Nakamura 2004a, Nakamura 2006]**:** il s'agit d'une source alternative prometteuse pour la transplantation autologue car ces cellules peuvent être facilement prélevées sur le patient de façon non invasive. Ces cellules isolées de la muqueuse buccale sont co-cultivées avec des fibroblastes 3T3 inactivés sur une membrane amniotique dépourvue de cellules. Ces cellules forment in vitro une structure histologique similaire à l'épithélium cornéen, et expriment les marqueurs spécifiques de l'épithélium cornéen : cytokératine 3 et 12, les marqueurs impliqués dans la fonction de barrière : ZO-1, désmoplakine, connexine 43, les marqueurs des CS limbiques: P63, intégrine beta-1, et ont une activité télomérase [Kinoshita 2002, Hayashida 2005, Ang 2006]. Les premières greffes de cellules dérivées des cellules épithéliales de la muqueuse buccale ont été faites en 2003 chez le lapin [Nakamura 2003a], et les premières greffes chez des patients ont été rapportées en 2004 [Nakamura 2004a]. La stabilité et la transparence de la surface oculaire et la survie de l'épithélium transplanté peut atteindre plus de 34 mois chez la plupart (67%) des patients [Inatomi 2006]. Cette méthode a néanmoins encore besoin d'être optimisée. Elle pourrait être très avantageuse en raison de son immunocompatibilité afin de remplacer l'allogreffe limbique en cas d'ICSL bilatérale. De plus, une étude récente a montré que la reconstitution de l'épithélium cornéen à partir des CS de la muqueuse buccale ne présente pas de risque tumorigène [Thepot 2010].

-CS épidermiques : ces CS isolées à partir de la peau de l'oreille de chèvre ont été cultivées et amplifiées in vitro. Elles se transforment en épithélium cornéen sur une membrane amniotique dénudée sans nécessité de co-culture avec des fibroblastes 3T3. L'épithélium cornéen ainsi obtenu est transplanté sur la surface oculaire des yeux de chèvres porteuse d'ICSL. La restauration de la transparence cornéenne a été obtenue chez 80% des animaux. L'expression faible de certains marqueurs spécifiques aux cellules épidermiques (cytokératine 1 et 10) par l'épithélium reconstitué 6 mois après la transplantation montre que cet épithélium est effectivement dérivé de CS épidermiques [Yang 2008].

- CS du follicule pileux: L'expérimentation chez la souris montre que ces CS peuvent se transdifférencier in vitro en cellules épithéliales cornéennes quand elles sont cultivées au sein d'un microenvironnement limbique spécifique (co-culture avec des fibroblastes stromaux du limbe sur une membrane de laminine-5) [Blazejewska 2009]. En utilisant ces cellules, la restauration de l'épithélium cornéen dans un modèle d'ICSL murin a montré des résultats encourageants [Meyer-Blazejewska 2011]. Elles sont donc proposées comme CS potentielles pour le traitement de l'ICSL.

-CS mésenchymateuses : elles représentent une population cellulaire particulière qui possède un caractère multipotent et peut être isolée dans la moelle osseuse ou le muscle squelettique [Caplan 2005, 2007]. La transplantation systémique de ces cellules chez le lapin ayant subi une brûlure de sa surface oculaire a montré des meilleurs résultats par rapport au groupe contrôle dans la réparation de l'épithélium et dans la récupération de la transparence cornéenne [Ye 2006]. La reconstitution de l'épithélium cornéen à partir de CS mésenchymateuses a été réalisée in vivo et in vitro chez le lapin [Gu 2009], in vitro au sein d'un microenvironnement spécifique limbique chez le lapin [Gu 2009] et également chez le rat [Jiang 2010]. L'utilisation de cet épithélium cornéen reconstitué sur des animaux présentant une ICSL a été bénéfique.

-CS de la pulpe dentaire. Monterio et al. ont montré que ces cellules partagent certaines caractéristiques importantes avec les CS limbiques [Monteiro 2009]. Dans un modèle lapin d'ICSL, la transplantation de CS de la

pulpe dentaire a abouti à la reformation de l'épithélium cornéen et au rétablissement de la transparence cornéenne [Gomes 2010].

-CS de l'épithélium conjonctival. Après lésion de la surface oculaire induisant une ICSL, il existe un envahissement de la cornée par l'épithélium conjonctival aboutissant à une cécité. Cependant, les CS de l'épithélium conjonctival peuvent être utilisées pour reconstituer in vitro l'épithélium cornéen au sein d'un microenvironnement spécifique limbique. Les résultats de la transplantation d'un greffon reconstitué chez le lapin à partir de ces CS sont similaires que ceux obtenus à partir de CS limbiques [Ono 2007, Ang 2010].

Note : *Malgré beaucoup de travaux sur le microenvironnement spécifique visant à conserver le caractère souche des CS in vitro, l'épithélium cornéen reconstitué ne contient probablement pas de CS, mais des progéniteurs de cellules épithéliales cornéennes en raison de la perte du caractère souche au cours du processus de croissance des CS in vitro [Kawakita 2008]. Ceci constitue une des raisons principales pour lesquelles les greffons d'épithélium reconstitué, même à partir de CS autologues, ne survivent généralement que quelques années après la greffe.*

III-6) Reconstitution in vivo d'un microenvironnement spécifique du limbe et traitements dérivés

L'invasion de la surface cornéenne par de l'épithélium conjonctival au cours d'ICSL prouve l'existence de CS actives au sein de l'épithélium conjonctival. Ces CS ont démontré leur capacité de transdifférenciation en cellules épithéliales cornéennes in vitro en présence d'un microenvironnement spécifique limbique [Ono 2007, Ang 2010]. Cependant une question demeure : Peut-on induire une trans-différenciation directement in situ en reproduisant un microenvironnement spécifique? En 2011, Amirjamshidi et al. ont démontré la faisabilité de cette hypothèse sur un modèle murin d'ICSL. Le microenvironnement spécifique a été reproduit par une application locale d'un surnagent de culture de fibroblastes limbiques humains à la surface oculaire pendant 3 semaines et ceci 2 heures par jour. Les résultats ont montré que l'épithélium repeuplant la surface cornéenne avait partiellement les caractéristiques d'un épithélium cornéen. (**Fig. 39)** [Amirjamshidi 2011].

Des études portant sur l'optimisation du microenvironnement inducteur de la transdifférenciation des CS conjonctivales en cellules épithéliales sont essentielles pour mettre au point cette technique chez l'homme. A la différence des thérapies cellulaires utilisant des greffons d'épithélium cornéen reconstitué in vitro ne fournissant qu'une source de cellules progénitrices épuisable au cours du temps, cette méthode non invasive utilisant in situ une source « naturelle » et non épuisable de CS épithéliales conjonctivales autologue, pourrait être la thérapie idéale pour la restauration à long terme de l'épithélium cornéen en cas d'ICSL.

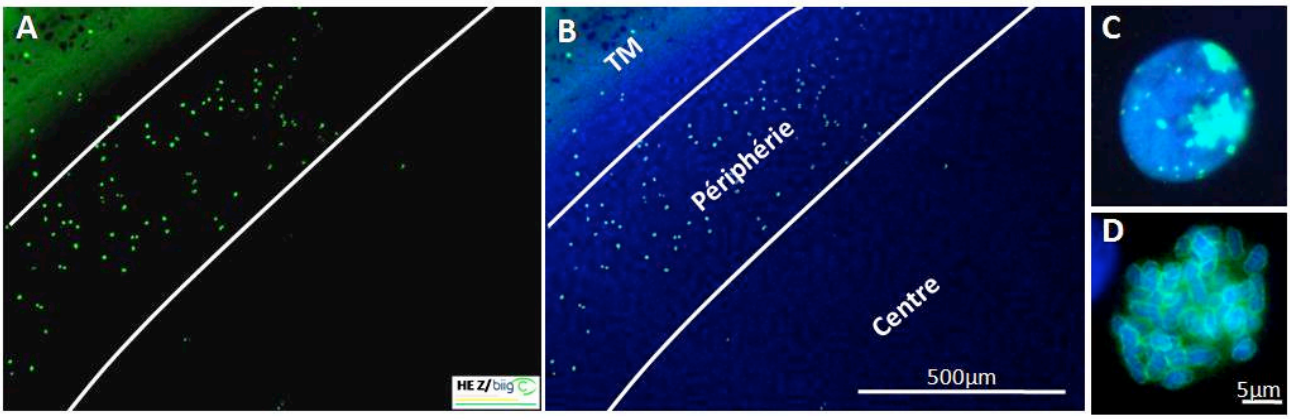


Figure 40. Capacité proliférative supérieure à la périphérie qu'au centre de l'endothélium cornéen humain. A) immunolocalisation de ki67 (vert) sur cornée montée à plat; B) merge avec la coloration des noyaux par Hoechst (bleu); c) localisation subcellulaire de Ki67 dans les nucléoles d'une CE avant la phase mitotique; D) localisation subcellulaire de ki67 au tour des chromosomes d'une CE pendant la phase mitotique. ***(Propre Observation de BiiGC/He et Thuret)***

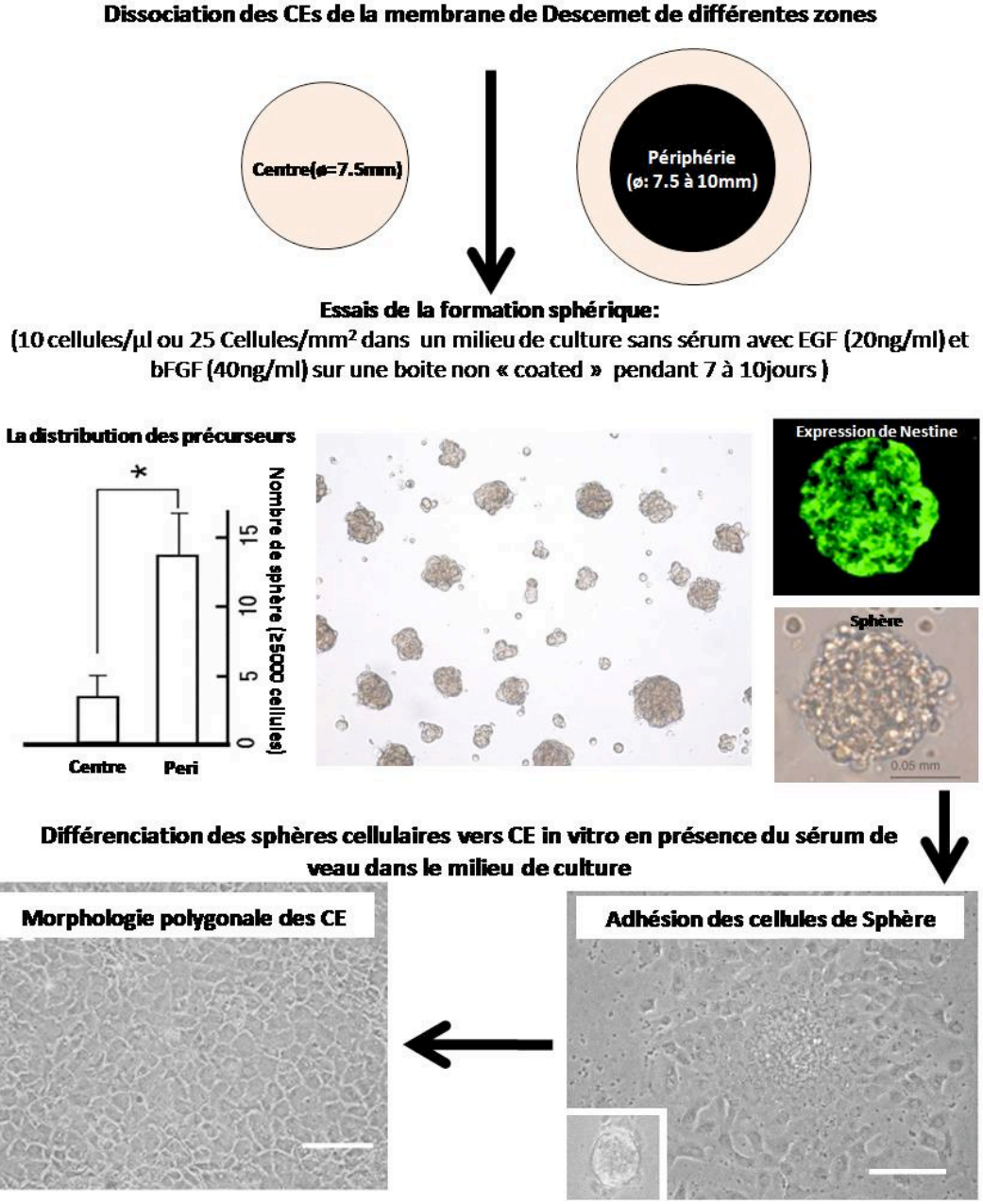


Figure 41. Distribution préférentielle des progéniteurs à la périphérie par rapport au centre de l'endothélium cornéen humain. Les CE sont isolées au centre et à la périphérie de la cornée humaine pour faire des essais de la formation sphérique afin de comparer le nombre de cellules souches/progénitrices sur le centre et la périphérie de l'endothélium par comptage du nombre de sphères formées en culture primaire. **(Adapté de Yokoo et al., 2005; Yamagami et al., 2007)**

IV) Etat actuel des connaissances et perspectives de recherche sur les CS endothéliales cornéennes

IV-1) Arguments suggérant le potentiel régénératif de l'endothélium cornéen chez l'homme

A) Hétérogénéité entre CE du centre et de la périphérie d'une cornée saine

Si l'endothélium cornéen était effectivement non régénératif, les CE d'une cornée saine devraient être homogènes sur toute la surface endothéliales puisque ces CE matures ont la même origine, se sont différenciées dans les mêmes conditions, ont subi les mêmes conditions physiologiques et les même stress au cours de la même période. Cependant, il existe des différences évidentes, expliquées ci-dessous, entre la périphérie et le centre de l'endothélium de cornées saines.

-**Morphologie nucléaire :** Les noyaux des CE centrales ont toujours une forme arrondie alors que ceux des CE la périphérie sont allongés **(Fig. 10)**. La taille des CE est toujours plus petite à la périphérie et la DCE logiquement plus élevée [Schimmelpfennig 1984, Daus 1989, Amann 2003].

-Capacité de prolifération : Les CE périphériques possèdent une plus grande capacité de prolifération ex vivo et in vitro que les CE centrales, même chez les sujets âgés [Bednarz 1998, Mimura 2006, Yamagami 2007]. En utilisant le marqueur de la prolifération, Ki67, notre laboratoire a observé, à partir des cornées provenant des donneurs âgés, une activité de la prolifération non négligeable de CE principalement sur la périphérie de l'endothélium après une période courte de 3 jours d'incubation de la cornée non lésée en OC à 31°C **(Fig. 40)**.

-Sénescence : Mimura et Joyce ont démontré que les CE centrales possèdent des caractéristiques de sénescence plus prononcées par rapport à celles de la périphérie [Mimura 2006]. Ceci est corrélé avec une différence d'aptitudes prolifératives entre le centre et la périphérie. La même équipe a montré ultérieurement que la sénescence des CE n'est pas due à la réplication, mais au stress environnemental inducteur de dommages sur l'ADN par oxydation [Joyce 2009]. En effet, les CE possèdent toutes la même longueur de télomères (10 à 15 kb) qui n'est pas modifié malgré l'âge et la localisation centrale ou périphérique [Konomi 2007]. Ceci confirme que les CE ne se divisent pas in vivo. Une des explications possible pourrait être une différence d'ordre chronologique : s'il existe des CS qui régénèrent lentement l'endothélium de façon centripète, les CE périphériques doivent être plus jeunes que celles du centre et sont exposées aux stress environnementaux moins longtemps que les cellules plus vieilles situées au centre de l'endothélium.

B) Observation de phénomènes pathologiques touchant les CE

-Migration centripète : ce type de migration des CE a été démontré par une marquage des chromosomes sexuels dans des greffons de patients greffés en sex-mismatch, dans au moins deux travaux étudiant la colonisation de la MD du donneur par les CE du receveur après kératoplastie transfixiante [Wollensak 1999, Lagali 2010].

-Survie accrue des kératoplasties transfixiantes chez les receveurs ayant une DCE élevée en préopératoire. L'exemple typique est le kératocône, qui est un modèle particulièrement intéressant puisque les phénomènes inflammatoires sont absents et les réactions de rejets rares, ce qui permet d'observer l'évolution naturelle des populations endothéliales du receveur et du donneur. La survie prolongée de ces greffons suggère fortement que les CE périphériques du receveur continuent d'alimenter en CE le centre du greffon [Thompson 2003, Williams 2008].

-Modèle iatrogénique de dystrophie endothéliale par destruction chronique des CE périphériques par les haptiques des implants de chambre antérieure à appui angulaire, la baisse progressive de la DCE conduisant à une décompensation oedémateuse de la cornée au bout de 10-15 ans démontre l'importance des CE périphériques dans l'homéostasie cornéenne [Gaynes 1985, Sugar 1985].

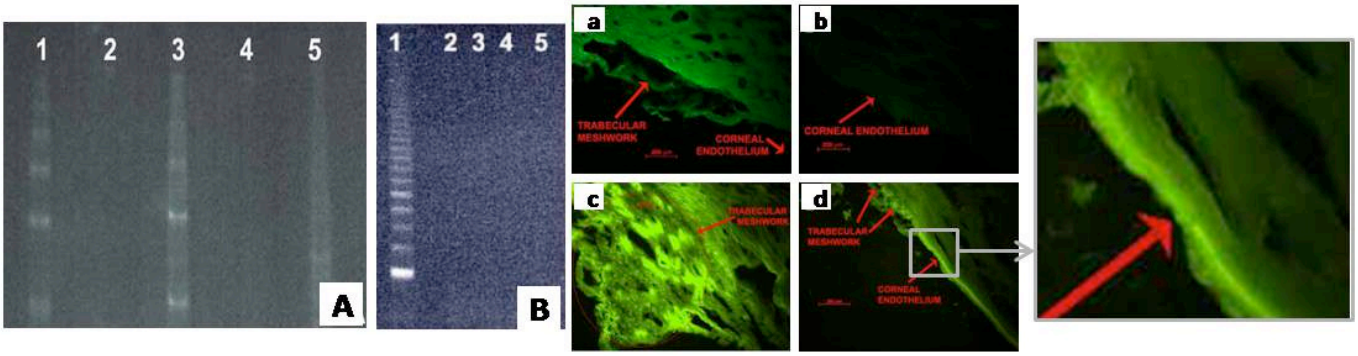


Figure 42. Activité de la télomérase et incorporation de BrdU sur l'endothélium cornéen. A et B : Activité de la télomérase. A) 1: contrôle positif; 2 et 3: zones centrale et périphérique d'une cornée provenant d'un donneur de 16 ans; 4, et 5: zones centrale et périphérique d'une cornée provenant d'un donneur de 66 ans. B) 1: contrôle positif; 2 et 3: zones centrale et périphérique d'une cornée provenant d'un donneur de 26 ans; 4 et 5) zones centrale et périphérique d'une cornée provenant d'un donneur de 55 ans; (zone centrale: diamètre< 8mm; zone périphérique: diamètre entre 8 et 12mm). a, b, c et d : incorporation de BrdU sur une cornée avec un endothélium intact ou lésé à 37°C dans Optisol pendant 48h. a et b: endothélium intact; c et d: endothélium lésé. a et c: Trabéculum; b et d: zone de transition **(D'après Whikehart et al., 2005)**

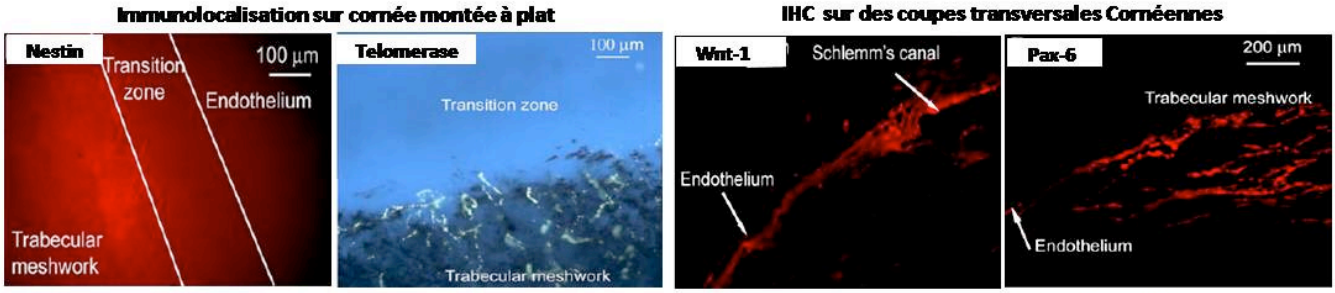


Figure 43. Exemples de marqueurs de cellules souches considérés comme positifs par McGowan et al., 2007. Selon notre expérience, ces marquages considérés comme positifs par les auteurs de cet article ne semblent pas être spécifiques, mais plutôt à un bruit de fond.

Marker/ preparation method	Number of corneas evaluated	Unwounded cornea	Wounded cornea (24-48 h)
Nestin	16		
Cross-section		TM, Schwalbe's line	TM, Schwalbe's line
Flat mount		Schwalbe's line	Schwalbe's line
Alkaline Phosphatase	6		
cross-section		TM	TM
flat mount		TM, Schwalbe's line	TM, Schwalbe's line
Telomerase	10		
cross-section		TM, Schwalbe's line, peripheral endothelium	TM, Schwalbe's line, peripheral endothelium
flat mount		no reaction	TM
Oct-3/4	8		
cross-section		no reaction	TM
flat mount		no reaction	TM
Wnt-1	8		
cross-section		no reaction	TM, Schwalbe's line
flat mount		no reaction	no reaction
Pax-6	7		
cross-section		no reaction	TM, Schwalbe's line, peripheral endothelium
flat mount		no reaction	no reaction
Sox-2	2		
cross-section		no reaction	TM, Schwalbe's line, endothelium
flat mount		no reaction	no reaction

Tableau 7. Marqueurs des cellules souches endothéliales cornéennes trouvés par McGowan et al., 2007. Les résultats résumés dans ce tableau sont probablement faussés à cause de résultats non fiables de l'immunolocalisation.

-Survie moindre des kératoplasties transfixiantes chez les receveurs ayant une DCE basse voir nulle en préopératoire : une diminution rapide de la DCE centrale de la greffe lorsque les CE périphériques du receveur sont absentes, par exemple, dans la dystrophie bulleuse du pseudo phake, est bien connue [Price 1991a, b].

C) Découverte in vitro de cellules progénitrices en nombre supérieur en périphérie

En utilisant la méthode de culture des microsphères chez l'homme, la présence de précurseurs/progéniteurs des CE sur la MD de la cornée a été rapportée par une équipe japonaise [Yokoo 2005]. La même équipe a ensuite mis en évidence la distribution non homogène des progéniteurs à forte capacité de régénération (sphère cellulaire contenant plus de 5000 cellules) sur la MD. Quatre fois plus de progéniteurs sont présents en périphérie (7,5mm-10mm) par rapport au centre (<7,5mm) de l'endothélium de cornées humaines (**Fig. 41)** [Yokoo 2005, Yamagami 2007]. En utilisant la même méthode chez le lapin, ils ont confirmé cette distribution non homogène en progéniteurs entre la périphérie et le centre de l'endothélium cornéen [Mimura 2005]. Une autre étude utilisant la même méthode chez les bovidés montre également l'existence d'une hiérarchie au sein des progéniteurs de l'endothélium [Huang 2010]. L'ensemble de ces arguments suggère que la régénération de l'endothélium se fait par une migration centripète des progéniteurs provenant des CS situées à l'extrême périphérie de l'endothélium ou sur les tissus adjacents.

IV-2) Nécessité de preuves directes des capacités régénératives des CE

A) Analyse critique des travaux existants

Il n'existe seulement à l'heure actuelle que deux articles publiés sur ce sujet et leur qualité est contestable. Ils tendent à montrer, uniquement de façon indirecte l'existence des capacités régénératives de l'endothélium cornéen in vivo.

-Article 1 [Whikehart 2005]

En 2005, Whikehart et al. (USA, Department of Vision Science, School of Optometry, The University of Albama at Birmingham) ont publié le premier article suggérant que des CS/progénitrices pourraient être séquestrées dans une niche située au sein de la zone de transition entre l'endothélium et le trabéculum. Deux méthodes principales ont été utilisées dans cet article : la détection d'une activité télomérase et l'incorporation de la BrdU sur les différentes zones de la surface postérieure cornéenne. Leurs principaux résultats sont synthétisés dans **la Fig. 42.**

Synthèse de leurs résultats : L'activité télomérase est principalement détectée dans la zone périphérique de l'endothélium mais jamais dans la zone centrale ni dans la zone de transition entre la ligne de Schwalbe et le trabéculum (sans toutefois que les données de matériel et méthode et des résultats qui leur permettent de l'affirmer soient très robustes dans cet article). Après une incubation pendant 48h de cornées intactes ou après lésion endothéliale calibrée dans le milieu Optisol-GS contenant du BrdU, l'incorporation de BrdU était limitée au trabéculum dans les cornées intactes, et étendue du trabéculum jusqu'à la périphérie endothéliale dans les cornées avec lésion endothéliale.
Synthèse de leurs conclusions : Les auteurs ont conclu que la niche de CS était probablement localisée dans la zone de transition. L'incorporation de BrdU en présence d'un processus de réparation tissulaire indique l'existence de CS et de cellules progénitrices. L'expression forte de BrdU dans la zone de transition confirme (d'après eux) la présence de CS.
-Critique 1 : La durée d'incubation avec le BrdU est contestable : seulement 48h en Optisol à 37°C sans réaliser ultérieurement une période de « dilution » du traceur chez les progéniteurs qui se répliquent. Le protocole utilisé met probablement en évidence toutes les cellules qui ont répliqué leur ADN, mais pas seulement les CS et/ou progénitrices
-Critique 2 : Le marquage fluorescent révélant l'incorporation de BrdU semble ne pas être localisé au niveau cellulaire, mais plutôt au niveau de la MD et des fibres collagènes du trabéculum.

-Critique 3 : Il paraît impossible de réaliser la mesure de l'activité de la télomérase sur la zone de transition selon leurs protocoles car celle ci mesure seulement 50 µm de large, et l'absence des datas pour cette zone pourtant cruciale pour justifier leur résultats et conclusions principales, font douter la fiabilité et la véracité de cet article.

-Article 2 McGowan 2007

En 2007, McGowan et al., appartenant à la même équipe que Whikehart ont publié un second article qui suggère, cette fois, que les CS sont localisées dans le limbe postérieur constitué du trabéculum et de la zone de transition. Ils ont testé différents marqueurs de CS sur un endothélium cornéen humain intact ou lésé artificiellement, en utilisant les techniques d'immunolocalisation sur endothélium de cornées entières et d'IHC sur des sections transversales congelées. Les résultats principaux sont synthétisés dans **le Tableau 7**, des exemples de marquage considéré comme positif sont montrés dans **la Figure 43.**

Synthèse de leurs résultats et conclusions : les auteurs ont mis en évidence l'expression de la nestine, de l'alkaline phosphatase et de la télomérase sur le trabéculum et la zone de transition sur l'endothélium intact et endommagé, et l'expression de l'Oct-3/4, Wnt-1, Pax-6, Sox-2 sur ces deux zones seulement sur l'endothélium lésé. A partir de ces résultats, ils ont conclu que les CS étaient localisées dans le trabéculum et au sein de la zone de transition.

Critique principale: Les marquages positifs considérés par les auteurs de cet article ne correspondent à aucun des compartiments subcellulaires, mais plutôt à des matrices extracellulaires telles que les fibres du trabéculum, le stroma cornéen, la MD ou d'autres structures non cellulaires.

B) Questions restant en suspens

A ce jour, aucune étude n'a démontré de façon certaine l'existence d'une régénération de l'endothélium cornéen, ou plus précisément l'existence et la localisation précise des CS endothéliales et de leur niche au sein de l'endothélium cornéen ou des tissus voisins. Il manque les preuves directes d'une régénération de l'endothélium en physiologie et dans les situations pathologiques. A la différence des tissus qui ont un cycle de régénération court tel que le sang, la peau, et l'épithélium cornéen, le cycle de régénération de l'endothélium cornéen, s'il existe réellement, doit être très lent, constituant une des difficultés principales dans cette démonstration.

Des questions cruciales restent en suspens :

1) Comment l'endothélium concilie-t-il apparente stabilité centrale et renouvellement périphérique ?
2) Comment étudier un phénomène aussi lent ?
3) Quelles sont les mécanismes limitant qui empêche une régénération efficace en cas de lésions ?
4) Quelle est l'origine des précurseurs de CE identifiés par les cultures des microspères? S'agit-il de CE qui ont gardé des capacités prolifératives résiduelles des CSE, de cellules progénitrices dérivées de CSA nichées dans les tissus voisins, ou bien de CSA spécifiques de l'endothélium cornéen ?
5) Ou se situe la niche des CS endothéliales.

IV-3) Thérapie du futur : la reconstitution de l'endothélium in vitro

Dans les dysfonctionnements endothéliaux cornéens, la future thérapie qui permettrait de remplacer les kératoplasties actuelles utilisant des greffons cornéens de pleine épaisseur ou lamellaire, est une greffe utilisant des greffons endothéliaux reconstitués par bioengineering. Les intérêts principaux de cette thérapie cellulaire seraient l'immunocompatibilité parfaite du greffon si la source cellulaire (les CS et progéniteurs endothéliaux ou alternatifs) pouvait être prélevée directement chez le patient, et sa grande disponibilité par rapport la rareté des greffons classiques dépendant du nombre limité de donneurs et de leur sélection drastique

Le greffon endothélial reconstitué idéal devrait présenter deux caractéristiques principales : une fonctionnalité parfaite c'est à dire une différenciation parfaite des CE et une réserve endothéliale importante pour une survie prolongé, autrement dit une DCE très élevée, supérieure à 3000 cellules/mm^2.

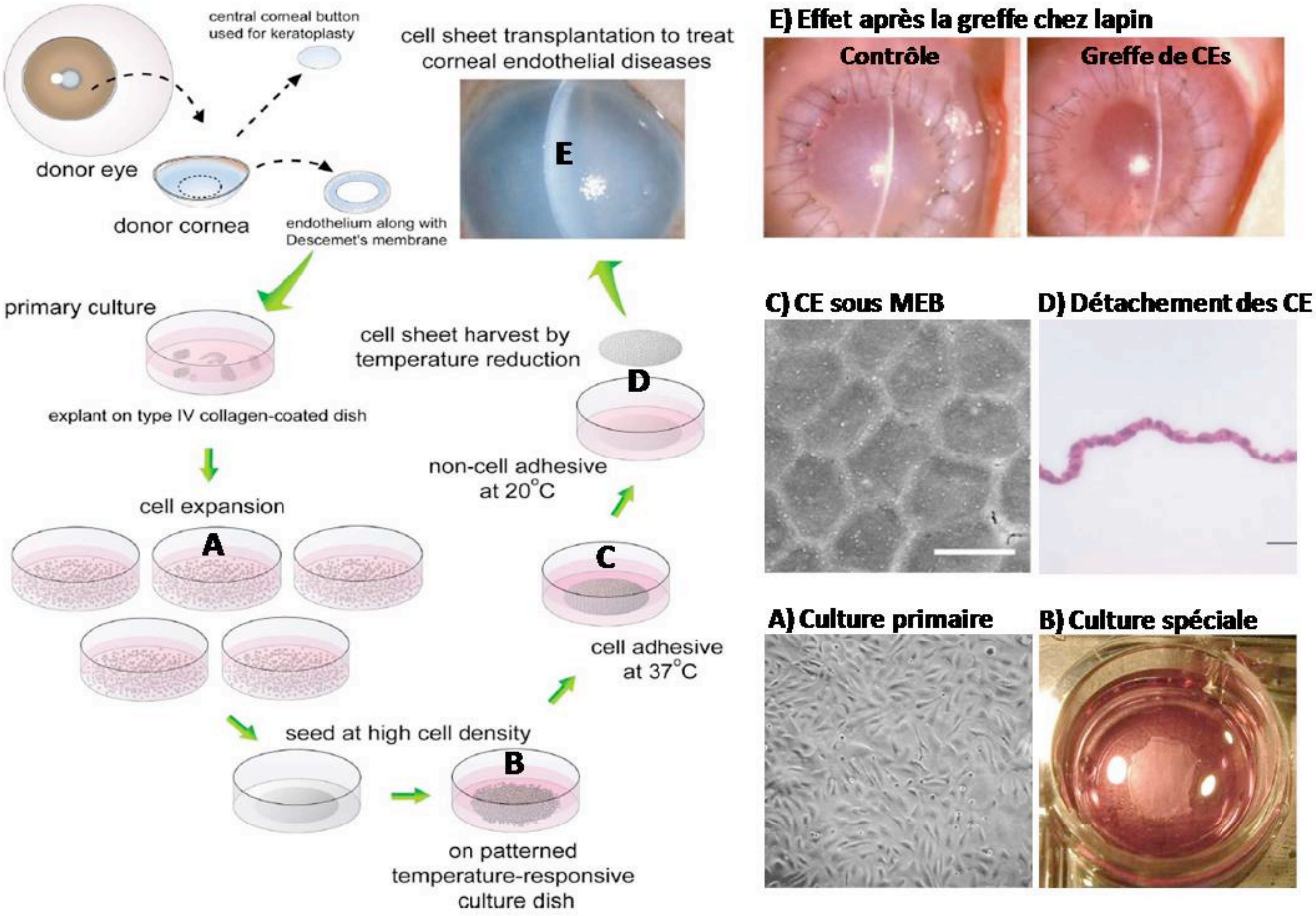


Figure 44. Reconstitution de l'endothélium cornéen humain in vitro sans membrane de support. A) Culture primaire de CE à partir de la collerette cornéenne; B) Après l'expansion des CE in vitro, elles sont transférées dans une boite de culture spéciale sur laquelle les CE adhérèrent à 37°C et sont décollées complètement à 20°C; C) Les CE dans la boite spéciale possèdent une forme hexagonale; D) A 20°C, l'endothélium reconstitué in vitro se détache complètement de la boite sans être fragmenté; E) La transplantation de cet endothélium reconstitué améliore la transparence de la cornée du lapin où l'endothélium a été détruit préalablement. **(D'après Sumide et al., 2006)**

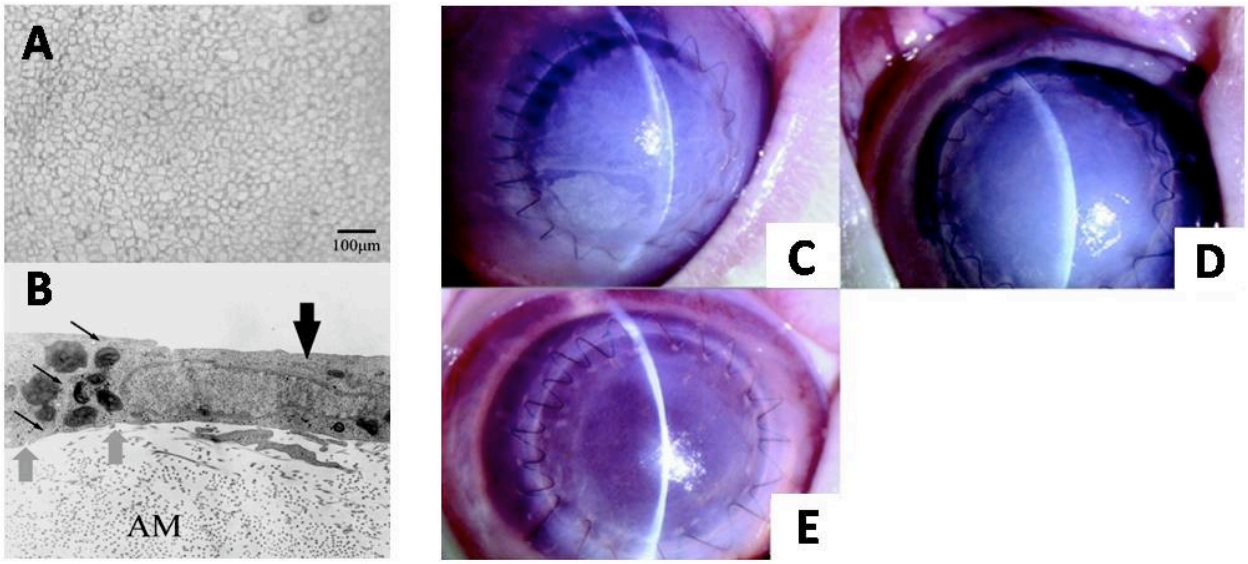


Figure 45, Reconstitution de l'endothélium cornéen humain sur membrane amniotique. Après expansion des CE en culture primaire, les CE sont semées à une concentration finale de 6000 cellules/mm² sur une membrane amniotique (MA) dénudée de cellules. A) Aspect de l'endothélium reconstitué sur la MA; B) Aspect de profil de l'endothélium reconstitué en MET; C, D et E) 7 jours après destruction de la membrane de Descemet chez le lapin; C) aucune intervention supplémentaire; D) seule la MA a été greffée; E) l'endothélium reconstitué a été greffé. **(D'après Ishino et al., 2004)**

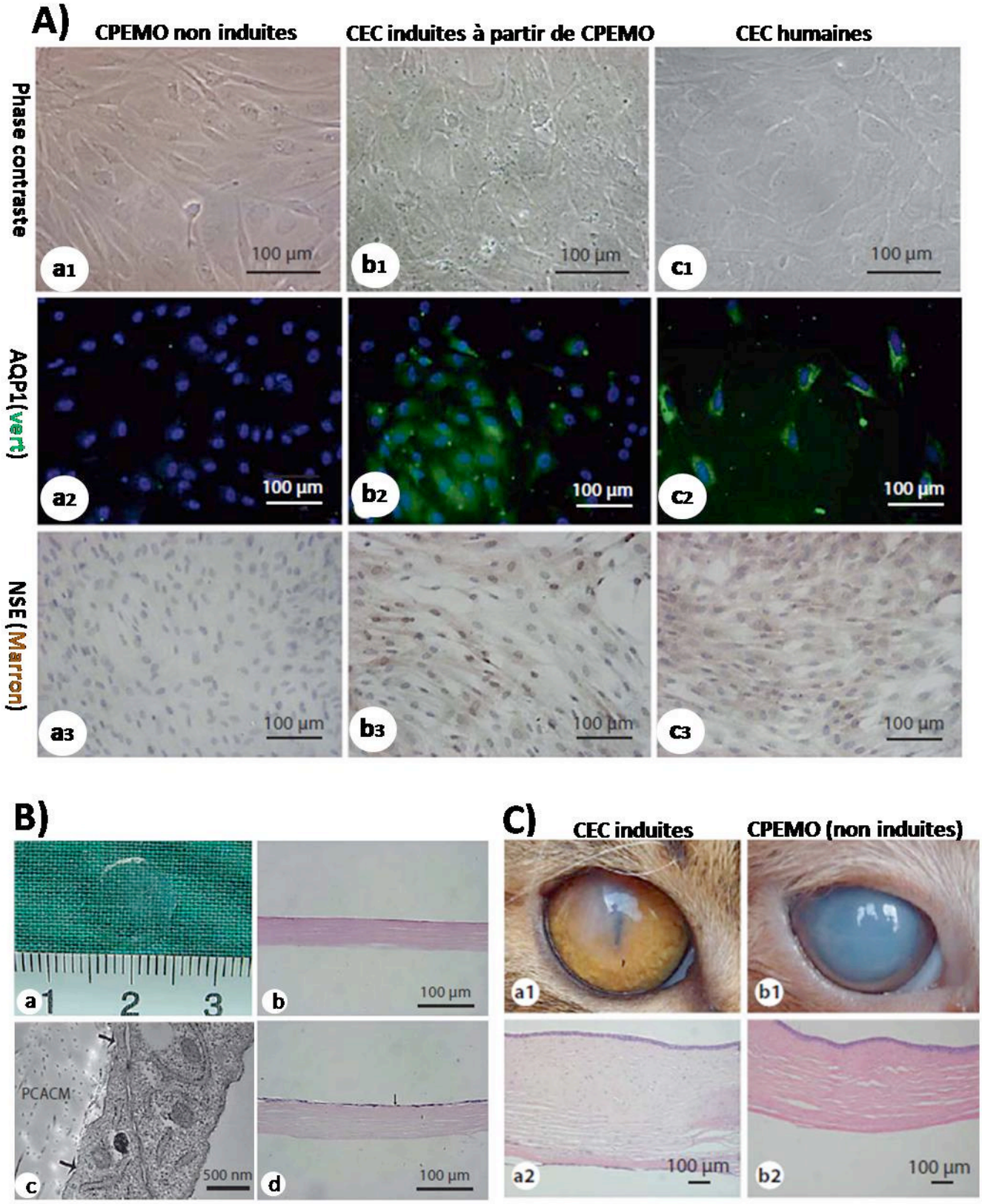


Figure 46. L'endothélium cornéen reconstitué en utilisant des CEC induites à partir des cellules progénitrices endothéliales dérivée de la moelle osseuse (CPEMO) des fœtus humains. A) La co-culture avec des CEC primaires humaines induit in vitro la transdifférenciation des CPEMO en CEC démontrée par l'induction de la morphologie similaire à CEC et l'expression des marqueurs de CEC. B) L'endothélium reconstitué avec les CEC induites à partir des CPEMO sur une lamelle (40 µm d'épaisseurs) stromale acellulaire provenant de la cornée porcine; a) la lamelle stromale acellulaire est transparente; b) histologie de la lamelle stromale acellulaire; observation de l'endothélium reconstitué avec CEC induites sur la lamelle stromale sous MET(c) et par la méthode histologie (d). C: La transparence (1) et l'histologie de la cornée transversale (2) 4 semaines après la greffe de l'endothélium reconstitué avec les CEC induites à partir des CPEMO (a) ; après la greffe de l'endothélium reconstitué avec les CPEMO non induites. **(D'après Shao et al.,2011)**

A) Reconstitution de l'endothélium in vitro

Reconstitution à partir des progéniteurs de CE

L'équipe japonaise d'Amanos a montré que l'endothélium cornéen de lapin mais aussi humain, contenait des progéniteurs ou précurseurs capables de se multiplier et de se différencier en CE in vitro [Mimura 2005, Yokoo 2005, Yamagami 2006, Yamagami 2007]. La reconstitution de l'endothélium cornéen a été obtenue à partir de ces précurseurs [Bohnke 1999, Engelmann 1999, Chen 2001, Yamagami 2006, Koizumi 2007, Koizumi 2008]. Les premières transplantations expérimentales des CE bovines cultivées in vitro chez lapin et chat datent de plus de 30 ans [Gospodarowicz 1979b, a]. Dans le travaux les plus récents, les CE et leurs progéniteurs situés sur la MD sont isolés et cultivés dans un milieu contenant du sérum fœtal de veau (SFV) supplémenté un facteur de croissance bFGF [Bohnke 1999, Sumide 2006, Koizumi 2007, Hitani 2008, Koizumi 2009], soit sans aucune membrane biologique (**Fig. 44**) [Sumide 2006, Hitani 2008], soit sur une membrane biologique. Cette membrane peut être soit une MD dénudée [Bohnke 1999, Joo 2000], une membrane de collagène de type I [Koizumi 2007, Koizumi 2008, Koizumi 2009], ou une membrane amniotique dénudée [Fu 2006, Wencan 2007] (**Fig. 45** [Ishino 2004]), ou encore sur une lamelle de stroma cornéen acellulaire [Choi 2010].

Malgré de nombreuses études portant sur la reconstitution de l'endothélium, il n'a jamais été encore possible de proposer une application thérapeutique clinique. Les raisons sont nombreuses : 1) la culture primaire de masse de CE reste difficile notamment en raison de la contamination des fibroblastes et de la différence de différenciation entre les CE in vivo et celles in vitro ; 2) la qualité de l'endothélium reconstitué reste imparfaite au niveau de sa fonctionnalité (différenciation imparfaite ou dédifférenciation) [Hitani 2008]; 3) la DCE obtenue est souvent < 2000 cellules/mm^2 et inconstante [Engelmann 1999, Chen 2001]; 4) l'endothélium reconstitué à partir d'un donneur correspond à une allogreffe.

La mise au point d'une thérapie cellulaire utilisant des greffons endothéliaux reconstitués par bio engineering nécessite donc : 1) la recherche de la source fiable de CE : soit CS spécifiques de l'endothélium cornéen s'il est possible de localiser leur niche et de mettre au point une méthode reproductible de recueil et de tri à partir de cornées de donneurs, soit CS alternatives, indispensable pour effectuer des greffes autologues à partir de CS adultes du propre patient ; 2) la recherche du microenvironnement permettant d'obtenir la différenciation ou la transdifférenciation des CS en CE cornéennes in vitro afin d'obtenir un greffon de qualité ; 3) un support de culture permettant l'implantation dans l'œil du receveur.

Première reconstitution par la transdifférenciation

La première étude de transdifférenciation en CE cornéenne à partir de cellules progénitrices endothéliales dérivées de la moelle osseuse (CPEMO) des fœtus humains de 15 à 18 semaines a été réalisée par une équipe chinoise en 2011 **(Fig. 46)** [Shao 2011]. Les auteurs ont induit la transdifférenciation des CPEMO en CE par une simple co-culture in vitro de CPEMO avec des CE humaines primaires provenant de la cornée de fœtus humains. L'induction d'une morphologie similaire à celle des CE et de l'expression des marqueurs spécifiques des CEC tel que la neurone-spécifique enolase (NSE) et l'aquaporine-1 (AQP1) semble indiquer une transdifférenciation effective. Ces CE induites à partir des CPEMO ont été repiquées à 2000 cellules/mm^2 sur une lamelle d'environ 40µm d'épaisseur de stroma cornéen porcin acellulaire afin de reconstituer un endothélium cornéen in vitro. Une kératoplastie lamellaire utilisant ce greffon endothélial reconstitué a ensuite été faite chez le chat. La cornée greffée a retrouvé une transparence bien meilleure par rapport aux témoins 4 semaines après l'opération. Cet article est probablement une étape encourageante dans l'étude de la reconstitution de l'endothélium cornéen à partir de différentes CS ou progéniteurs autologues. Cependant, la transparence et l'épaisseur de la cornée greffée ne sont pas parfait et la DCE impossible à mesurer in vivo. Ces résultats mitigés peuvent être expliqués par une différenciation imparfaite des CE in vitro (comparer les figures 5 et 46). Cette différenciation imparfaite n'est pas réservée aux CE obtenues par transdifférenciation in vitro, mais semble commune à toute culture in vitro d'endothélium cornéen, suggérant l'importance majeure du microenvironnement qu'il faut également reconstituer in vitro.

B) Nécessité d'études du microenvironnement

A l'image du rôle primordial du micro environnement limbique dans la reconstitution de l'épithélium cornéen à partir de divers types de CS, la recherche du microenvironnement permettant la différenciation ou la transdifférenciation de CS en CE cornéennes in vitro est un enjeu fondamental qui conditionne directement les possibilités de reconstitution d'un greffon endothélial bioartificiel.

Concernant l'épithélium, la reconstitution in vitro d'un greffon épithélial à partir de CS nécessite un microenvironnement assurant non seulement la reformation de l'épithélium (ou la différenciation), mais aussi le maintien du caractère souche des CS. Le turn over rapide de l'épithélium nécessite en effet de restaurer non seulement les cellules différenciées mais aussi les cellules souches pour assurer la pérennité de la greffe.

Par contre, le caractère résistant de l'endothélium et la baisse très lente de la DCE in vivo ne rende pas absolument indispensable la présence de CS/progénitrices sur le greffon endothélial. Seule semble compter l'obtention d'une différenciation complète et d'une DCE élevée, tout deux gages d'un endothélium focntionnel.

Les éléments principaux reconstituant le microenvironnement spécifique nécessaire à l'induction de la différenciation en CE paraissent être 1) une membrane adhérente qui servira à l'induction de la différenciation et de support solide au transfert pendant le processus chirurgical de greffe; 2) la co-culture avec des cellules matures spécifiques qui serviront à fournir tous les facteurs de croissances, cytokines et les substances solubles de la matrice extracellulaire nécessaires à l'induction de la différenciation ; 3) des paramètres physiques, notamment la PIO qui joue probablement un rôle très important dans la constitution du cytosquelette caractéristique des CE **(Fig. 6)**; 4) les autres éléments caractéristiques du microenvironnement de l'endothélium cornéen in vivo, tel que TGF beta et les autres facteurs solubles présents dans l'humeur aqueuse.

CONCLUSION ET PERSPECTIVES

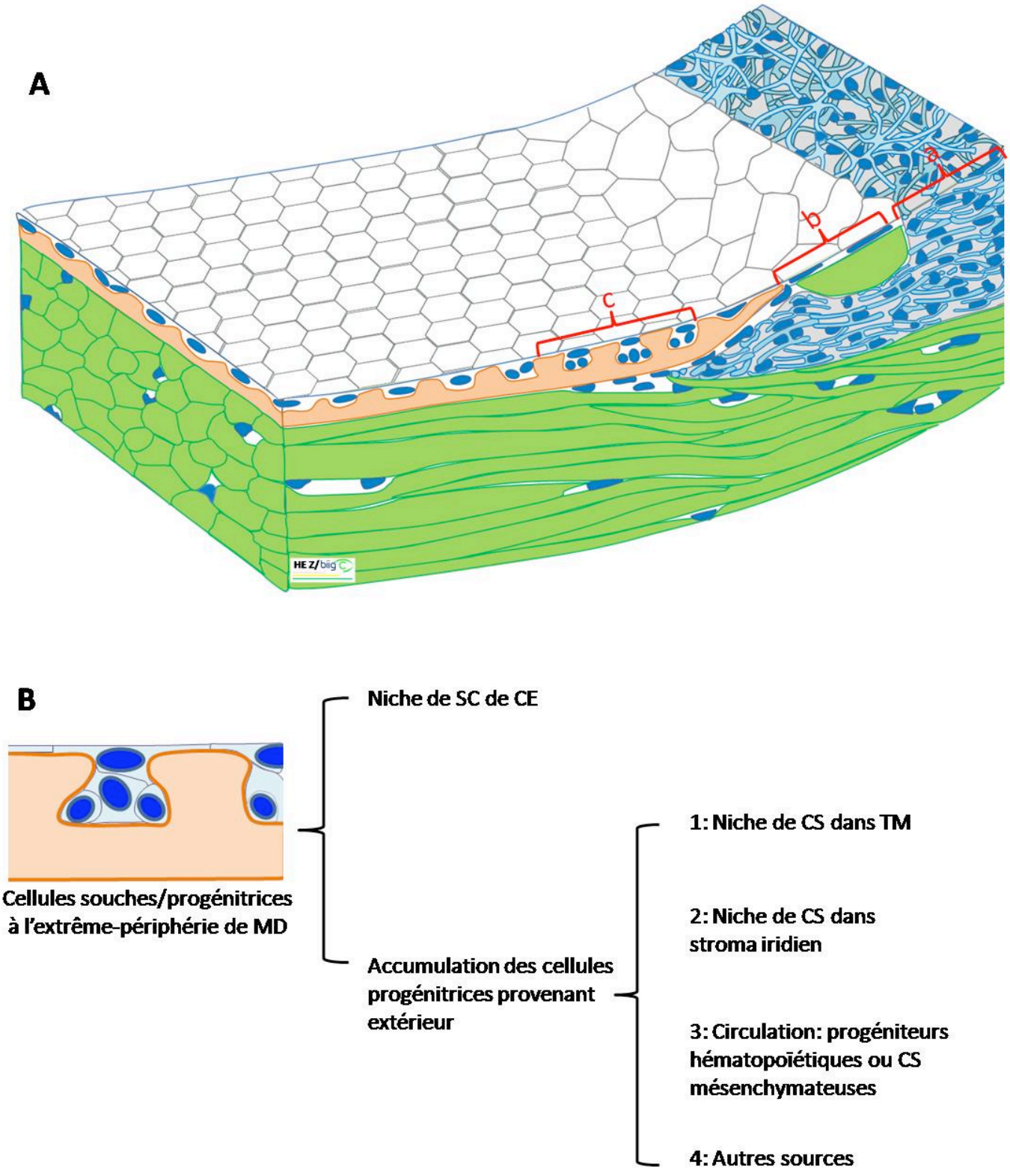
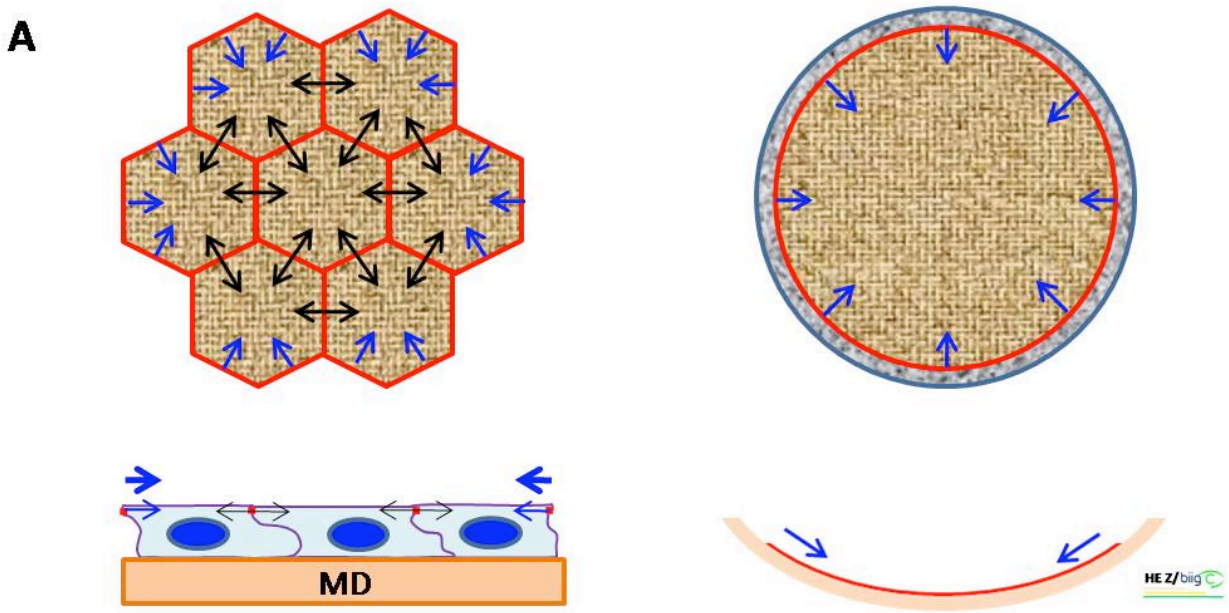


Figure 47. Conclusion sur les CS de CE: hypothèse de la provenance de la source cellulaire pour CE cornéennes chez humain. (BiiGC/HE)

A. a) McGowan et al., ont suggéré la niche de CS situé dans trabéculum (TM) en 2007 (Résultats non pertinents) ; b) Whikehart et al. ont sugéré la niche de CS dans la zone de transition en 2005 (Résultats non pertinents); c) Nos résultats ont montré une accumulation des cellules souches/progénitrices à l'extrême périphérie de l'endothélium.

B. Nos hypothèse sur ces cellules souches/progénitrices de l'extrême périphérie de l'endothélium cornéen.



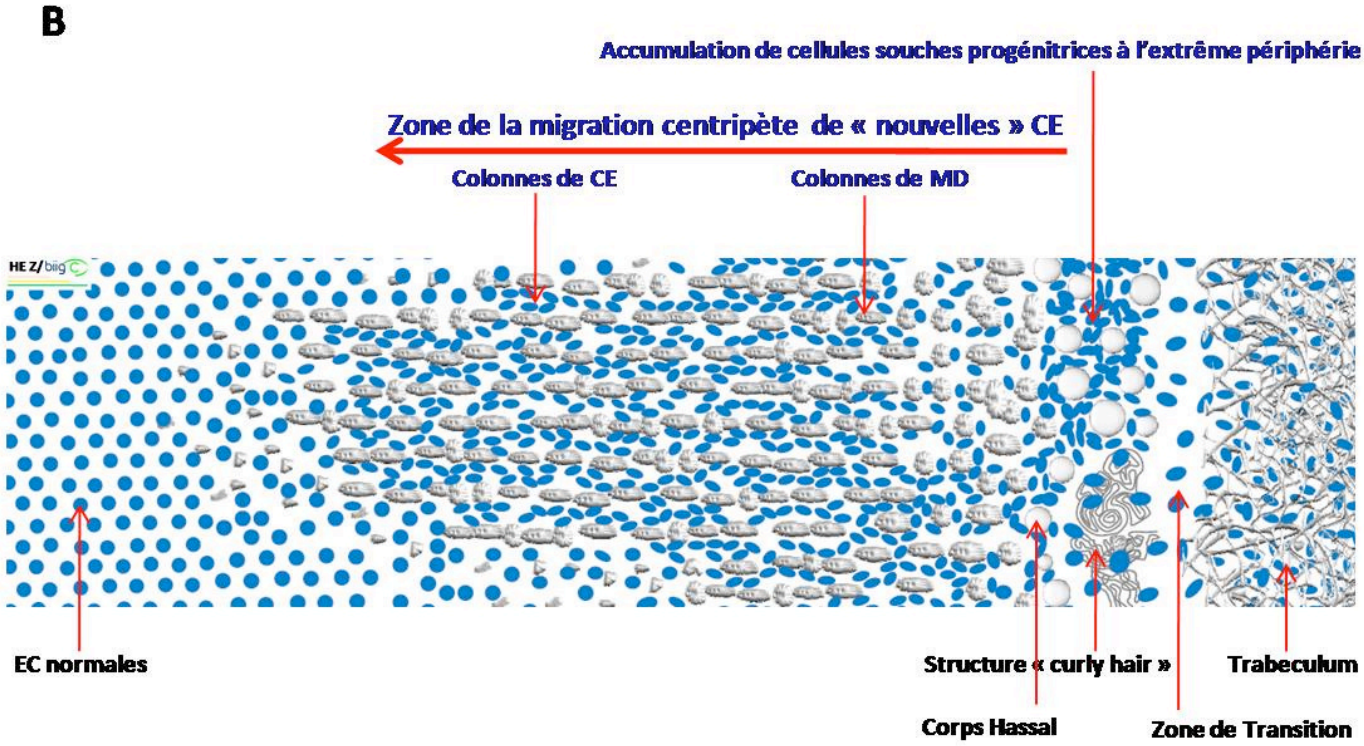


Figure 48. Conclusion sur la régénération de CE: hypothèse de la migration centripète des « nouvelles » CE provenant de l'extrême périphérie de l'endothélium cornéen humain. (Source de BiiGC/HE)

A. Hypothèse du mécanisme/de la force qui conduit la migration centripète des CE. A gauche, un modèle en illustrant 7 CE montre que la force rétractive à la surface de CE induit une force rétractive du bord extérieur des CE périphériques vers le centre; A droite, la généralisation de cette force rétractive sur l'endothélium entier suggère une force rétractive centripète au bord d'endothélium; les flèches de double têtes indiquent une force équilibrée, et les flèches de simple tête indiquent la force rétractive centripète.

B. Observation des colonnes de CE accompagné des colonnes de MD suggèrent une migration centripète de CE. Les caractères en bleu sont des propres observations de BiiGC; Les caractère en noir sont des observation décrites dans la littérature.

Les multiples avancées récentes dans les techniques chirurgicales des greffes endothéliales ont indéniablement démontré les nombreux avantages du remplacement sélectif de cette population cellulaire indispensable à la fonction visuelle. Elles sont cependant toutes dépendantes de la disponibilité de greffons cornéens, si possible à très haute DCE, donc du don de cornée. La simplicité apparente du tissu à remplacer – une monocouche de cellules jointives sur une membrane de collagène IV- incite naturellement à envisager le développement d'endothélium bioartificiel par des techniques de bioengineering. Ceci permettrait de s'affranchir des contraintes actuelles du don de cornée (disponibilité et qualité variables). A l'image de ce qui été rendu possible pour l'épithélium cornéen en 25 ans, depuis la découverte des cellules souches épithéliales par Cotsarelis jusqu'aux premières applications cliniques de greffe d'épithélium reconstitués in vitro, plusieurs étapes essentielles sont nécessaires pour envisager la culture in vitro d'un endothélium cornéen humain prêt à la greffe : identification des cellules souches endothéliales, repérage de leur niche, compréhension de leur microenvironnement, mise au point des méthodes d'extraction et de culture, mise au point du contrôle qualité des cultures obtenues, caractérisation de la survie au long cours de ces cellules, et , bien entendu de leurs capacités de déturgescence cornéenne.

Nos travaux ont tenté d'apporter quelques pierres à ce vaste chantier :

Sur un plan technique, nous sommes convaincus que l'étude de montage à plat de cornées intactes n'a pas d'équivalent pour l'observation de la population des CE dans son ensemble. Cette technique est indispensable pour comprendre les phénomènes de migration cellulaire, mesurer la DCE et la morphométrie, et repérer des cellules rares au milieu d'une population en apparence homogène. L'immunolocalisation de protéines sur montage à plat constitue de ce fait une méthode incontournable. Elle souffrait jusqu'à présent de l'absence d'utilisation systématique faute de protocoles bien définis. Nos travaux ont permis d'optimiser des protocoles pour la détection de protéines du cycle cellulaire en soulignant plusieurs aspects fondamentaux. L'immunolocalisation dans les CE de cornées entières montées à plat est une technique hybride entre IHC et ICC. Les protéines des CE semblent exposées à la sur-fixation par les fixateurs aldéhydiques aux concentrations pourtant largement utilisées dans la littérature. La structure endothélio-Descemetique facilite le détachement des CE lors de l'utilisation des techniques de démasquages antigéniques habituelles. Nous proposons désormais l'utilisation de 0,5% de formaldéhyde ou le méthanol à température ambiante, ces 2 fixateurs seuls permettant de révéler la plupart des Ag étudiés. L'utilisation d'un démasquage antigénique par du SDS est susceptible d'améliore la qualité des immunomarquages. Ces outils optimisés devraient permettre de standardiser les résultats inter laboratoire. Ils devraient permettre l'étude de n'importe quel type d'Ag.

Cette technique optimiser nous a permis d'étudier l'expression de protéines du cycle cellulaire dans les CE de 2 types de cornées non étudiées jusque là : cornées fraiches étudiées immédiatement après prélèvement et cornées en organoculture. Nous avons ainsi pu préciser la localisation subcellulaire de plusieurs protéines. Les résultats les plus innovants concerne les cyclines D1, A, PCNA, MCM2 et p21 dont les patterns d'expression (ou l'absence d'expression pour p21) n'avaient pas été rapportés aussi précisément ; Nous avons également pu montrer que Ki67 mais pas PCNA ni MCM2 pouvaient être utilisés comme marqueur de prolifération des CE ; enfin, si nos résultats sont cohérents avec les données de la littérature sur le statut des CE dans le cycle cellulaire (bloquée en phase G1) des différences notables (p21, cycline D1) pourraient être due aux particularités de nos cornées (fraiches ou en OC) alors que les travaux antérieurs auxquels nous nous referons ont tous été réalisés sur des cornées conservées en Optisol à 4°C. L'ensemble de nos marquages suggère un arrêt du cycle lié à des dommages à l'ADN.

Toujours en utilisant l'observation systématique de l'ensemble de l'endothélium sur des montages à plat, mais cette fois en confrontant microscopie optique et électronique à balayage, nous avons décrit une série de caractéristiques originales de l'extrême périphérie et de la périphérie de l'endothélium de cornées humaines : des clusters de cellules et une organisation en colonnes radiaires qui permettent de revisiter la microanatomie et de cette région et suggèrent que les CE migrent vers le centre de la cornée pendant toute la vie du sujet, à partir de sites très périphériques, potentiellement des niches de CS ou des amas de progéniteurs (**Figure 46 et 47**).

En parallèle nous avons développé deux autres outils spécifiques pour l'étude de l'endothélium cornéen dans deux domaines différents : un dispositif d'électrotransfert de gène dans l'endothélium de cornées humaines **(article publié dans Ophthalmic Research IF 1,29)** et un panel de mosaïques endothéliales virtuelles microlithogravées destinée à la formation des techniciens des banques de cornées et au contrôle qualité de leur comptages endothéliaux (article **en review Optics Letters, IF 3,316).**

Les perspectives de nos travaux sont nombreuses, focalisés sur la poursuite de la caractérisation des cellules de l'extrême périphérie de l'endothélium cornéen :

1) Poursuite des immunomarquages pour d'autres marqueurs de cellules d'amplification transitoire, progéniteurs ou cellules souches (**Figure 46**)

2) Développement de techniques confirmatives de l'expression protéiques (Luminex, WB à partir de très faibles quantités de protéines)

3) Comparaison des profils transcriptionnels des CE centrales, de la périphérie et de l'extrême périphérie endothéliale par microarray pour réunir des arguments en faveur du caractère souche des cellules de l'extrême périphérie

4) Développement de méthode de digestion enzymatique permettant d'isoler les cellules des clusters localisées en profondeur de l'extrême périphérie, de les trier et de les cultiver in vitro.

5) Etablissement de cultures des différents types cellulaires de cette région pour établir des co-cultures favorable à la survie et la différenciation des hypothétiques cellules souches endothéliales ou de la transdifférenciation d'autres CSA adultes.

En attendant d'aboutir dans un futur proche à l'isolement des CS endothéliales ou de CSA transdifférentiable en CE dans un microenvironnement contrôlé, le don de cornée reste, rappelons-le, indispensable aux patients, soit directement en fournissant des greffons soit indirectement en permettant l'avancé des travaux de recherche qui aboutiront, n'en doutons pas, aux greffes de demain.

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The specialized microenvironment or niche where stem cells reside provides regulatory input governing stem cell function. We tested the hypothesis that targeting the niche might improve stem cell-based therapies using three mouse models that are relevant to clinical uses of hematopoietic stem (HS) cells. We and others previously identified the osteoblast as a component of the adult HS cell niche and established that activation of the parathyroid hormone (PTH) receptor on osteoblasts increases stem cell number. Here we show that pharmacologic use of PTH increases the number of HS cells mobilized into the peripheral blood for stem cell harvests, protects stem cells from repeated exposure to cytotoxic chemotherapy and expands stem cells in transplant recipients. These data provide evidence that the niche may be an attractive target for drug-based stem cell therapeutics.
Center for Regenerative Medicine, Massachusetts General Hospital, Harvard Medical School, 185 Cambridge Street, Boston, Massachusetts 02114, USA.

Ahmad S, Kolli S, Lako M, Figueiredo F and Daniels J T. Stem cell therapies for ocular surface disease. *Drug Discov Today* 2010;7-8:306-13

Transparency of the cornea on the front surface of the eye is essential for vision. A variety of blinding ocular surface diseases involve the cornea. This review focusses on vision loss caused by disruption of the integrity and function of the outermost corneal layer (the epithelium) and the stem-cell-based therapeutic strategies in use and under development to restore sight in affected patients.
North East England Stem Cell Institute, Central Parkway, Newcastle upon Tyne NE13BZ, UK.

Al-Hajj M, Wicha M S, Benito-Hernandez A, Morrison S J and Clarke M F. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci U S A* 2003;7:3983-8

Breast cancer is the most common malignancy in United States women, accounting for >40,000 deaths each year. These breast tumors are comprised of phenotypically diverse populations of breast cancer cells. Using a model in which human breast cancer cells were grown in immunocompromised mice, we found that only a minority of breast cancer cells had the ability to form new tumors. We were able to distinguish the tumorigenic (tumor initiating) from the nontumorigenic cancer cells based on cell surface marker expression. We prospectively identified and isolated the tumorigenic cells as CD44(+)CD24(-/low)Lineage(-) in eight of nine patients. As few as 100 cells with this phenotype were able to form tumors in mice, whereas tens of thousands of cells with alternate phenotypes failed to form tumors. The tumorigenic subpopulation could be serially passaged: each time cells within this population generated new tumors containing additional CD44(+)CD24(-/low)Lineage(-) tumorigenic cells as well as the phenotypically diverse mixed populations of nontumorigenic cells present in the initial tumor. The ability to prospectively identify tumorigenic cancer cells will facilitate the elucidation of pathways that regulate their growth and survival. Furthermore, because these cells drive tumor development, strategies designed to target this population may lead to more effective therapies.
Department of Internal Medicine, Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI 48109, USA.

Alison M R and Islam S. Attributes of adult stem cells. *J Pathol* 2009;2:144-60

While cultured embryonic stem (ES) cells can be harvested in abundance and appear to be the most versatile of cells for regenerative medicine, adult stem cells also hold promise, but the identity and subsequent isolation of these comparatively rare cells remains problematic in most tissues, perhaps with the notable exception of the bone marrow. The ability to continuously self-renew and produce the differentiated progeny of the tissue of their location are their defining properties. Identifying surface molecules (markers) that would aid in stem cell isolation is a major goal. Considerable overlap exists between different putative organ-specific stem cells in their repertoire of gene expression, often related to self-renewal, cell survival and cell adhesion. More robust tests of 'stemness' are now being employed, using lineage-specific genetic marking and tracking to show production of long-lived clones and multipotentiality in vivo. Moreover, the characterization of normal stem cells in specific tissues may provide a dividend for the treatment of cancer. The successful treatment of neoplastic disease may well require the specific targeting of neoplastic stem cells, cells that may well have many of the characteristics of their normal counterparts.
Centre for Diabetes and Metabolic Medicine, St. Bartholomew's and the London School of Medicine and Dentistry, London, UK. m.alison@qmul.ac.uk

Alvarez-Dolado M, Pardal R, Garcia-Verdugo J M, Fike J R, Lee H O, Pfeffer K, Lois C, Morrison S J and Alvarez-Buylla A. Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 2003;6961:968-73

Recent studies have suggested that bone marrow cells possess a broad differentiation potential, being able to form new liver cells, cardiomyocytes and neurons. Several groups have attributed this apparent plasticity to 'transdifferentiation'. Others, however, have suggested that cell fusion could explain these results. Using a simple method based on Cre/lox recombination to detect cell fusion events, we demonstrate that bone-marrow-derived cells (BMDCs) fuse spontaneously with neural progenitors in vitro. Furthermore, bone marrow transplantation demonstrates that BMDCs fuse in vivo with hepatocytes in liver, Purkinje neurons in the brain and cardiac muscle in the heart, resulting in the formation of multinucleated cells. No evidence of transdifferentiation without fusion was observed in these tissues. These observations provide the first in vivo evidence for cell fusion of BMDCs with neurons and cardiomyocytes, raising the possibility that cell fusion may contribute to the development or maintenance of these key cell types.
Department of Neurological Surgery, University of California at San Francisco, San Francisco, California 94143-0520, USA.

Amann J, Holley G P, Lee S B and Edelhauser H F. Increased endothelial cell density in the paracentral and peripheral regions of the human cornea. *Am J Ophthalmol* 2003;5:584-90

PURPOSE: To systematically investigate the central, paracentral, and peripheral endothelial cell density (ECD) in normal human corneas. DESIGN: Observational case series and experimental study. METHODS: Noncontact specular microscopy was undertaken to determine the ECD of the central, paracentral (2.7 +/- 0.2 mm from center) and peripheral (4.7 +/- 0.2 mm from center) regions of the cornea of 48 normal eyes. The ECDs of central and peripheral regions were also determined with contact specular microscopy in 21 normal eyes and a group of 30 Optisol-GS eye bank corneas were evaluated with alizarin red stain. Histologic ECD of 13 Optisol-GS stored corneas were also determined. RESULTS: Paracentral and peripheral ECD measured with the noncontact specular microscope were 5.8% (P <.01) and 9.6% (P <.001) increased compared with central ECD. Superior peripheral ECD was increased compared with the other three peripheral quadrants (P <.05) and was 15.9% higher than central ECD. Contact specular microscopy showed an increase of 8.9% in the peripheral ECD from the center. Alizarin red stained corneas confirmed the specular microscopy numbers with a 9.2% increase in the paracentral region, and a 17.2% increase in the peripheral region. Histological cross sections of human corneas also showed a 22.9% increase in peripheral ECD compared with the central region. CONCLUSIONS: The human cornea has an increased ECD in the paracentral and peripheral regions of cornea compared with the central region. The superior peripheral region of the corneal endothelium has the largest increase in ECD. These data on normal endothelial cell distribution in the human cornea are especially significant as they relate to new surgical techniques and endothelial wound repair.
Emory University Eye Center, Atlanta, Georgia 30322, USA.

Amirjamshidi H, Milani B Y, Sagha H M, Movahedan A, Shafiq M A, Lavker R M, Yue B Y and Djalilian A R. Limbal fibroblast conditioned media: a non-invasive treatment for limbal stem cell deficiency. *Mol Vis* 2011;658-66

PURPOSE: Limbal fibroblasts are known to regulate the maintenance and differentiation of the corneal epithelium including the limbal epithelial stem cells. This study examined the effect of limbal fibroblast conditioned media in a mouse model of limbal stem cell deficiency. METHODS: Limbal stem cell deficiency was created in C57/Bl6 mice by performing a limbus to limbus epithelial debridement. The mice were treated topically for 3 weeks with conditioned media derived from human limbal fibroblasts. The control mice were treated with skin fibroblast conditioned media or Dulbecco's serum-free medium. RESULTS: The mice treated with limbal fibroblast conditioned media demonstrated substantial growth of corneal type epithelial cells on the corneal surface with less conjunctival goblet cells. By contrast, the control treated corneas were found to be covered primarily by conjunctival type epithelium. CONCLUSIONS: Cell culture media conditioned by limbal fibroblasts appear to contain factor(s) that are therapeutically beneficial in a model of limbal stem cell deficiency. The current results further support the notion that the essential limbal stem cell niche is provided by limbal fibroblasts and suggest a new, non-invasive option in the treatment of limbal stem cell deficiency.
Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, IL, USA.

Ang L P, Nakamura T, Inatomi T, Sotozono C, Koizumi N, Yokoi N and Kinoshita S. Autologous serum-derived cultivated oral epithelial transplants for severe ocular surface disease. *Arch Ophthalmol* 2006;11:1543-51

OBJECTIVE: To evaluate the use of autologous serum (AS)-derived cultivated oral epithelial transplants for the treatment of severe ocular surface disease. METHODS: We used AS from 10 patients with severe ocular surface disease and total limbal stem cell deficiency to develop autologous cultivated oral epithelial equivalents. These were compared with epithelial equivalents derived from conventional fetal bovine serum-supplemented medium. Surgery involved removal of the corneal pannus and surrounding diseased tissue and transplantation of the AS-derived epithelial equivalents. The oral equivalents were analyzed by review of histologic and immunohistochemical findings. RESULTS: Oral epithelial sheets cultivated in AS- and fetal bovine serum-supplemented media were similar in morphology, and both formed basement membrane assembly proteins important for maintaining graft integrity. Complete corneal epithelialization was achieved within 2 to 5 days postoperatively. The ocular surface remained stable without major complications in all eyes during a mean +/- SD follow-up of 12.6 +/- 3.9 months. The visual acuity improved by more than 2 lines in 9 of 10 eyes, with transplanted oral epithelium surviving up to 19 months. CONCLUSION: The successful use of an AS-derived oral epithelial equivalent to treat severe ocular surface disease represents an important advance in the pursuit of completely autologous xenobiotic-free bioengineered ocular equivalents for clinical transplantation.
Department of Ophthalmology, Kyoto Prefectural University of Medicine, and Research Center for Regenerative Medicine, Doshisha University, Kyoto, Japan.

Ang L P, Tanioka H, Kawasaki S, Yamasaki K, Do T P, Thein Z M, Koizumi N, Nakamura T, Yokoi N, Komuro A, Inatomi T, Nakatsukasa M and Kinoshita S. Cultivated human conjunctival epithelial transplantation for total limbal stem cell deficiency. *Invest Ophthalmol Vis Sci* 2010;2:758-64

PURPOSE: To determine the feasibility of cultivated conjunctiva as a viable epithelial sheet for transplantation and corneal resurfacing in eyes with limbal stem cell deficiency (LSCD). METHODS: Human corneal epithelial (HCE) and human conjunctival epithelial (HCjE) cells were cultivated on human amniotic membrane (AM) to confluence and then air lifted to allow further stratification and differentiation. Denuded AM and cultivated HCE and cultivated HCjE cells were then transplanted into 18 eyes of rabbits with induced LSCD. The cultivated and engrafted epithelia were examined by transmission electron microscopy (TEM) and immunohistochemistry. Two weeks after transplantation, the eyes were examined by slit lamp biomicroscopy and scored on epithelial integrity, corneal haze, and corneal neovascularization. RESULTS: Both cultivated and engrafted HCjE sheets demonstrated confluent epithelial sheets with five to six layers of well-stratified epithelium. TEM examination of engrafted HCjE revealed numerous microvilli, desmosomes, and hemidesmosomes, identical with in vivo corneal epithelium. Immunohistochemical analysis of both HCjE and HCE cells showed the presence of CK3, CK4, and CK12, with absence of Muc5AC. Clinical outcomes for eyes receiving HCjE transplants and HCE transplants were comparable, with most having transparent, smooth corneas, free of epithelial defects. CONCLUSIONS: The study showed that microscopically, HCjE cells have features similar to HCE cells, with clinically equivalent outcomes. The ex vivo cultivation of conjunctiva to form transplantable epithelial sheets for corneal replacement is a promising new treatment modality in patients with LSCD.
The Eye and Cornea Transplant Centre, Singapore.

Arai F and Suda T. Quiescent stem cells in the niche. 2008;

Quiescence of stem cells is critical to ensure lifelong tissue maintenance and to protect the stem cell pool from premature exhaustion under conditions of various stresses. The long-term maintenance of stem cells largely depends on the interaction with their specific microenvironments, niches. Detailed studies in recent years elucidated that the interaction of stem cells with their specialized microenvironment "niche" is critical to sustain stem cell pools in tissues over long periods and that the stem cell niche regulates stem cell-specific properties, including self-renewal, multi-potentiality, and relative quiescence in cell cycle. Here we discuss quiescent stem cells in the adult hematopoietic system and describe the regulatory mechanisms of hematopoietic stem cell (HSC) quiescence in bone marrow. Niches for HSCs have been identified in the endosteal region "osteoblastic niche" and a perivascular area "vascular niche". Although the functional differences of these two niches still need to be investigated, HSCs are maintained via a complex interaction of cells, cytokines, adhesion molecules, and extracellular matrix. Quiescent stem cells in the hypoxic niche might be sensitive to reactive oxygen species (ROS) and show glycolysis-dependent metabolism.

Avilion A A, Nicolis S K, Pevny L H, Perez L, Vivian N and Lovell-Badge R. Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev* 2003;1:126-40

Each cell lineage specified in the preimplantation mammalian embryo depends on intrinsic factors for its development, but there is also mutual interdependence between them. OCT4 is required for the ICM/epiblast lineage, and at transient high levels for extraembryonic endoderm, but also indirectly through its role in regulating Fgf4 expression, for the establishment and proliferation of extraembryonic ectoderm from polar trophectoderm. The transcription factor SOX2 has also been implicated in the regulation of Fgf4 expression. We have used gene targeting to inactivate Sox2, examining the phenotypic consequences in mutant embryos and in chimeras in which the epiblast is rescued with wild-type ES cells. We find a cell-autonomous requirement for the gene in both epiblast and extraembryonic ectoderm, the multipotent precursors of all embryonic and trophoblast cell types, respectively. However, an earlier role within the ICM may be masked by the persistence of maternal protein, whereas the lack of SOX2 only becomes critical in the chorion after 7.5 days postcoitum. Our data suggest that maternal components could be involved in establishing early cell fate decisions and that a combinatorial code, requiring SOX2 and OCT4, specifies the first three lineages present at implantation.
Division of Developmental Genetics, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

Beckstead J H. A simple technique for preservation of fixation-sensitive antigens in paraffin-embedded tissues. *J Histochem Cytochem* 1994;8:1127-34

Immunohistochemistry is a powerful tool for tissue diagnosis and research. Although the frozen section has remained the gold standard for this important approach to evaluation of antigens in tissues, there is widespread acknowledgment of many limitations. Routine paraffin-embedded sections ware widely used for morphological examination of tissues but are not optimal for antigen preservation. In this study, paraffin-embedded tissues fixed with a simple buffer containing zinc as the primary fixative were compared with tissues fixed with routine formalin, zinc-formalin, paraformaldehyde, ethanol, a variety of commercial (non-formalin-containing) fixatives that have been recommended for

reduced toxicity and improved antigen survival, and frozen sections. Human lymphoid tissues and a group of antibodies to antigens (CD1, CD4, CD7, CD8, CD19) usually preserved only in frozen tissue were used as a model system. Fixation in a simple solution of zinc acetate and zinc chloride in a Tris-Ca acetate buffer resulted in antigen preservation comparable to that in frozen sections with antibodies to these cell surface markers. Morphological preservation was comparable to formalin-fixed sections. The work presents a new method that represents the closest approach yet to a technique that combines optimal antigenic survival with the convenience and morphological preservation of traditional formalin-fixed tissue embedded in paraffin.
Department of Pathology, Oregon Health Sciences University, Portland 97201.

Bednarz J, Rodokanaki-von Schrenck A and Engelmann K. Different characteristics of endothelial cells from central and peripheral human cornea in primary culture and after subculture. *In Vitro Cell Dev Biol Anim* 1998;2:149-53

Several methods for isolation and cultivation of human corneal endothelial cells have been described during the last few decades. In contrast to the situation in vivo, the cultured cells show mitogenic activity but often lose their typical morphological appearance. In this paper, we describe a technique to isolate and cultivate morphologically unchanged endothelium from the human cornea. This method revealed different characteristics of endothelial cells according to their position within the human cornea. Endothelial cells isolated from the central part have a morphology similar to that of cells in vivo (i.e., they are densely packed and show no mitogenic activity). In contrast, endothelial cells derived from the peripheral part of the cornea are characterized by mitogenic activity but their cell-to-cell attachment seems to be less tight than in vivo. The significance of these two different endothelial cell types for wound healing in the human cornea is discussed.
University of Hamburg, Department of Ophthalmology, Germany.

Bendayan M and Zollinger M. Ultrastructural localization of antigenic sites on osmium-fixed tissues applying the protein A-gold technique. *J Histochem Cytochem* 1983;1:101-9

The protein A-gold immunocytochemical technique has been modified to allow labeling of cellular antigenic sites on osmium-fixed or postfixed tissues. Several strong oxidizing agents have been found able to restore protein antigenicity on osmicated tissue thin sections. According to the fine structural preservation and intensities of labeling, pretreatment with sodium metaperiodate gave optimal results. Pancreatic secretory proteins (and/or proproteins) as well as insulin (and/or proinsulin) were localized over perfectly preserved rough endoplasmic reticulum (rER), Golgi apparatus, and secretory granules of the corresponding pancreatic cells; carbamyl phosphate synthetase and catalase were revealed over liver mitochondria and peroxisomes, respectively. In addition to the higher resolution in the labeling obtained using osmium-fixed tissues, the present modification confers an additional advantage to the protein A-gold technique by allowing labeling on tissues processed for routine electron microscopy.

Blazejewska E A, Schlotzer-Schrehardt U, Zenkel M, Bachmann B, Chankiewitz E, Jacobi C and Kruse F E. Corneal limbal microenvironment can induce transdifferentiation of hair follicle stem cells into corneal epithelial-like cells. *Stem Cells* 2009;3:642-52

The aim of this study was to investigate the transdifferentiation potential of murine vibrissa hair follicle (HF) stem cells into corneal epithelial-like cells through modulation by corneal- or limbus-specific microenvironmental factors. Adult epithelial stem cells were isolated from the HF bulge region by mechanical dissection or fluorescence-activated cell sorting using antibodies to alpha6 integrin, enriched by clonal expansion, and subcultivated on various extracellular matrices (type IV collagen, laminin-1, laminin-5, fibronectin) and in different conditioned media derived from central and peripheral corneal fibroblasts, limbal stromal fibroblasts, and 3T3 fibroblasts. Cellular phenotype and differentiation were evaluated by light and electron microscopy, real-time reverse transcription-polymerase chain reaction, immunocytochemistry, and Western blotting, using antibodies against putative stem cell markers (K15, alpha6 integrin) and differentiation markers characteristic for corneal epithelium (K12, Pax6) or epidermis (K10). Using laminin-5, a major component of the corneo-limbal basement membrane zone, and conditioned medium from limbal stromal fibroblasts, clonally enriched HF stem and progenitor cells adhered rapidly and formed regularly arranged stratified cell sheets. Conditioned medium derived from limbal fibroblasts markedly upregulated expression of cornea-specific K12 and Pax6 on the mRNA and protein level, whereas expression of the epidermal keratinocyte marker K10 was strongly downregulated. These findings suggest that adult HF epithelial stem cells are capable of differentiating into corneal epithelial-like cells in vitro when exposed to a limbus-specific microenvironment. Therefore, the HF may be an easily accessible alternative therapeutic source of autologous adult stem cells for replacement of the corneal epithelium and restoration of visual function in patients with ocular surface disorders.
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Bohnke M, Eggli P and Engelmann K. Transplantation of cultured adult human or porcine corneal endothelial cells onto human recipients in vitro. Part II: Evaluation in the scanning electron microscope. *Cornea* 1999;2:207-13

PURPOSE: To evaluate the morphology of endothelial monolayers, which have been regrafted onto the denuded Descemet's membrane, with scanning electron microscopy (SEM). METHODS: Material derived from each of the experimental groups described in part I of this investigation was evaluated in the current study. Recipient corneas, denuded of their native endothelium by mechanical, chemical, or physical debridement, were examined to assess the effectiveness of each technique in killing and removing cells. Porcine or human donor corneal endothelial cells maintained in monolayer culture for up to 10 passages then were seeded onto the denuded Descemet's membranes of recipients in the absence or presence of fibroblast growth factor (FGF). The monolayers thereby established were examined in the SEM, and the morphologic status of individual cells compared with that manifested in normal human donor corneas maintained for 4 weeks in organ culture (reference control). Isolated and cultured human keratocytes regrafted onto the denuded Descemet's membranes of recipient corneas served as nonendothelial control specimens. Tissue was processed for examination in the SEM according to standard techniques. RESULTS: Each of the three methods used to strip recipient corneas of their native endothelium was effective and elicited no gross structural damage to Descemet's membrane. Some small focal defects within this latter layer were, however, observed, these being encountered at higher frequency after mechanical debridement than after chemical or physical stripping. Porcine or human endothelial cells seeded onto the denuded Descemet's membranes of recipient corneas formed stable monolayers. The morphologic status of regrafted cells corresponded to that manifested in monolayer cultures before seeding, porcine ones always being more differentiated than their human counterparts. Poorly differentiated human endothelial cells had a slender, elongated, fibroblast-like appearance, whereas more highly differentiated ones manifested broad, flat, polygonal profiles. Monolayers covered the entire corneal surface and impinged to a variable degree onto the trabecular meshwork, at which juncture cells always assumed a less well-differentiated morphology. FGF consistently effected an increase in differentiation status, and as this became augmented, the capacity of monolayers to violate the corneal-trabecular meshwork border was correspondingly repressed. Seeded keratocytes formed dense, multilayered sheaths, resembling retrocorneal membranes, across the entire corneal surface, trabecular meshwork, and iris root. The surface characteristics of the constituent cells were quite distinct from those manifested by endothelial cells, even the least well-differentiated ones. CONCLUSION: Regrafting of human corneal endothelial cells onto the denuded Descemet's membranes of recipients resulted in the formation of stable monolayers. Because the morphologic status of seeded cells closely mimicked that manifested in monolayer cultures before transplantation, it may be anticipated that efforts to refine and optimize culturing conditions would yield improvements in this parameter after regrafting. If these expectations can be realized, then the possibility of successfully establishing a "new" and functional endothelium on recipient corneas destined for clinical grafting may well be brought to fruition in the not-too-distant future.
Department of Ophthalmology, University of Hamburg, Germany.

Bonnet D and Dick J E. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997;7:730-7

On the subject of acute myeloid leukemia (AML), there is little consensus about the target cell within the hematopoietic stem cell hierarchy that is susceptible to leukemic transformation, or about the mechanism that underlies the phenotypic, genotypic and clinical heterogeneity. Here we demonstrate that the cell capable of initiating human AML in non-obese diabetic mice with severe combined immunodeficiency disease (NOD/SCID mice) - termed the SCID leukemia-initiating cell, or SL-IC - possesses the differentiative and proliferative capacities and the potential for self-renewal expected of a leukemic stem cell. The SL-ICs from all subtypes of AML analyzed, regardless of the heterogeneity in maturation characteristics of the leukemic blasts, were exclusively CD34++ CD38-, similar to the cell-surface phenotype of normal SCID-repopulating cells, suggesting that normal primitive cells, rather than committed progenitor cells, are the target for leukemic transformation. The SL-ICs were able to differentiate in vivo into leukemic blasts, indicating that the leukemic clone is organized as a hierarchy.
Department of Genetics, Research Institute, Hospital for Sick Children, University of Toronto, Ontario, Canada.

Boyd A S, Higashi Y and Wood K J. Transplanting stem cells: potential targets for immune attack. Modulating the immune response against embryonic stem cell transplantation. *Adv Drug Deliv Rev* 2005;13:1944-69

The curative promise of stem cells and their descendants for tissue regeneration and repair is currently the subject of an intense research effort worldwide. If it proves feasible to differentiate stem cells into specific tissues reliably and safely, this approach will be invaluable in the treatment of diseases that lead to organ degeneration or failure, providing an alternative or supplementary source of tissue for transplantation. Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of a pre-implantation blastocyst that can produce all cells and tissues of the foetus. In recent years, several laboratories have described the directed differentiation of ES cells into multiple mature cell types including: cardiomyocytes; haemopoietic cells; hepatocytes; neurones; muscle cells and both endocrine and exocrine cells of the pancreas. How the immune system of the host will respond when these ES cell-derived mature cells are transplanted is ill defined. This review will focus on the potential mechanisms that the immune system could use to target ES cell-derived transplants and how unwanted responses might be prevented.
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Burry R W. Controls for immunocytochemistry: an update. *J Histochem Cytochem* 2011;1:6-12

Immunocytochemistry is a highly productive method in biomedical research used to identify proteins and other macromolecules in tissues and cells. Control samples are required to show label localization is correct, but the understanding and use of immunocytochemistry controls have been inconsistent. A new classification of immunocytochemical controls is proposed that will help in understanding this most important component of the experiment. The three types of controls required for immunocytochemistry are primary antibody controls that show the specificity of the primary antibody binding to the antigen, secondary antibody controls that show the label is specific to the primary antibody, and label controls that show the labeling is the result of the label added and not the result of endogenous labeling. Publications containing immunocytochemical results must give details of how these controls were performed.
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Caplan A I. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. *Tissue Eng* 2005;7-8:1198-211

Adult stem cells provide replacement and repair descendants for normal turnover or injured tissues. These cells have been isolated and expanded in culture, and their use for therapeutic strategies requires technologies not yet perfected. In the 1970s, the embryonic chick limb bud mesenchymal cell culture system provided data on the differentiation of cartilage, bone, and muscle. In the 1980s, we used this limb bud cell system as an assay for the purification of inductive factors in bone. In the 1990s, we used the expertise gained with embryonic mesenchymal progenitor cells in culture to develop the technology for isolating, expanding, and preserving the stem cell capacity of adult bone marrow-derived mesenchymal stem cells (MSCs). The 1990s brought us into the new field of tissue engineering, where we used MSCs with site-specific delivery vehicles to repair cartilage, bone, tendon, marrow stroma, muscle, and other connective tissues. In the beginning of the 21st century, we have made substantial advances: the most important is the development of a cell-coating technology, called painting, that allows us to introduce informational proteins to the outer surface of cells. These paints can serve as targeting addresses to specifically dock MSCs or other reparative cells to unique tissue addresses. The scientific and clinical challenge remains: to perfect cell-based tissue-engineering protocols to utilize the body's own rejuvenation capabilities by managing surgical implantations of scaffolds, bioactive factors, and reparative cells to regenerate damaged or diseased skeletal tissues.
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Caplan A I. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J Cell Physiol* 2007;2:341-7

Adult mesenchymal stem cells (MSCs) can be isolated from bone marrow or marrow aspirates and because they are culture-dish adherent, they can be expanded in culture while maintaining their multipotency. The MSCs have been used in preclinical models for tissue engineering of bone, cartilage, muscle, marrow stroma, tendon, fat, and other connective tissues. These tissue-engineered materials show considerable promise for use in rebuilding damaged or diseased mesenchymal tissues. Unanticipated is the realization that the MSCs secrete a large spectrum of bioactive molecules. These molecules are immunosuppressive, especially for T-cells and, thus, allogeneic MSCs can be considered for therapeutic use. In this context, the secreted bioactive molecules provide a regenerative microenvironment for a variety of injured adult tissues to limit the area of damage and to mount a self-regulated regenerative response. This regenerative microenvironment is referred to as trophic activity and, therefore, MSCs appear to be valuable mediators for tissue repair and regeneration. The natural titers of MSCs that are drawn to sites of tissue injury can be augmented by allogeneic MSCs delivered via the bloodstream. Indeed, human clinical trials are now under way to use allogeneic MSCs for treatment of myocardial infarcts, graft-versus-host disease, Crohn's Disease, cartilage and meniscus repair, stroke, and spinal cord injury. This review summarizes the biological basis for the in vivo functioning of MSCs through development and aging.
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Cartwright P, McLean C, Sheppard A, Rivett D, Jones K and Dalton S. LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. *Development* 2005;5:885-96

Murine ES cells can be maintained as a pluripotent, self-renewing population by LIF/STAT3-dependent signaling. The downstream effectors of this pathway have not been previously defined. In this report, we identify a key target of the LIF self-renewal pathway by showing that STAT3 directly regulates the expression of the Myc transcription factor. Murine ES cells express elevated levels of Myc and following LIF withdrawal, Myc mRNA levels collapse and Myc protein becomes phosphorylated on threonine 58 (T58), triggering its GSK3beta dependent degradation. Maintained expression of stable Myc (T58A) renders self-renewal and maintenance of pluripotency independent of LIF. By contrast, expression of a dominant negative form of Myc antagonizes self-renewal and promotes differentiation. Transcriptional control by STAT3 and suppression of T58 phosphorylation are crucial for regulation of Myc activity in ES cells and therefore in promoting self-renewal. Together, our results establish a mechanism for how LIF and STAT3 regulate ES cell self-renewal and pluripotency.
University of Georgia, Rhodes Center, 425 River Road, Athens, GA 30602-2771, USA.

Caussinus E and Gonzalez C. Induction of tumor growth by altered stem-cell asymmetric division in Drosophila melanogaster. *Nat Genet* 2005;10:1125-9

Loss of cell polarity and cancer are tightly correlated, but proof for a causative relationship has remained elusive. In stem cells, loss of polarity and impairment of asymmetric cell division could alter cell fates and thereby render daughter cells unable to respond to the mechanisms that control proliferation. To test this hypothesis, we generated Drosophila melanogaster larval neuroblasts containing mutations in

various genes that control asymmetric cell division and then assayed their proliferative potential after transplantation into adult hosts. We found that larval brain tissue carrying neuroblasts with mutations in raps (also called pins), mira, numb or pros grew to more than 100 times their initial size, invading other tissues and killing the hosts in 2 weeks. These tumors became immortal and could be retransplanted into new hosts for years. Six weeks after the first implantation, genome instability and centrosome alterations, two traits of malignant carcinomas, appeared in these tumors. Increasing evidence suggests that some tumors may be of stem cell origin. Our results show that loss of function of any of several genes that control the fate of a stem cell's daughters may result in hyperproliferation, triggering a chain of events that subverts cell homeostasis in a general sense and leads to cancer.
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Cegielski M, Calkosinski I, Dziegiel P and Zabel M. The search for stem cells of the epithelium in pulmonary alveoli. *Folia Morphol (Warsz)* 2004;2:221-3

In recent years significant progress has been witnessed in the identification of stem cells, which have now also been identified in the lungs. The aim of this was to induce post-pneumonia alveolar regeneration to facilitate the identification of stem cells. The studies were performed on Buffalo strain rats. Pneumonia was induced in the animals by a sub-pleural injection of carragenin. On days 4, 5 and 10 of the experiment both the control and experimental animals received intraperitoneal injections of bromodeoxyuridine (BrdU). Twenty-four hours after the last BrdU injection the rats were sacrificed and samples of the lungs were taken for examination. In order to detect proliferating cells in the paraffin sections, BrdU incorporation was detected with monoclonal antibodies. In pilot experiments BrdU incorporation was demonstrated in individual alveolar cells of variable distribution and of variable intensity in the colour reaction. The results have confirmed the existence of stem cells in pulmonary alveoli but their closer characterisation requires further studies with other techniques to detect pulmonary stem cells.
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Challen G A and Little M H. A side order of stem cells: the SP phenotype. *Stem Cells* 2006;1:3-12

A defining property of murine hematopoietic stem cells (HSCs) is low fluorescence after staining with Hoechst 33342 and Rhodamine 123. These dyes have proven to be remarkably powerful tools in the purification and characterization of HSCs when used alone or in combination with antibodies directed against stem cell epitopes. Hoechst low cells are described as side population (SP) cells by virtue of their typical profiles in Hoechst red versus Hoechst blue bivariate fluorescent-activated cell sorting dot plots. Recently, excitement has been generated by the findings that putative stem cells from solid tissues may also possess this SP phenotype. SP cells have now been isolated from a wide variety of mammalian tissues based on this same dye efflux phenomenon, and in many cases this cell population has been shown to contain apparently multipotent stem cells. What is yet to be clearly addressed is whether cell fusion accounts for this perceived SP multipotency. Indeed, if low fluorescence after Hoechst staining is a phenotype shared by hematopoietic and organ-specific stem cells, do all resident tissue SP cells have bone marrow origins or might the SP phenotype be a property common to all stem cells? Subject to further analysis, the SP phenotype may prove invaluable for the initial isolation of resident tissue stem cells in the absence of definitive cell-surface markers and may have broad-ranging applications in stem cell biology, from the purification of novel stem cell populations to the development of autologous stem cell therapies.
Institute for Molecular Bioscience, Queensland Bioscience Precinct, 306 Carmody Road, The University of Queensland, St. Lucia, Brisbane, QLD, 4072, Australia.

Chan R W and Gargett C E. Identification of label-retaining cells in mouse endometrium. *Stem Cells* 2006;6:1529-38

Human and mouse endometrium (lining of the uterus) undergo cycles of growth and regression as part of each reproductive cycle. A well-known method to identify somatic stem/progenitor cells and their location in the stem cell niche is the label-retaining cell (LRC) approach. We hypothesized that mouse endometrium contains small populations of both epithelial and stromal somatic stem/progenitor cells that may be detected by the LRC technique. The overall objective of this study was to identify and quantify LRCs in mouse endometrium, to determine their location, and to identify their niche in this highly regenerative tissue. Endometrium was labeled for 3 days with bromodeoxyuridine (BrdU) in postnatal day 3 (P3) mice prior to gland development and prepubertal (P19) mice after glands had formed, followed by chase periods of up to 12 weeks. After an 8-week chase, 3% of epithelial nuclei immunostained with BrdU antibody and were considered epithelial LRCs. These were primarily located in the luminal epithelium. Epithelial LRCs did not express estrogen receptor-alpha (ER-alpha). Stromal LRCs (6%) were found adjacent to luminal epithelium, at the endometrial-myometrial junction, and near blood vessels after a 12-week chase. Stromal LRCs were stem cell antigen-1, CD45(-), and some (16%) expressed ER-alpha, indicating their capacity to respond to estrogen and transmit paracrine signals to epithelial cells for endometrial epithelium regeneration. Both epithelial LRCs and some stromal LRCs, mainly located at the endometrial-myometrial junction, were recruited into the cell cycle after estrogen-stimulated endometrial regeneration, indicating a functional response to proliferative signals. This study has demonstrated for the first time the presence of both epithelial and stromal LRCs in mouse endometrium, suggesting that these stem-like cells may be responsible for endometrial regeneration.
Centre for Women's Health Research, Monash University Department of Obstetrics and Gynaecology, Monash Medical Centre, Clayton, VIC, Australia.

Chang C Y, Green C R, McGhee C N and Sherwin T. Acute wound healing in the human central corneal epithelium appears to be independent of limbal stem cell influence. *Invest Ophthalmol Vis Sci* 2008;12:5279-86

PURPOSE: In the adult cornea, epithelial cells are maintained by limbal stem cells (LSCs) that cycle slowly and give rise to transient amplifying (TA) cells. These migrate centripetally, differentiate outward to the surface, and are then lost by desquamation. This study was conducted to analyze the contribution of human central corneal epithelial cells toward corneal epithelial regeneration. METHODS: A human corneal organotypic culture model was used to assess corneal healing in vitro in 12 matched cornea pairs. Two types of ablation were studied: (1) A ring-shaped, transepithelial, excimer laser (193 nm) ablation, of 7 mm outer diameter and 3 mm inner diameter, to a depth of 80 mum-sparing the central and peripheral corneal epithelium; and (2) an ablation pattern identical to that in (1) with ablation of the limbal epithelium in addition. Corneal healing was followed using time-lapse dark-field microscopy for up to 12 hours, and the corneas were analyzed by using immunohistochemical markers for cell proliferation and stem cells. RESULTS: In the donut model, corneal epithelial repair originated from both the limbus and the central corneal epithelium with the average rate of epithelial recovery from the center being similar to the rate from the periphery (0.06 +/- 0.01 mm/h vs. 0.07 +/- 0.03 mm/h, P = 0.44). When the area of recovery was calculated relative to original edge circumferences, the central epithelial rate tended to be faster than the peripheral (0.06 +/- 0.02 mm(2)/mm/h vs. 0.04 +/- 0.01 mm(2)/mm/h, P = 0.04). Similar rates in epithelial recovery were identified in centripetal and centrifugal directions in both the donut and donut+limbus ablation models. Central epithelial cell density increased 36% over the control cornea within 12 hours after surgery, but there was no change at the periphery. Cell proliferation, assessed using Ki67 and BrdU labeling, was observed across the entire cornea. Expression of the putative stem cell markers p63 and ABCG2 was clearly evident in the basal layer of the limbus. However, weaker labeling was also observed in the central epithelium. Connexin 43 (Cx43), a differentiation marker, was mainly absent in the normal untreated limbal basal cells, but more Cx43-positive cells were labeled in the basal layer of the limbus after wounding. CONCLUSIONS: After wounding, the capacity for epithelial cell proliferative and migration appears to be as active in the central cornea as in the periphery/limbus. Central and peripheral epithelial recovery remains equal even after ablation of the limbus. Central human corneal epithelial cells are therefore capable of corneal epithelial regeneration, at least in the first 12 hours after wounding.
Department of Ophthalmology, Faculty of Medical and Health Sciences, University of Auckland, Auckland, New Zealand.

Chen J J and Tseng S C. Abnormal corneal epithelial wound healing in partial-thickness removal of limbal epithelium. *Invest Ophthalmol Vis Sci* 1991;8:2219-33

Limbal basal epithelium is thought to possess corneal epithelial stem cells that are the ultimate source of corneal epithelial proliferation and differentiation during corneal epithelial wound healing. Destruction of the limbal epithelium results in corneal conjunctivalization and vascularization, suggesting that the limbal epithelium also may be a barrier between corneal and conjunctival epithelia. In this experiment, a total corneal epithelial debridement using combined n-heptanol and mechanical scraping was created immediately (one-step) or 5 weeks (two-step) after 15 or 30 sec n-heptanol treatment at the limbus. All defects healed in 1-2 weeks. The severity of corneal vascularization, as judged by external photography, followed the ascending order of 30-sec two-step and 15-sec two-step less than 15-sec one-step less than 30-sec one-step (P less than 0.005). Immunofluorescence studies using monoclonal antibodies AM-3 and AE-5 showed mixed expression of corneal and conjunctival epithelial phenotypes on the corneal surface in the one-step subgroups. By contrast, the two-step subgroups had a normal corneal epithelial phenotype. Impression cytology was used to map goblet-cell distribution on the perilimbal corneal surface. The specimens taken from superior, temporal, and inferior bulbar areas were evaluated by a scoring system at different times. The extent of goblet cells invading onto the corneal surface also followed the same ascending order (P = 0.005). A transient goblet-cell surge was noted, and the extent was related to the extent of corneal vascularization. It is thus evident that in vivo n-heptanol treatment for different durations can result in different extents of corneal conjunctivalization and vascularization. The authors concluded that the capability of the remaining limbal basal epithelium to recover its original full-thickness stratified layers determines the strength of the limbal barrier.
Department of Ophthalmology, Univeristy of Miami School of Medicine, Bascom Palmer Eye Institute, FL 33101.

Chen K H, Azar D and Joyce N C. Transplantation of adult human corneal endothelium ex vivo: a morphologic study. *Cornea* 2001;7:731-7

PURPOSE: To investigate the feasibility of transplanting untransformed human corneal endothelial cells as a treatment strategy and possible alternative for penetrating keratoplasty by growing donor cells in culture and then transplanting them to denuded Descemet's membrane of recipient corneas. METHODS: Corneas from adult donors (50-80 years old) were obtained from eye banks. To grow corneal endothelial cells, Descemet's membrane with associated cells was dissected from the stroma. Endothelial cells were released by ethylenediaminetetraacetic acid treatment, grown in medium containing multiple growth factors, and identified as being of endothelial origin by morphology and by reverse-transcription polymerase chain reaction for keratin 12 and collagen type VIII. In transplantation experiments, cultured cells were seeded onto denuded Descemet's membrane of a second donor cornea at 5 x 10(5) cells/mL. The recipient cornea was incubated in organ culture for as long as 2 weeks. The morphology and ultrastructure of the endothelium were evaluated 7 and 14 days after transplantation by transmission electron microscopy, and by immunolocalization of zonula occludins-1 (ZO-1). Endothelial cell density was calculated in transplants by counting ZO-1-stained cells. RESULTS: Corneal endothelial cells cultured from adult donors consistently grew well in culture medium. Cells were identified as corneal endothelium by characteristic morphology and messenger RNA expression. Morphologic and ultrastructural studies of corneas containing transplanted endothelial cells demonstrated that with time there was an increase in endothelial cell-Descemet's membrane adhesion, in the extent of cell-cell contacts and lateral interdigitation, and in formation of a single cell layer. ZO-1 staining revealed tight junction formation similar to that of corneas in vivo. Mean endothelial cell density in transplanted corneas was 1,895 cells/mm(2) (range, 1,503-2,159 cells/mm(2)). CONCLUSION: Untransformed adult human corneal endothelial cells can be efficiently and consistently cultured and transplanted onto denuded Descemet's membrane. Transplanted cells in organ culture exhibit morphologic characteristics and cell densities similar to corneal endothelial cells in vivo. These results provide evidence for the feasibility of developing methods for in vivo transplantation of untransformed corneal endothelial cells cultured from adult donor tissue.
Schepens Eye Research Institute and the Department of Ophthalmology, Harvard Medical School, 20 Staniford St., Boston, MA 02114-2500, U.S.A.

Chen Y T, Li W, Hayashida Y, He H, Chen S Y, Tseng D Y, Kheirkhah A and Tseng S C. Human amniotic epithelial cells as novel feeder layers for promoting ex vivo expansion of limbal epithelial progenitor cells. *Stem Cells* 2007;8:1995-2005

Human amniotic epithelial cells (HAECs) are a unique embryonic cell source that potentially can be used as feeder layers for expanding different types of stem cells. In vivo, HAECs uniformly expressed pan-cytokeratins (pan-CK) and heterogeneously expressed vimentin (Vim). The two phenotypes expressing either pan-CK(+)/Vim(+) or pan-CK(+)/Vim(-) were maintained in serum-free media with high calcium. In contrast, all HAECs became pan-CK(+)/Vim(+) in serum-containing media, which also promoted HAEC proliferation for at least eight passages, especially supplemented with epidermal growth factor and insulin. Mitomycin C-arrested HAEC feeder layers were more effective in promoting clonal growth of human limbal epithelial progenitors than conventional 3T3 murine feeder layers. Cells in HAEC-supported clones were uniformly smaller, sustained more proliferation, and expressed less CK12 and connexin 43 but higher levels of stem cell-associated markers such as p63, Musashi-1, and ATP-binding cassette subfamily G2 than those of 3T3-supported clones. Subculturing of clonally expanded limbal progenitors from HAEC feeder layers, but not from 3T3 feeder layers, gave rise to uniformly p63-positive epithelial progenitor cells as well as nestin-positive neuronal-like progenitors. Collectively, these results indicated that HAECs can be used as a human feeder layer equivalent for more effective ex vivo expansion of adult epithelial stem cells from the human limbus. Disclosure of potential conflicts of interest is found at the end of this article.
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Cheng C Y, Sun C C, Yu W H, Hsieh H L, Ma D H, Pang J H and Yang C M. Novel laminin 5 gamma 2-chain fragments potentiating the limbal epithelial cell outgrowth on amniotic membrane. *Invest Ophthalmol Vis Sci* 2009;10:4631-9

PURPOSE: Matrix metalloproteases (MMPs)-mediated extracellular matrix (ECM) degradation potentially releases cryptic motility factors involved in somatic stem cell migration and epithelial outgrowth. The authors previously demonstrated that MMP-9 is upregulated in limbal epithelial cells cultivated on amniotic membrane (AM). Here, the authors further investigated whether plasminogen activator (PA)/plasmin regulates MMP-9 activity in this model implicated in the processing of laminin 5 (Ln5), a component of amniotic basement membrane. METHODS: Limbal epithelial cells migrated from limbal explants were expanded on intact AM. The activities and proteins of uPA and MMP-9 in limbal epithelial cells were determined by fibrin and gelatin zymography, reverse transcription-polymerase chain reaction (RT-PCR), Western blotting, and immunofluorescent staining. Specific pharmacological inhibitors including MMPs inhibitor GM6001, MMP-2/-9 inhibitor, and uPA inhibitor B428 were used to determine whether the PA/plasmin/MMP-9 axis induces cell growth via Ln5 in this model. RESULTS: These data showed that MMP-9 activity was attenuated by a selective uPA inhibitor, B428. Furthermore, MMP-9 activity was enhanced by exogenous addition or pre-incubation with plasmin. These results demonstrated that PA/plasmin regulates MMP-9 expression. An interesting proteolytic fragment of Ln5 gamma 2-chain was suppressed by pretreatment with GM6001, B428, or neutralizing antibodies of MMP-9 and uPA, indicating that Ln5 gamma 2-chain is processed by uPA/MMP-9. Moreover, the extent of limbal outgrowth was also retarded by B428. CONCLUSIONS: This study suggested that MMP-9 activity was upregulated by PA/plasmin, which in turn processed Ln5 gamma 2-chain to facilitate limbal outgrowth on intact AM.
Department of Physiology, Chang Gung University, Tao-Yuan, Taiwan.

Choi J S, Williams J K, Greven M, Walter K A, Laber P W, Khang G and Soker S. Bioengineering endothelialized neo-corneas using donor-derived corneal endothelial cells and decellularized corneal stroma. *Biomaterials* 2010;26:6738-45

Corneal transplantation is a common transplant procedure performed to improve visual acuity by replacing the opaque or distorted host tissue by clear healthy donor tissue. However, its clinical utility is limited due to a lack of high quality donor corneas. Bioengineered neo-corneas, created using an expandable population of human donor-derived corneal endothelial cells (HCEC), could address this current shortage. The objectives of this study were to establish HCEC isolation and culture protocols and to investigate the feasibility of bioengineering corneal tissue constructs by seeding the cells on decellularized human corneal stroma. HCECs were removed from the discarded corneas of eye donors by enzymatic digestion. Cells were expanded and evaluated for their expression of Na(+)/K(+)-ATPase and zona occludens-1 (ZO-1). Donor corneal stromas were cut to 120-200 microm thickness slices using a microtome and then decellularized. Extracellular matrix components and mechanical properties of the scaffolds were measured after decellularization. To engineer neo-corneas,

130 HCEC/mm(2) were seeded on decellularized human corneal stromas. The resulting constructs were placed in growth medium for 14 days and then analyzed using scanning electron microscopy (SEM), histology, and immunocytochemistry. Seeded cells retain expression of the functional markers Na(+)/K(+)-ATPase and ZO-1 and constructs have biomechanical properties similar to those of normal corneas. These results indicate that construction of neo-corneas, using HCECs derived from discarded donor corneas and decellularized thin-layer corneal stromas, may create a new source of high quality corneal tissue for transplantation.
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Chun Y S, Chaudhari P and Jang Y Y. Applications of patient-specific induced pluripotent stem cells; focused on disease modeling, drug screening and therapeutic potentials for liver disease. *Int J Biol Sci* 2010;7:796-805

The recent advances in the induced pluripotent stem cell (iPSC) research have significantly changed our perspectives on regenerative medicine by providing researchers with a unique tool to derive disease-specific stem cells for study. In this review, we describe the human iPSC generation from developmentally diverse origins (i.e. endoderm-, mesoderm-, and ectoderm- tissue derived human iPSCs) and multistage hepatic differentiation protocols, and discuss both basic and clinical applications of these cells including disease modeling, drug toxicity screening/drug discovery, gene therapy and cell replacement therapy.
Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21231, USA.

Connon C J, Kawasaki S, Liles M, Koizumi N, Yamasaki K, Nakamura T, Quantock A J and Kinoshita S. Gene expression and immunolocalisation of a calcium-activated chloride channel during the stratification of cultivated and developing corneal epithelium. *Cell Tissue Res* 2006;1:177-82

The spatial and temporal localisation of a calcium-activated chloride channel (CLCA) and its mRNA was investigated, during the in vivo and in vitro development of stratified epithelia, by fluorescence immunohistochemistry and quantitative polymerase chain reaction in embryonic chicken corneas and the expansion of excised human corneal stem cells on amniotic membrane. Single-layered human epithelial cultures on amniotic membrane and early day embryonic chicken corneas expressed relatively little human CLCA2 or its chicken homologue. However, as the epithelium in both models matured and the number of cell-layers increased, the gene expression level and protein staining intensity increased, primarily within the basal cells of both the cultured and embryonic tissues. These results demonstrate that human CLCA2 protein and mRNA expression are elevated during epithelial stratification, suggesting that this protein plays a role in the growth of multi-layered corneal epithelia during both natural development and tissue cultivation.
Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan. connoncj@cf.ac.uk

Copeland R A, Jr. and Char D H. Limbal autograft reconstruction after conjunctival squamous cell carcinoma. *Am J Ophthalmol* 1990;4:412-5

Two patients who had squamous cell carcinoma with extensive limbal and corneal involvement were treated with surgery and cryotherapy. Rarely large areas of the cornea are involved by this tumor. Visual prognosis in such patients is poor. In these two patients, autologous limbal transplants were effective in restoring an excellent corneal surface and good visual function. This technique may be useful in the reconstruction of eyes with extensive neoplastic involvement of the corneoscleral limbus and cornea.
Department of Ophthalmology, Francis I. Proctor Foundation, University of California, San Francisco 94143.

Coster D J, Aggarwal R K and Williams K A. Surgical management of ocular surface disorders using conjunctival and stem cell allografts. *Br J Ophthalmol* 1995;11:977-82

AIMS: The aim of this work was to investigate different surgical options for the repair of the ocular surface, using various extensions of the procedure of limbal stem cell allotransplantation. METHODS/RESULTS: Straightforward lamellar limbal transplantation was performed in one patient with contact lens induced limbal stem cell failure. A second patient with a neoplastic corneal lesion underwent limbal allotransplantation, followed later by a second procedure in which 360 degrees of limbus and the entire ocular surface was transplanted. A third patient who had suffered extensive chemical burns was treated by penetrating keratoplasty to restore central corneal clarity, followed later by a lamellar allograft comprising a 360 degrees annulus of peripheral cornea to repair the ocular surface. A fourth patient with long standing, chronic trachomatous eye disease underwent allotransplantation of the upper lid tarsal plate and conjunctiva, with reconstruction of the fornix. Finally, a child with Goldenhar's syndrome underwent reconstruction of the medial fornix with autologous buccal mucosa, followed by a lamellar corneal and conjunctival allograft. A stable ocular surface has been achieved in each case and there have been no obvious rejection episodes. CONCLUSION: Limbal allotransplantation can be extended to engraftment of the entire superficial cornea, limbus, conjunctiva, and tarsal plate in patients with a range of pathologies. We have described the surgical management of five cases which demonstrate the potential of the technique, but which raise questions which still need to be explored.
Department of Ophthalmology, Flinders University of South Australia, Adelaide, Australia.

Cotsarelis G, Cheng S Z, Dong G, Sun T T and Lavker R M. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell* 1989;2:201-9

Despite the obvious importance of epithelial stem cells in tissue homeostasis and tumorigenesis, little is known about their specific location or biological characteristics. Using 3H-thymidine labeling, we have identified a subpopulation of corneal epithelial basal cells, located in the peripheral cornea in a region called limbus, that are normally slow cycling, but can be stimulated to proliferate in response to wounding and to a tumor promotor, TPA. No such cells can be detected in the central corneal epithelium, suggesting that corneal epithelial stem cells are located in the limbus. A comparison of various types of epithelial stem cells revealed a common set of features, including their preferred location, pigment protection, and growth properties, which presumably play a crucial role in epithelial stem cell function.
Department of Dermatology, University of Pennsylvania School of Medicine, Philadelphia 19104.

Coulombel L. [Adult stem cells: seing is not being]. *Med Sci (Paris)* 2003;6-7:683-94

Recent unexpected observations in adult rodents that stem/progenitor cells located in the bone marrow, but also in other tissues, could, after their transplantation to an irradiated host contribute to the regeneration of damaged organs such as brain, liver, pancreas or muscle, have raised much hope for future therapeutic applications. These data have also initially been interpreted as a proof of a possible transdifferentiation or plasticity of adult stem cells located in these tissues. Additional experiments rigorously analyzed have tempered initial enthusiasm, by showing that if marrow cells do migrate in damaged muscles and liver, their contribution to organ repair is low, and in some cases, explained by cell fusion. Nevertheless, among bone marrow cells, two categories of stem cells now emerge that have a potentially tremendous interest in cell therapy, if we succeed in understanding how to purify, amplify and differentiate these more efficiently and reproducibly.
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Coulombel L. [Adult stem cells: their scientific interest and therapeutic future]. *Gynecol Obstet Fertil* 2007;9:806-10

Fascinating and provocative findings have shaken the stem cell field during these past years, which may be exploited in the future in cell replacement therapies. Continuous renewal of blood, skin, and gut cells, has long be attributed to stem cells, but it was more unexpected to identify cells that fulfil the requirements for stem-progenitor cells in many tissues with a slow turnover such as heart, kidney, muscle and brain. However, despite their lack of risk and immunological barrier, adult stem cells are yet of poor therapeutic value in many diseases, because they are available in scarce number, are poorly amplified, and loose potential with ageing, among many obstacles. Thus, the identification in adult, and more recently fetal tissues, of cells with a high proliferative capacity and multi-lineage differentiation potential has

been wellcome, although their existence is still a matter of controversy. An alternative would be to activate stem cells in situ, by acting on components of the niche as recently exemplified in the hematopoetic system. Finally, as fiction meets reality, it may become possible to reprogram human adult cells in pluripotent ES cells-like, as recently demonstrated in mice.
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Curran R C and Gregory J. Demonstration of immunoglobulin in cryostat and paraffin sections of human tonsil by immunofluorescence and immunoperoxidase techniques. Effects of processing on immunohistochemical performance of tissues and on the use of proteolytic enzymes to unmask antigens in sections. *J Clin Pathol* 1978;10:974-83

A fluorescein isothiocyanate (FITC) technique and one based on peroxidase-antiperoxidase (PAP) were used to study the distribution of immunoglobulin (Ig) in cryostat and paraffin sections of human tonsil. Trypsin and other proteolytic enzymes were used to 'unmask' the antigen in paraffin sections. The effects of processing, and particularly of fixation, on the immunohistochemical response of tissues were studied. The FITC and PAP methods detected Ig in paraffin and cryostat sections equally well. The distribution of the antigen was the same with both methods but the PAP method was the more informative. Formaldehyde-sucrose solution proved more suitable for fixing tissues for immunohistochemistry than glutaraldehyde. Trypsin revealed antigen in parraffin sections more efficiently than pepsin, papain, or pronase. Surface Ig (s-Ig) could be demonstrated in trypsinised paraffin sections but less effectively than in cryostat sections. Trypsinised paraffin sections were, however, more suitable for intracellular Ig (c-Ig) than cryostat sections although the performance of cryostat sections could be improved by prior fixation with a coagulative fixative.

Curran R C and Gregory J. Effects of fixation and processing on immunohistochemical demonstration of immunoglobulin in paraffin sections of tonsil and bone marrow. *J Clin Pathol* 1980;11:1047-57

A number of fixatives were tested to determine their suitability for use with the unlabelled antibody peroxidase-antiperoxidase (PAP) method for demonstrating immunoglobulin in paraffin sections of tonsil and trephine samples of bone marrow. It was found that tonsil fixed in 'isotonic' solutions of formaldehyde reacted with the PAP method only after the sections had been trypsinised. Several other fixatives, including Bouin's fluid, Carnoy's fluid, and solutions containing mercuric chloride, gave tissues which reacted without trypsinisation of sections, and particularly good results were obtained with formol saline to which acetic acid (2-10%) had been added. A combination of acetic acid (10%)-formol saline and formol sublimate also gave excellent results with bone marrow. The influence on the PAP method of a number of steps in the processing of tissues and sections was also examined.

Daniels J T, Notara M, Shortt A J, Secker G, Harris A and Tuft S J. Limbal epithelial stem cell therapy. *Expert Opin Biol Ther* 2007;1:1-3

Restoring vision in patients suffering from previously intractable blinding ocular surface disease has become possible with the advent of techniques for ex vivo expansion and transplantation of limbal epithelial stem cells onto the cornea. This approach represents one of the few adult stem cell therapies presently in the clinic. This article highlights several key research areas where progress will be made to specifically understand the biology and therapeutic potential of limbal epithelial stem cells, which may have an applicability to the understanding of other adult stem cell populations.
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Daus W, Volcker H E, Meysen H and Bundschuh W. [Vital staining of the corneal endothelium--increased possibilities of diagnosis]. *Fortschr Ophthalmol* 1989;4:259-64

When the endothelium is examined with vital staining techniques, followed by endothelial cell mapping the cell distribution can be demonstrated over the entire corneal surface. To determine the normal cell distribution, 30 corneas were examined of 30 patients who were 19-90 years of age. In all cases an inhomogeneous cell distribution was found for different areas of the corneas. Usually, all corneas showed a region with maximal cell density in the corneal periphery. Three age-related distribution patterns were distinguished: (1) in younger patients (age 19-30 years, n = 5) a region of very high cell density in the corneal periphery was typical with the cell density decreasing towards the center of the cornea; (2) in the middle-age group (age 30-60 years, n = 10), the peripheral region of high cell density was narrower and there was fluctuation in the cell density towards the center of the cornea; in the elderly (age 60 years, n = 15), the cell density varied markedly in all areas. However, the area of highest cell density was still in the periphery.

Davanger M and Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. *Nature* 1971;5286:560-1

Daya S M, Watson A, Sharpe J R, Giledi O, Rowe A, Martin R and James S E. Outcomes and DNA analysis of ex vivo expanded stem cell allograft for ocular surface reconstruction. *Ophthalmology* 2005;3:470-7

PURPOSE: To investigate the outcome of a new technique of ex vivo expanded stem cell allograft for limbal stem cell deficiency (LSCD), and to characterize the ocular surface genotype after surgery. DESIGN: Retrospective noncomparative case series. PARTICIPANTS: Ten eyes of 10 patients with profound LSCD arising from ectodermal dysplasia (3 eyes), Stevens-Johnson syndrome (3 eyes), chemical injury (2 eyes), thermal injury (1 eye), and rosacea blepharoconjunctivitis (1 eye). INTERVENTION: Allogeneic corneal limbal stem cells were cultured on plastic and transplanted to the recipient eye after removal of conjunctival pannus. Amniotic membrane was applied in a bandage capacity. The procedure was combined with other reconstructive surgery in 2 cases. Nine patients received systemic cyclosporin A immunosuppression, and the DNA genotype was investigated with surface impression cytology. MAIN OUTCOME MEASURES: Parameters of LSCD, including vascularization, conjunctivalization, inflammation, epithelial defect, photophobia, and pain. RESULTS: The mean follow-up period was 28 months (range, 12-50). Seven of 10 eyes (70%) had improved parameters of LSCD at final follow-up and were considered successes. Four (40%) had improved visual acuity, including 3 having had further procedures for visual rehabilitation. Three patients failed to improve-1 with a thermal burn and lid deformity, 1 with Stevens-Johnson syndrome and severe dry eye, and 1 with ectodermal dysplasia who developed an epithelial defect at 26 months. DNA analysis of the first 7 cases showed no ex vivo donor stem cell DNA present beyond 9 months. CONCLUSIONS: Ex vivo expanded stem cell allograft is a useful technique for restoring the ocular surface in profound LSCD. The absence of donor DNA beyond 9 months suggests that ongoing immunosuppression may be unnecessary and raises questions regarding the origin of the host corneal epithelium.
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De Coppi P, Bartsch G, Jr., Siddiqui M M, Xu T, Santos C C, Perin L, Mostoslavsky G, Serre A C, Snyder E Y, Yoo J J, Furth M E, Soker S and Atala A. Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotechnol* 2007;1:100-6

Stem cells capable of differentiating to multiple lineages may be valuable for therapy. We report the isolation of human and rodent amniotic fluid-derived stem (AFS) cells that express embryonic and adult stem cell markers. Undifferentiated AFS cells expand extensively without feeders, double in 36 h and are not tumorigenic. Lines maintained for over 250 population doublings retained long telomeres and a normal karyotype. AFS cells are broadly multipotent. Clonal human lines verified by retroviral marking were induced to differentiate into cell types representing each embryonic germ layer, including cells of adipogenic, osteogenic, myogenic, endothelial, neuronal and hepatic

lineages. Examples of differentiated cells derived from human AFS cells and displaying specialized functions include neuronal lineage cells secreting the neurotransmitter L-glutamate or expressing G-protein-gated inwardly rectifying potassium channels, hepatic lineage cells producing urea, and osteogenic lineage cells forming tissue-engineered bone.
Wake Forest Institute for Regenerative Medicine, Wake Forest University School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157-1094, USA.

de Paiva C S, Chen Z, Corrales R M, Pflugfelder S C and Li D Q. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells* 2005;1:63-73

ABCG2, a member of the ATP binding cassette (ABC) transporters, has been identified as a molecular determinant for bone marrow stem cells and proposed as a universal marker for stem cells. This study investigates ABCG2 expression and its potential as a marker that identifies human limbal epithelial stem cells. ABCG2 expression was evaluated by immunofluorescent and immunohistochemical staining, laser scanning confocal microscopy, flow cytometry, and semiquantitative reverse transcription-polymerase chain reaction. Cells selected from primary limbal epithelial cultures by flow cytometry with ABCG2 monoclonal antibody (mAb) or Hoechst 33342 dye staining were evaluated for their gene expression and colony-forming efficiency (CFE). ABCG2 protein was mainly located in the basal cells of limbal epithelia but not in the limbal suprabasal and corneal epithelia. ABCG2 staining was also observed in primary limbal epithelial cultures. Limbal epithelia express higher levels of ABCG2 and DeltaNp63 mRNAs than corneal epithelia. Labeling with ABCG2 mAb yielded 2.5%-3.0% positive cells by flow cytometry. The ABCG2-positive cells exhibited greater CFE on a 3T3 fibroblast feeder layer than ABCG2-negative cells. A side population (SP) was detected by the Hoechst 33342 exclusion assay. SP cells displayed stronger expression of ABCG2 and DeltaNp63 mRNA and greater CFE than the non-SP cells. In conclusion, these findings demonstrate that ABCG2 transporter was exclusively expressed by limbal basal cells and that the ABCG2-positive and SP cells possess enriched stem cell properties, suggesting for the first time that ABCG2 could serve as a marker to identify the putative limbal epithelial stem cells.
Ocular Surface Center, Cullen Eye Institute, Department of Ophthalmology, Baylor College of Medicine, 6565 Fannin St., NC-205, Houston, Texas 77030, USA.

Denis D, Burguiere O and Burillon C. A biometric study of the eye, orbit, and face in 205 normal human fetuses. *Invest Ophthalmol Vis Sci* 1998;12:2232-8

PURPOSE: To develop models for assessing the differential growth of the fetal face along its horizontal and vertical axes, as well as of the orbit and the eye. METHODS: Normal human fetuses (n = 205), whose gestational ages ranged from 15.5 to 41 weeks of amenorrhea, were examined. Orbitofacial parameters were as follows: outer canthal distance, inner canthal distance, palpebral fissure length, and oropalpebral distance. Ocular parameters were corneal diameter and axial length. Correlations were tested among all parameters. Linear and polynomial regression analyses of gestational age and the orbitofacial and ocular parameters were used to develop models of growth. Differential patterns of growth in the face were investigated. RESULTS: The best correlation was found between palpebral fissure length and oropalpebral distance. The increase of each of the parameters studied could be accurately described by a linear model. Sex had no detectable effect on these parameters. Compared with the skull, the face had a more rapid growth along the vertical axis. The palpebral fissure developed more rapidly than the eye. CONCLUSIONS: The parameters that were studied in the fetal face, orbit, and eye follow a roughly linear growth curve.
Hopital La Timone, Marseille, France.

Di Iorio E, Barbaro V, Ruzza A, Ponzin D, Pellegrini G and De Luca M. Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proc Natl Acad Sci U S A* 2005;27:9523-8

The p63 gene generates transactivating and N-terminally truncated transcripts (DeltaNp63) initiated by different promoters. Alternative splicing gives rise to three different C termini, designated alpha, beta, and gamma. In the ocular epithelium, the corneal stem cells, which are segregated in the basal layer of the limbus, contain the alpha isoform but not beta or gamma. Holoclones derived from the limbus are rich in alpha, meroclones contain little, and paraclones contain none. In normal resting corneal epithelium, p63 of all isoforms is absent. Upon corneal wounding, cells originating from the limbus and containing alpha migrate progressively through the epithelium of the peripheral and central cornea. In the absence of an attached limbus, no alpha isoform appears in the corneal epithelium. When migrating cells containing the alpha isoform appear in the wounded corneal epithelium, they are confined to the basal layer, but the suprabasal cells, not only of the cornea but of the limbus as well, contain mRNA encoding beta and gamma. These data support the concept that the alpha isoform of p63 is necessary for the maintenance of the proliferative potential of limbal stem cells and their ability to migrate over the cornea. The beta and gamma isoforms, being suprabasal and virtually absent from the resting limbus, are not stem cell markers but are likely to play a role in epithelial differentiation specifically during the process of corneal regeneration.
Epithelial Stem Cell Research Center, Veneto Eye Bank Foundation, 30122 Venice, Italy.

Dolby D E. The action of pepsin on protein fractions from horse antisera to diphtheria toxin. *Biochem J* 1964;1:112-9

Dua H S, Shanmuganathan V A, Powell-Richards A O, Tighe P J and Joseph A. Limbal epithelial crypts: a novel anatomical structure and a putative limbal stem cell niche. *Br J Ophthalmol* 2005;5:529-32

BACKGROUND/AIMS: There is substantial evidence that mammalian epithelial stem cells are located within well defined niches. Although the corneoscleral limbus is acknowledged as the site of corneal epithelial stem cells no anatomical niche for such cells has yet been described. The authors undertook to re-evaluate the microanatomy of the limbus in order to identify possible sites that may represent a stem cell niche. METHODS: Systematic serial 5-7 microm sections of human corneoscleral segments obtained from cadaver donors, were examined. The sections were stained with haematoxylin and eosin or toludine blue. Sections with specific areas of interest were further examined immunohistologically for the corneal epithelial marker cytokeratin 14 and the "stem cell" marker ABCG2 transporter protein. RESULTS: Distinct anatomical extensions from the peripheral aspect of the limbal palisades were identified. These consist of a solid cord of cells extending peripherally or circumferentially. The cells stained positive for CK14 and ABCG2. CONCLUSIONS: A novel anatomical structure has been identified at the human limbus, which demonstrates characteristics of being a stem cell niche. The authors have termed this structure the limbal epithelial crypt.
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Emerson S G. Ex vivo expansion of hematopoietic precursors, progenitors, and stem cells: the next generation of cellular therapeutics. *Blood* 1996;8:3082-8

Department of Medicine, University of Pennsylvania, Philadelphia, 19104, USA.

Emsley J G, Mitchell B D, Kempermann G and Macklis J D. Adult neurogenesis and repair of the adult CNS with neural progenitors, precursors, and stem cells. *Prog Neurobiol* 2005;5:321-41

Recent work in neuroscience has shown that the adult central nervous system contains neural progenitors, precursors, and stem cells that are capable of generating new neurons, astrocytes, and oligodendrocytes. While challenging previous dogma that no new

neurons are born in the adult mammalian CNS, these findings bring with them future possibilities for the development of novel neural repair strategies. The purpose of this review is to present current knowledge about constitutively occurring adult mammalian neurogenesis, to highlight the critical differences between "neurogenic" and "non-neurogenic" regions in the adult brain, and to describe the cardinal features of two well-described neurogenic regions-the subventricular zone/olfactory bulb system, and the dentate gyrus of the hippocampus. We also provide an overview of currently used models for studying neural precursors in vitro, mention some precursor transplantation models, and emphasize that, in this rapidly growing field of neuroscience, one must take caution with respect to a variety of methodological considerations for studying neural precursor cells both in vitro and in vivo. The possibility of repairing neural circuitry by manipulating neurogenesis is an intriguing one, and, therefore, we also review recent efforts to understand the conditions under which neurogenesis can be induced in non-neurogenic regions of the adult CNS. This work aims toward molecular and cellular manipulation of endogenous neural precursors in situ, without transplantation. We conclude this review with a discussion of what the function might be of newly generated neurons in the adult brain and provide a summary of current thinking about the consequences of disturbed adult neurogenesis and the reaction of neurogenic regions to disease.
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Engelmann K, Drexler D and Bohnke M. Transplantation of adult human or porcine corneal endothelial cells onto human recipients in vitro. Part I: Cell culturing and transplantation procedure. *Cornea* 1999;2:199-206

PURPOSE: To develop a method for grafting endothelial cells isolated from organ-cultured adult human corneas onto the denuded Descemet's membrane of human recipients. METHODS: Adult human or porcine corneal endothelial cells were isolated and maintained in monolayer cultures before seeding. Recipient corneas were stripped of their own endothelium by one of three different methods (mechanical, chemical, or physical) and the completeness of removal assessed after vital staining. The utility of each method was evaluated by monitoring the quality of attachment of the seeded-cell population. The seeding density of transplanted cells required for optimal results also was determined and the final numeric cell density achieved on recipient corneas after culturing for 7-20 days ascertained. The influence of incubating source cells with fibroblast growth factor (FGF), both on this latter parameter and on cell morphology, also was evaluated. The functional integrity of regrafted endothelium was assessed in 24-h perfusion experiments. RESULTS: The seeding of between 150,000 and 700,000 cells onto recipient corneas, followed by gentle centrifugation to improve attachment, yielded maximal final numeric cell densities of 3,450/mm2 and 1,850/mm2 in porcine and human lines, respectively. Recipient corneas were most effectively denuded of their own endothelium by freezing-and-thawing. The newly established endothelial monolayer remained stable for up to 20 days in organ culture (longest period monitored). FGF treatment did not enhance the final numeric density of cells attained on recipient corneas, but it did have a beneficial effect on their morphology. Only those recipient corneas that exhibited a well-differentiated monolayer of seeded endothelial cells underwent stromal deswelling near to physiologic levels. CONCLUSION: A practical working model has been developed, whereby recipient corneas stripped of their own endothelium can be furnished with a "new," near-normal endothelium by appropriate manipulations of the seeded-cell population. This now paves the way for a realistic tackling of the problem of endothelial cell paucity in donor corneas destined for transplantation.
University of Hamburg, Department of Ophthalmology, Germany.

Fazel S, Cimini M, Chen L, Li S, Angoulvant D, Fedak P, Verma S, Weisel R D, Keating A and Li R K. Cardioprotective c-kit+ cells are from the bone marrow and regulate the myocardial balance of angiogenic cytokines. *J Clin Invest* 2006;7:1865-77

Clinical trials of bone marrow stem/progenitor cell therapy after myocardial infarction (MI) have shown promising results, but the mechanism of benefit is unclear. We examined the nature of endogenous myocardial repair that is dependent on the function of the c-kit receptor, which is expressed on bone marrow stem/progenitor cells and on recently identified cardiac stem cells. MI increased the number of c-kit+ cells in the heart. These cells were traced back to a bone marrow origin, using genetic tagging in bone marrow chimeric mice. The recruited c-kit+ cells established a proangiogenic milieu in the infarct border zone by increasing VEGF and by reversing the cardiac ratio of angiopoietin-1 to angiopoietin-2. These oscillations potentiated endothelial mitogenesis and were associated with the establishment of an extensive myofibroblast-rich repair tissue. Mutations in the c-kit receptor interfered with the mobilization of the cells to the heart, prevented angiogenesis, diminished myofibroblast-rich repair tissue formation, and led to precipitous cardiac failure and death. Replacement of the mutant bone marrow with wild-type cells rescued the cardiomyopathic phenotype. We conclude that, consistent with their documented role in tumorigenesis, bone marrow c-kit+ cells act as key regulators of the angiogenic switch in infarcted myocardium, thereby driving efficient cardiac repair.
Division of Cardiovascular Surgery, Toronto General Hospital, University Health Network, University of Toronto, Toronto, Ontario, Canada.

Feldman S T, Gjerset R, Gately D, Chien K R and Feramisco J R. Expression of SV40 virus large T antigen by recombinant adenoviruses activates proliferation of corneal endothelium in vitro. *J Clin Invest* 1993;4:1713-20

Infection with the Ad5-SVR4 virus was used to introduce the large T antigen encoding region of the SV40 virus into bovine and human corneal endothelial cells. Expression of large T antigen occurred in 40% of bovine corneal endothelial cells after a 24-h incubation time versus 12% after 8 h of incubation. By 48 h after infection, almost all (92.8%) bovine corneal endothelial cells expressed large T antigen. Bovine and human corneal endothelial cells which expressed large T antigen proliferated and the characteristic morphologic features of corneal endothelium were maintained. This method may enable growth of enough corneal endothelium to perform studies to elucidate the biochemical mechanisms involved in regulating endothelial cell function.
Department of Ophthalmology, University of California, San Diego, La Jolla 92093.

Finley J C, Grossman G H, Dimeo P and Petrusz P. Somatostatin-containing neurons in the rat brain: widespread distribution revealed by immunocytochemistry after pretreatment with pronase. *Am J Anat* 1978;3:483-8

Immunocytochemical staining after controlled proteolytic treatment of the sections with pronase revealed widespread distribution of neuronal cell bodies with somatostatin-like immunoreactivity (SLI) in the rat forebrain. SLI-positive neurons were found in regions of the neocortex, the pyriform cortex, the cingulate cortex, the striatum, the olfactory tract and tubercle, the nucleus accumbens, the septum, and the hypothalamus. These results are consistent with previous radioimmunoassay findings and suggest the presence of large somatostatin-like (possibly precursor) molecules in the neurons stained for SLI after pronase treatment.

Friedman A H and Deutsch-Sokol R H. Sugiura's sign. Perilimbal vitiligo in the Vogt-Koyanagi-Harada syndrome. *Ophthalmology* 1981;11:1159-65

Fu Y, Fan X Q, Luo M and Chen P. [Amniotic membrane as a carrier for cultivated and labeled corneal endothelial cell transplantation]. *Zhonghua Yan Ke Za Zhi* 2006;10:925-9

OBJECTIVE: To investigate the feasibility of using amniotic membrane (AM) as a carrier for cultured corneal endothelial cells transplantation. METHODS: Rabbit corneal endothelial cells were cultivated, passaged, and labeled with the fluorescent tracker CM-DiI, then transplanted onto denuded AM. The cell density, morphology, histology and ultrastructure of the cultured cells on AM were examined by light microscope, fluorescent microscopy and scanning electron microscope. To determine whether these composite endothelial sheets were functional in vivo, the sheets were transplanted onto rabbit corneas whose deep lamellar with Descemet's membrane and endothelial cells had been completely removed. The deep layer excised rabbit corneas without any transplantation or with transplantation of denuded AM were

treated as controls. After transplantation, the corneal appearance and corneal thickness were examined. The grafts were examined for cell density, morphology and histology 4 weeks after the surgery. RESULTS: A monolayer of endothelial cells were confluent on the AM 5 - 7 days after seeding. The density of the endothelial cells was (3202.84 +/- 347.77)/mm(2). Morphologically, the sheets consisted of a fairly continuous layer of flat polygonal endothelial cells that appeared uniform in size with tight junctions. The labeled cells were red in color under fluorescent microscopy. After transplantation, the corneas that received cultured endothelial cells had little edema and retained their thinness and transparency, while the corneas of controls showed prominent edema and opacity, indicating that these cells were transplanted cells rather than the cells migrated from peripheral cornea. Four weeks after surgery, the labeled endothelial cells were still found in the cornea with red fluorescence. CONCLUSIONS: AM maintains cultured endothelial cells' morphology and function both in vitro and in vivo. AM could serve as a carrier for corneal endothelial cells transplantation. These results provide a promising method for the treatment of diseases caused by corneal endothelial disorders.
Department of Ophthalmology, Ninth People's Hospital, Shanghai Jiao Tong University, Shanghai 200011, China.

Gage P J, Rhoades W, Prucka S K and Hjalt T. Fate maps of neural crest and mesoderm in the mammalian eye. *Invest Ophthalmol Vis Sci* 2005;11:4200-8

PURPOSE: Structures derived from periocular mesenchyme arise by complex interactions between neural crest and mesoderm. Defects in development or function of structures derived from periocular mesenchyme result in debilitating vision loss, including glaucoma. The determination of long-term fates for neural crest and mesoderm in mammals has been inhibited by the lack of suitable marking systems. In the present study, the first long-term fate maps are presented for neural crest and mesoderm in a mammalian eye. METHODS: Complementary binary genetic approaches were used to mark indelibly the neural crest and mesoderm in the developing eye. Component one is a transgene expressing Cre recombinase under the control of an appropriate tissue-specific promoter. The second component is the conditional Cre reporter R26R, which is activated by the Cre recombinase expressed from the transgene. Lineage-marked cells were counterstained for expression of key transcription factors. RESULTS: The results established that fates of neural crest and mesoderm in mice were similar to but not identical with those in birds. They also showed that five early transcription factor genes are expressed in unique patterns in fate-marked neural crest and mesoderm during early ocular development. CONCLUSIONS: The data provide essential new information toward understanding the complex interactions required for normal development and function of the mammalian eye. The results also underscore the importance of confirming neural crest and mesoderm fates in a model mammalian system. The complementary systems used in this study should be useful for studying the respective cell fates in other organ systems.
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Gain P, Thuret G, Kodjikian L, Gavet Y, Turc P H, Theillere C, Acquart S, Le Petit J C, Maugery J and Campos L. Automated tri-image analysis of stored corneal endothelium. *Br J Ophthalmol* 2002;7:801-8

BACKGROUND: Endothelial examination of organ culture stored corneas is usually done manually and on several mosaic zones. Some banks use an image analyser that takes account of only one zone. This method is restricted by image quality, and may be inaccurate if endothelial cell density (ECD) within the mosaic is not homogeneous. The authors have developed an analyser that has tools for automatic error detection and correction, and can measure ECD and perform morphometry on multiple zones of three images of the endothelial mosaic. METHODS: 60 human corneas were divided into two equal groups: group 1 with homogeneous mosaics, group 2 with heterogeneous ones. Three standard microscopy video images of the endothelium, graded by quality, were analysed either in isolation (so called mono-image analysis) or simultaneously (so called tri-image analysis), with 50 or 300 endothelial cells (ECs) counted. The automated analysis was compared with the manual analysis, which concerned 10 non-adjacent zones and about 300 cells. For each analysis method, failures and durations were studied according to image quality. RESULTS: All corneas were able to undergo analysis, in about 2 or 7.5 minutes for 50 and 300 ECs respectively. The tri-image analysis did not increase analysis time and never failed, even with mediocre images. The tri-image analysis of 300 ECs was always most highly correlated with the manual count, particularly in the heterogeneous cornea group ($r=0.94$, $p<0.001$) and prevented serious count errors. CONCLUSIONS: This analyser allows reliable and rapid analysis of ECD, even for heterogeneous endothelia mosaics and mediocre images.
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Garaud J C, Eloy R, Moody A J, Stock C and Grenier J F. Glucagon- and glicentin-immunoreactive cells in the human digestive tract. *Cell Tissue Res* 1980;1:121-36

The distribution and cellular location of substances reacting with anti-glucagon or anti-glicentin sera, i.e., glucagon-like and glicentin-like immunoreactivities, were studied in the human digestive tract using the immunofluorescence and immunoperoxidase methods. Both types of immunoreactivity were (1) absent in the antrum, (2) abundant in cells located at the periphery of pancreatic islets, (3) unevenly present in cells scattered in the epithelium of the small intestinal mucosa, the glicentin-immunoreactive cells being particularly abundant in the ileum. In the pancreas, and, when simultaneously present, in the intestine, both glucagon and glicentin immunoreactivities were located in the same cells. The precise ultrastructural location of each immunoreactivity was readily made using colloidal gold and ferritin tracers on ultrathin sections of glutaraldehyde-osmium fixed and epoxy resin-embedded tissues. In the pancreas, both glucagon and glicentin immunoreactivities were found in the granules of the A-type cells; the glucagon immunoreactivity was only present in the core of the granule, whereas the glicentin immunoreactivity was found either in the peripheral halo only, or throughout the entire granule. In the small intestine, both immunoreactivities were located inside the granules of the L-type cells. Quantitative specificity tests suggested that the glucagon- and the glicentin-like substances of the pancreas differ from those found in the intestine.

Gaynes B I and Oshinskie L J. Pseudophakic bullous keratopathy. *J Am Optom Assoc* 1985;10:794-6

Although cataract surgery alone carries a risk of iatrogenic endothelial damage, IOL implantation will further increase the risk of endothelial compromise and resulting bullous keratopathy. PBK is an important consideration in the differential diagnosis of corneal disease in the pseudophakic eye and with proper treatment visual loss due to PBK is usually reversible. While seen most frequently with iris clip implants, all pseudophakes are susceptible to PBK and should be carefully observed for this disease entity.

Gershoni J M, Roitburd-Berman A, Siman-Tov D D, Tarnovitski Freund N and Weiss Y. Epitope mapping: the first step in developing epitope-based vaccines. *BioDrugs* 2007;3:145-56

Antibodies are an effective line of defense in preventing infectious diseases. Highly potent neutralizing antibodies can intercept a virus before it attaches to its target cell and, thus, inactivate it. This ability is based on the antibodies' specific recognition of epitopes, the sites of the antigen to which antibodies bind. Thus, understanding the antibody/epitope interaction provides a basis for the rational design of preventive vaccines. It is assumed that immunization with the precise epitope, corresponding to an effective neutralizing antibody, would elicit the generation of similarly potent antibodies in the vaccinee. Such a vaccine would be a 'B-cell epitope-based vaccine', the implementation of which requires the ability to backtrack from a desired antibody to its corresponding epitope. In this article we discuss a range of methods that enable epitope discovery based on a specific antibody. Such a reversed immunological approach is the first step in the rational design of an epitope-based vaccine. Undoubtedly, the gold standard for epitope definition is x-ray analyses of crystals of antigen:antibody complexes. This method provides atomic resolution of the epitope; however, it is not readily applicable to many antigens and antibodies, and requires a very high degree of sophistication and expertise. Most other methods rely on the ability to monitor the binding of the antibody to antigen fragments or mutated variations. In mutagenesis of the antigen, loss of binding due to point modification of an amino acid residue is often considered an indication of an epitope component. In addition, computational combinatorial methods for epitope mapping are also useful. These methods rely

on the ability of the antibody of interest to affinity isolate specific short peptides from combinatorial phage display peptide libraries. The peptides are then regarded as leads for the definition of the epitope corresponding to the antibody used to screen the peptide library. For epitope mapping, computational algorithms have been developed, such as Mapitope, which has recently been found to be effective in mapping conformational discontinuous epitopes. The pros and cons of various approaches towards epitope mapping are also discussed.
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Ghods A J, Irvin D, Liu G, Yuan X, Abdulkadir I R, Tunici P, Konda B, Wachsmann-Hogiu S, Black K L and Yu J S. Spheres isolated from 9L gliosarcoma rat cell line possess chemoresistant and aggressive cancer stem-like cells. *Stem Cells* 2007;7:1645-53

The rat 9L gliosarcoma is a widely used syngeneic rat brain tumor model that closely simulates glioblastoma multiforme when implanted in vivo. In this study, we sought to isolate and characterize a subgroup of cancer stem-like cells (CSLCs) from the 9L gliosarcoma cell line, which may represent the tumor-initiating subpopulation of cells. We demonstrate that these CSLCs form clonal-derived spheres in media devoid of serum supplemented with the mitogens epidermal growth factor and basic fibroblast growth factor, express the NSC markers Nestin and Sox2, self-renew, and differentiate into neuron-like and glial cells in vitro. More importantly, these cells can propagate and recapitulate tumors when implanted into the brain of syngeneic Fisher rats, and they display a more aggressive course compared with 9L gliosarcoma cells grown in monolayer cultures devoid of mitogens. Furthermore, we compare the chemosensitivity and proliferation rate of 9L gliosarcoma cells grown as a monolayer to those of cells grown as floating spheres and show that the sphere-generated cells have a lower proliferation rate, are more chemoresistant, and express several antiapoptosis and drug-related genes, which may prove to have important clinical implications. Disclosure of potential conflicts of interest is found at the end of this article.
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Gomes J A, Geraldes Monteiro B, Melo G B, Smith R L, Cavenaghi Pereira da Silva M, Lizier N F, Kerkis A, Cerruti H and Kerkis I. Corneal reconstruction with tissue-engineered cell sheets composed of human immature dental pulp stem cells. *Invest Ophthalmol Vis Sci* 2010;3:1408-14

PURPOSE: To determine the outcome of the use of a tissue-engineered cell sheet composed of human undifferentiated immature dental pulp stem cells (hIDPSC) for ocular surface reconstruction in an animal model of total limbal stem cell deficiency (LSCD). METHODS: LSCD was induced by the application of 0.5 M NaOH to the right eye of rabbits for 25 seconds (mild chemical burn [MCB]) and for 45 seconds (severe chemical burn [SCB]). After 1 month, a superficial keratectomy was performed to remove the fibrovascular pannus that covered the animals' burned corneas. A tissue-engineered hIDPSC sheet was transplanted onto the corneal bed and then covered with deepithelialized human amniotic membrane (AM). In the respective control groups, the denuded cornea was covered with AM only. After 3 months, a detailed analysis of the rabbit eyes was performed with regard to clinical aspect, histology, electron microscopy, and immunohistochemistry. RESULTS: Corneal transparency of the rabbit eyes that underwent hIDPSC transplantation was improved throughout the follow-up, while the control corneas developed total conjunctivalization and opacification. Rabbits from the MCB group showed clearer corneas with less neovascularization. The clinical data were confirmed by histologic analysis that showed healthy uniform corneal epithelium, especially in the MCB group. The presence of hIDPSC was detected using an anti-hIDPSC antibody. The corneal tissue also showed positive immunostaining with anti-human antibodies. In the control corneas, none of these antigens were detected. CONCLUSIONS: Overall, these data showed that transplantation of a tissue-engineered hIDPSC sheet was successful for the reconstruction of corneal epithelium in an animal model of LSCD.
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Goodell M A, Brose K, Paradis G, Conner A S and Mulligan R C. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med* 1996;4:1797-806

Hematopoietic stem cells (HSC) are multipotent cells that reside in the bone marrow and replenish all adult hematopoietic lineages throughout the lifetime of the animal. While experimenting with staining of murine bone marrow cells with the vital dye, Hoechst 33342, we discovered that display of Hoechst fluorescence simultaneously at two emission wavelengths revealed a small and distinct subset of whole bone marrow cells that had phenotypic markers of multipotential HSC. These cells were shown in competitive repopulation experiments to contain the vast majority of HSC activity from murine bone marrow and to be enriched at least 1,000-fold for in vivo reconstitution activity. Further, these Hoechst-stained side population (SP) cells were shown to protect recipients from lethal irradiation at low cell doses, and to contribute to both lymphoid and myeloid lineages. The formation of the Hoechst SP profile was blocked when staining was performed in the presence of verapamil, indicating that the distinctly low staining pattern of the SP cells is due to a multidrug resistance protein (mdr) or mdr-like mediated efflux of the dye from HSC. The ability to block the Hoechst efflux activity also allowed us to use Hoechst to determine the DNA content of the SP cells. Between 1 and 3% of the HSC were shown to be in S-G2M. This also enabled the purification of the G0-G1 and S-G2M HSC had a reconstitution capacity equivalent to quiescent stem cells. These findings have implications for models of hematopoietic cell development and for the development of genetic therapies for diseases involving hematopoietic cells.
Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Cambridge 02142, USA.

Gospodarowicz D, Greenburg G and Alvarado J. Transplantation of cultured bovine corneal endothelial cells to rabbit cornea: clinical implications for human studies. *Proc Natl Acad Sci U S A* 1979a;1:464-8

Rabbit corneas denuded of their endothelium were coated with bovine corneal endothelial cells (from steers) previously maintained in tissue culture for short (20 generations) or prolonged (200 generations) periods. When grafted back into female rabbits, the corneal buttons remained clear and showed no edema. In contrast, denuded corneas coated with bovine keratocytes and grafted into rabbits became opaque and edematous within 7 days and remained so thereafter. Bovine corneal endothelial cells of the grafted corneas, which had remained clear for over 100 days, proliferated actively when put back into tissue culture. The corneal endothelial cells of the graft were characteristic of the male (XY). The chromosome number of the endothelium of the recipient rabbit was 2n = 44 with sex chromosomes characteristic of the female (XX). Results of the karyotype analysis show that there was no invasion of the corneal button by the recipient endothelium and, conversely, no invasion of the recipient endothelium by the endothelium on the corneal button. These results demonstrate that cultured corneal endothelial cells remain functional in vitro and can replace a damaged or nonfunctional endothelium in vivo.

Gospodarowicz D, Greenburg G and Alvarado J. Transplantation of cultured bovine corneal endothelial cells to species with nonregenerative endothelium. The cat as an experimental model. *Arch Ophthalmol* 1979b;11:2163-9

The in vivo transplantation of cultured bovine corneal endothelial cells has been attempted in the cat. Cat corneas denuded of their endothelium were coated with bovine corneal endothelial cells previously maintained in tissue culture. When grafted back into cat recipients, the corneal buttons remained clear with no edema. Alizarin red staining of the endothelial side of the corneal transplant demonstrated that the coated bovine corneal endothelial cells reorganized themselves into a highly organized cell monolayer within eight days in vivo. In contrast, corneas denuded of their endothelium became opaque and edematous within seven days and remained so thereafter. These results demonstrate that cultured corneal endothelial cells remain functional in vitro and can replace a damaged or nonfunctional endothelium i

Grueterich M, Espana E M and Tseng S C. Ex vivo expansion of limbal epithelial stem cells: amniotic membrane serving as a stem cell niche. *Surv Ophthalmol* 2003;6:631-46

Identification, maintenance, and expansion of stem cells for subsequent transplantation has become a new strategy for treating many diseases in most medical subspecialties. The stem cells of the corneal epithelium are located in the limbal basal layer and are the ultimate source for constant corneal epithelial renewal. Like those in other tissues, limbal stem cells are supported by a unique stromal microenvironment called the stem cell niche, which consists of certain extracellular matrix components, cell membrane-associated molecules, and cytokine dialogues. Destructive loss of limbal stem cells or dysfunction of their stromal environment renders many corneas with a clinical entity called limbal stem cell deficiency, which is characterized by variable extents of conjunctival ingrowth depending on the severity of limbal damage. A new strategy of treating limbal stem cell deficiency is to transplant a bio-engineered graft by expanding limbal epithelial stem cells ex vivo on amniotic membrane. This review summarizes the published literature data collectively explaining how amniotic membrane is an ideal biological substrate that can help maintain and support the expansion of limbal epithelial stem cells.
Department of Ophthalmology, Ludwig Maximilians University, Munich, Germany.

Gu S, Xing C, Han J, Tso M O and Hong J. Differentiation of rabbit bone marrow mesenchymal stem cells into corneal epithelial cells in vivo and ex vivo. *Mol Vis* 2009;99-107

PURPOSE: To examine whether bone marrow mesenchymal stem cells (MSCs) could be differentiated into corneal epithelial cells in vivo and ex vivo. METHODS: In vivo, BrdU labeled rabbit MSCs (Rb-MSCs) were suspended in the fibrin gels and transplanted onto the surface of the damaged rabbit corneas. Histology and molecular phenotype were studied on postoperative day 28. In vitro, labeled Rb-MSCs were cultured for three days in two different systems: (1) Group A: Rb-MSCs were co-cultured with rabbit limbal stem cells (Rb-LSCs) by the Transwell culture system. A suspension of Rb-LSCs was added to the upper membrane surface, and the inserts were positioned in the culture wells, which were incubated with Rb-MSCs; (2) Group B: Supernatant medium that had first been used to culture Rb-LSCs and then filtered with a 0.45 mum filter was used to culture Rb-MSCs. For both groups, immunofluorescence and flow cytometric analysis were used to examine the expression of cytokeratin 3 (CK3) in differentiated Rb-MSCs. RESULTS: In vivo, the data showed that following transplantation of Rb-MSCs, the rabbit's damaged corneal surface was successfully reconstructed and that some Rb-MSCs participated in the healing of the injured corneal epithelium and expressed CK3. In vitro, the data showed that Rb-MSCs rapidly differentiated into cells with a morphological and molecular phenotype of corneal epithelial-like cells. For both groups, the differentiated Rb-MSCs were positive for corneal epithelial-specific marker CK3. In Group A, flow cytometry analysis showed that at day one, only 3.46+/-1.9% of cells expressed CK3. This increased to 7.24+/-3.80% at day two and decreased slightly (5.50+/-3.33%) at day three. The proportion of CK3 in Group B was 4.09+/-1.84% at day one, rising to 9.31+/-5.92% after 24 h, but falling (4.37+/-2.61%) at day three. The mean differences are significant between each group and the negative control, but was not significant between Group A and Group B. CONCLUSIONS: MSCs could differentiate into corneal epithelial-like cells in vivo and ex vivo.
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Guan K, Nayernia K, Maier L S, Wagner S, Dressel R, Lee J H, Nolte J, Wolf F, Li M, Engel W and Hasenfuss G. Pluripotency of spermatogonial stem cells from adult mouse testis. *Nature* 2006;7088:1199-203

Embryonic germ cells as well as germline stem cells from neonatal mouse testis are pluripotent and have differentiation potential similar to embryonic stem cells, suggesting that the germline lineage may retain the ability to generate pluripotent cells. However, until now there has been no evidence for the pluripotency and plasticity of adult spermatogonial stem cells (SSCs), which are responsible for maintaining spermatogenesis throughout life in the male. Here we show the isolation of SSCs from adult mouse testis using genetic selection, with a success rate of 27%. These isolated SSCs respond to culture conditions and acquire embryonic stem cell properties. We name these cells multipotent adult germline stem cells (maGSCs). They are able to spontaneously differentiate into derivatives of the three embryonic germ layers in vitro and generate teratomas in immunodeficient mice. When injected into an early blastocyst, SSCs contribute to the development of various organs and show germline transmission. Thus, the capacity to form multipotent cells persists in adult mouse testis. Establishment of human maGSCs from testicular biopsies may allow individual cell-based therapy without the ethical and immunological problems associated with human embryonic stem cells. Furthermore, these cells may provide new opportunities to study genetic diseases in various cell lineages.
Department of Cardiology and Pneumology, Heart Center, Georg-August-University of Gottingen, Robert-Koch-Str. 40, 37075 Gottingen, Germany.

Hao Y, Ma D H, Hwang D G, Kim W S and Zhang F. Identification of antiangiogenic and antiinflammatory proteins in human amniotic membrane. *Cornea* 2000;3:348-52

PURPOSE: To identify the potential antiangiogenic and antiinflammatory proteins expressed in human amniotic membrane tissue. METHODS: Human amniotic epithelial and mesenchymal cells were isolated from human amniotic membranes by sequential trypsin and collagenase digestion. Total RNAs were harvested from freshly obtained human amniotic epithelial and mesenchymal cells. Antiangiogenic and antiinflammatory proteins were detected by the reverse transcriptase-polymerase chain reaction (RT-PCR) technique and further confirmed by DNA sequencing of PCR-amplified transcripts. The distribution of tissue inhibitors of metalloproteinase (TIMPs) were studied further by immunohistochemistry performed on paraffin-embedded amniotic membrane tissue. RESULTS: RT-PCR results showed that both human amniotic epithelial and mesenchymal cells express interleukin-1 receptor antagonist, all four TIMPs, collagen XVIII, and interleukin-10. Thrombospondin-1 was expressed in all of the epithelial cell specimens and in one out of five mesenchymal cell specimens. Furthermore, immunohistochemistry studies performed on freshly prepared amniotic membrane confirmed that all members of the TIMP family were present in epithelial and mesenchymal cells as well as in the compact layer of the amniotic stroma. In cryopreserved amniotic membranes, positive staining was seen in residual amniotic cells and stroma. CONCLUSIONS: Human amniotic membrane epithelial and mesenchymal cells express various antiangiogenic and antiinflammatory proteins. Some of those proteins also were found in amniotic membrane stroma. These findings may explain in part the antiangiogenic and antiinflammatory effects of amniotic membrane transplantation.
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Hausen P and Dreyer C. Urea reactivates antigens in paraffin sections for immunofluorescent staining. *Stain Technol* 1982;5:321-4

Hay E D. Development of the vertebrate cornea. *Int Rev Cytol* 1980;263-322

Hayashi K, Sueishi K, Tanaka K and Inomata H. Immunohistochemical evidence of the origin of human corneal endothelial cells and keratocytes. *Graefes Arch Clin Exp Ophthalmol* 1986;5:452-6

Human corneal endothelial cells and keratocytes were immunohistochemically examined using antisera against neuronal tissue antigens, namely, S-100 protein and neuron-specific enolase (NSE), and intermediate filaments such as vimentin, neurofilament, and glial fibrillary acidic protein (GFAP). The corneal endothelium and keratocytes showed a positive immunoreaction to neuron-specific enolase and S-100 protein antisera, and these cells were also stained with antiserum against vimentin, a main intermediate filament of mesenchymal cells. These immunohistochemical findings provide additional evidence that the corneal endothelium and keratocytes originate from neural crest cells and then differentiate into mesenchymal cells.

Hayashi R, Yamato M, Sugiyama H, Sumide T, Yang J, Okano T, Tano Y and Nishida K. N-Cadherin is expressed by putative stem/progenitor cells and melanocytes in the human limbal epithelial stem cell niche. *Stem Cells* 2007;2:289-96

Corneal epithelial stem cells are known to be localized to the basal layer of the limbal epithelium, providing a model system for epithelial stem cell biology; however, the mechanisms regarding the maintenance of these stem cells in their specialized niche remain poorly understood. N-cadherin is a member of the classic cadherin family and has previously been demonstrated to be expressed by hematopoietic stem cells. In the present study, we demonstrate that N-cadherin is expressed by putative stem/progenitor cells, as well as melanocytes, in the human limbal epithelial stem cell niche. In addition, we demonstrate that upon in vitro culture using 3T3 feeder layers, loss of N-cadherin expression occurs with cell proliferation. These results indicate that N-cadherin may be a critical cell-to-cell adhesion molecule between corneal epithelial stem/progenitor cells and their corresponding niche cells in the limbal epithelium.
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Hayashida Y, Nishida K, Yamato M, Watanabe K, Maeda N, Watanabe H, Kikuchi A, Okano T and Tano Y. Ocular surface reconstruction using autologous rabbit oral mucosal epithelial sheets fabricated ex vivo on a temperature-responsive culture surface. *Invest Ophthalmol Vis Sci* 2005;5:1632-9

PURPOSE: Autologous stem cell transplantation for total limbal stem cell deficiency is immunologically preferable, to avoid allograft rejection. This study was undertaken to investigate the possibility of a novel tissue engineering approach for ocular surface reconstruction, using autologous oral mucosal epithelial stem cells expanded ex vivo on temperature-responsive cell culture surfaces. METHODS: Rabbit oral mucosal epithelial cells cultured on temperature-responsive culture surfaces with mitomycin-C-treated 3T3 feeder cells for 2 weeks produced confluent epithelial cell sheets. Putative progenitor cell populations were estimated by colony-forming assays. Autologous transplantation of these cell sheets to surgically manipulated eyes was performed, and ocular surface reconstruction and cell phenotypic modulation were examined. RESULTS: All cultured oral epithelial cells were nonenzymatically harvested as transplantable intact cell sheets by reducing culture temperature to 20 degrees C. Oral epithelial cells were stratified in three to five cell layers more similar to corneal epithelium than to oral mucosal epithelium. Colony-forming assays and immunofluorescence for p63, beta1-integrin, and connexin 43 indicated retention of viable stem and/or progenitor cell populations in cell sheets. Autologous transplantation to rabbit corneal surfaces successfully reconstructed the corneal surface, with restoration of transparency. Four weeks after transplantation, epithelial stratification was similar to that in the corneal epithelium, although the keratin expression profile retained characteristics of the oral mucosal epithelium. CONCLUSIONS: Cell sheet harvest technology enables fabrication of viable, transplantable, tissue-engineered epithelial cell sheets that retain putative progenitor cells from autologous oral mucosal epithelial cells. Promising clinical capabilities for autologous tissue-engineered epithelial cell sheets for ocular surface reconstruction are indicated.
Department of Ophthalmology, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan.

Helander K G. Kinetic studies of formaldehyde binding in tissue. *Biotech Histochem* 1994;3:177-9

Specimens of rabbit liver were fixed for various periods up to 6 days in buffered 14C-formaldehyde. Binding of the isotope reached a plateau after fixation for approximately 24 hr; the half-maximal binding level was reached after approximately 100 min. Formaldehyde binding at 37 C was faster than at 25 C, and faster at pH 7.0 than at pH 4.0. During rinsing of the fixed tissue in water for up to 26 days there was a progressive decrease in isotope content to 10-20% of the pre-rinse level, indicating that formaldehyde fixation is a reversible process.
Laboratory of Membrane Biology, University of California, Los Angeles 90073.

Henderson T R, Coster D J and Williams K A. The long term outcome of limbal allografts: the search for surviving cells. *Br J Ophthalmol* 2001;5:604-9

BACKGROUND/AIMS: Limbal allotransplantation is increasingly being used for ocular surface repair in patients with limbal stem cell dysfunction. However, it is uncertain whether donor cells survive long term on the ocular surface and whether patients maintain the early benefits of the procedure. The aims of this study were to investigate the long term outcome of clinical limbal allografts and to correlate outcome with donor cell survival. METHODS: Five patients who had undergone allotransplantation-four keratolimbal allografts and one tarsoconjunctival allograft-from 3-5 years previously, and for whom residual frozen donor ocular tissue was available, were reviewed. Survival of donor cells lifted from the recipient ocular surface by impression cytology was investigated by DNA fingerprinting using primers detecting variable nucleotide tandem repeat sequences. Recipient buccal cells and scleral samples from the remnant donor eye were used to genotype recipients and donors, respectively. Polymerase chain reaction products were sized by Genescan analysis. RESULTS: An objective long term benefit from the procedure (improved Snellen acuity, reduced frequency of epithelial defects, reduced vascularisation, and scarring) was recorded for four patients. Some subjective benefit was also reported. However, in no instances were donor cells recovered from the ocular surface at 3-5 years post-graft. Initial experiments to examine sensitivity indicated that any surviving donor cells must have constituted less than 2.5% of cells sampled. CONCLUSION: Limbal stem cell allotransplantation can provide long term benefits, as measured by objective criteria. However, such benefits do not necessarily correlate with survival of measurable numbers of donor cells on the ocular surface.
Department of Ophthalmology, Flinders University of South Australia, Adelaide, Australia.

Higa K, Shimmura S, Miyashita H, Shimazaki J and Tsubota K. Melanocytes in the corneal limbus interact with K19-positive basal epithelial cells. *Exp Eye Res* 2005;2:218-23

The human corneal limbus is identified by the distinct features of the palisades of Vogt (POV), which contain pigment granules that are aligned with the microplicae of the epithelium. Although it is presumed that pigments are produced by melanocytes, the characterization of melanocytes in the limbus has not been clearly documented. We examined human limbal tissues by whole mounts and serial histological sections to localize epithelial cells containing melanin granules. Most of the pigmented cells observed by immunohistochemistry were K19 (+) cells in the basal limbal epithelium. A superimposed image revealed that melanin granules were oriented towards the apex of each K19 (+) cell, acting as a pigmented cap facing the ocular surface. Melanocytes were identified by MART1, an antigen specific to melanocyte-lineage cells. Melanocytes were shown to exist as sporadic cells with dendritic processes that extend to surrounding epithelial cells. Melanocytes were also found in light-pigmented donor tissue when visualized by the tyrosinase assay using the enzyme substrate DOPA. Since tyrosinase activity was not found in epithelial cells, the production of melanin is exclusively the role of melanocytes that comprised 5.3+/-2.7% of the total cells in cytospin samples (N=3). Melanocytes and K19 (+) epithelial cells may form a functional network similar to the melanin unit of the skin.
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Hitani K, Yokoo S, Honda N, Usui T, Yamagami S and Amano S. Transplantation of a sheet of human corneal endothelial cell in a rabbit model. *Mol Vis* 2008;1-9

PURPOSE: To develop a novel method for constructing a sheet of human corneal endothelial cells (HCECs) and examine the properties of the HCEC sheet. METHODS: HCECs were cultured on a cell culture insert for a week; ethylenediamine tetraacetic acid was applied from the bottom of the cell culture insert to attenuate the attachment of HCECs. The sheet of HCECs was constructed by bluntly detaching the cell sheet with a spatula. HCEC cell sheets were placed on the posterior surface of excised rabbit corneal buttons and transplanted onto the corneal beds of donor rabbits. In two eyes from the HCEC sheet group, cultured HCECs were labeled with PKH26 to observe the localization of HCECs after transplantation. RESULTS: Cultured HCECs could be bluntly detached en bloc from the bottom of a culture insert. Immunostaining for ZO-1, Na+, K+-ATPase, laminin, fibronectin, and type IV collagen was positive in the cell sheet. The average cell density in a HCEC sheet was 2,425 cells/mm(2). After HCEC sheet transplantation, corneal edema decreased much earlier in the HCEC group than in the control group. In the HCEC sheet group, the monolayer of continuous cells attached to the posterior surface of the transplanted rabbit cornea and the posterior surface of transplanted cornea was covered with PKH26-labeled cells. The average endothelial cell

density in the HCEC sheet group seven days postoperatively was 2,244 cells/mm(2). CONCLUSIONS: This technique for producing an HCEC sheet might be useful in regenerative medicine for the cornea and reconstruction of the corneal endothelium.
Department of Ophthalmology, University of Tokyo Graduate School of Medicine, Tokyo, Japan.

Holland E J. Epithelial transplantation for the management of severe ocular surface disease. *Trans Am Ophthalmol Soc* 1996;677-743

PURPOSE: First, to present a new classification of epithelial transplantation procedures for ocular surface disease; second, to present our experience with a keratolimbal allograft procedure for limbal stem cell deficiency; and third, to make recommendations for the indications and postoperative management of epithelial transplantation procedures. METHODS: A review of all epithelial transplantation procedures was performed. A classification of these procedures based on the source of donor tissue and the tissue transplanted was proposed. In addition, a review of 25 eyes of 21 patients who underwent a keratolimbal allograft was completed. Ocular surface stability, improvement of visual acuity, success of subsequent keratoplasties, and preoperative risk factors were evaluated. Results were compared with those of other epithelial transplantation procedures for ocular surface disease. On the basis of the results of published studies, as well as ours, a recommendation for the indication of the various procedures was made. RESULTS: Epithelial transplantation for ocular surface disease can be classified as one of the following procedures: conjunctival autograft (CAU), conjunctival allograft (CAL), conjunctival limbal autograft (CLAU), cadaveric conjunctival limbal allograft (c-CLAL), living related conjunctival limbal allograft (lr-CLAL), or keratolimbal allograft (KLAL). Evaluation of our keratolimbal allograft patients revealed that 18 of 25 eyes (72%) developed a stable ocular surface. Fifteen eyes (60%) demonstrated a significant improvement in visual acuity. Persistent epithelial defects and symblephara were successfully managed with this procedure. Six of 13 (46%) subsequent keratoplasties were successful. Patients with limbal deficiency due to Stevens-Johnson syndrome had a significantly worse outcome. Patients with preoperative conjunctival keratinization also had a significantly worse outcome. Indications for epithelial transplantation are as follows: For patients with unilateral cicatrizing conjunctival disease, the first option should be CAU. For patients with unilateral limbal deficiency, CLAU is the procedure of choice. For patients with bilateral disease lr-CLAL should be considered first. If this procedure is not available, then consideration of KLAL is warranted. CONCLUSIONS: Classification of the various epithelial transplantation procedures based on anatomy is useful for an accurate comparison and discussion of the procedures. KLAL is a useful technique in the management of severe ocular surface disease due to limbal deficiency. However, patients with preoperative conjunctival keratinization have a poor prognosis. Consideration of a CLAU or a lr-CLAL should be made for ocular surface disease on the basis of whether the disease is unilateral or bilateral. The importance of HLA and ABO typing, as well as the protocol for immunosuppression in the allograft procedures for limbal deficiency, needs further study.

Huang A J, Tseng S C and Kenyon K R. Paracellular permeability of corneal and conjunctival epithelia. *Invest Ophthalmol Vis Sci* 1989;4:684-9

The paracellular permeability of normal rabbit cornea and conjunctiva was studied in vivo and in vitro. After intravenous administration, horseradish peroxidase was found to percolate to the intercellular space of conjunctival epithelia and was restricted by the tight junctions of the superficial epithelium. Only minimal tracer was present in the limbus and cornea. The difference between corneal and conjunctival paracellular pathways was further compared in vitro by tissue perfusion studies using various tracers from subepithelial space to apical surface. The intact full-thickness cornea was permeable to mannitol (MW 182) but not to inulin or dextran. The conjunctiva was permeable to mannitol, inulin and FITC-dextran (MW 20,000). The quantitative permeability to 3H-mannitol (X10(-8) cm/sec) of adult rabbit cornea was 0.12 +/- 0.02, which is about 55-fold and 50-fold lower than that of conjunctiva (6.78 +/- 0.21) and peritoneum (6.12 +/- 0.63), respectively. Removal of the corneal epithelium increased the permeability 40-fold; however, removal of the endothelium had little effect on the solute permeation. When both corneal epithelium and endothelium were debrided, the bare stroma became edematous and the permeability increased 70-fold. The permeability of 1-week-old rabbit cornea was 1.32 +/- 0.18, which decreased to 0.46 +/- 0.06 in 2-week-old rabbits, and became similar to the adult level at 4 weeks of age. When Tenon's capsule was included in the perfusion, the conjunctival permeability decreased 2.5-fold. With the apposition of bare corneal stroma to the conjunctiva and Tenon's capsule, the permeability decreased further (4-fold).(ABSTRACT TRUNCATED AT 250 WORDS)
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Huang L, Harkenrider M, Thompson M, Zeng P, Tanaka H, Gilley D, Ingram D A, Bonanno J A and Yoder M C. A hierarchy of endothelial colony-forming cell activity displayed by bovine corneal endothelial cells. *Invest Ophthalmol Vis Sci* 2010;8:3943-9

PURPOSE: To test the hypothesis that the robust expansion of bovine corneal endothelial cells (BCECs) in vitro is due to the presence of individual endothelial cells with various levels of proliferative potential. METHODS: BCECs and bovine vascular endothelial cells (ECs) derived from aorta, coronary artery, and pulmonary artery were cultivated in optimized medium. These cell populations were confirmed by morphologic features, functional assays, and gene expression profiles. Moreover, ECs were plated in a single-cell clonogenic assay to evaluate colony-forming ability. RESULTS: Both corneal and vascular ECs were confirmed to be pure populations of endothelium uncontaminated with hematopoietic cells. A complete hierarchy of endothelial colony-forming cells (ECFCs) was identified in BCECs by a single-cell clonogenic assay. The distribution of the various types of ECFCs was similar to the control ECs removed from the systemic vessels. CONCLUSIONS: Cultured BCECs display clonal proliferative properties similar to those of vascular ECs.
Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Indiana, USA.

Inaba K, Tanishima T and Hirosawa K. Electron microscopic observations of Descemet's membrane of peripheral cornea. *Jpn J Ophthalmol* 1986;1:1-13

The posterior portion of the Descemet's membrane was studied by scanning and transmission electron microscopy; the materials comprised 87 human peripheral corneas with ages from 2 to 98 years, 5 monkey corneas and 4 rabbit corneas. In some specimens, the endothelium was removed by ultrasonication. After removal of the endothelium, "curly structures" were recognized on the surface of the Descemet's membrane, where the membrane showed a gradual thinning. These structures appeared along the whole circumference of the cornea with variable width in the human specimens, but in monkey and rabbit corneas, the extent of these structures was less than in the human cornea. The "curly structures" were not encountered in young subjects, and they increased with age. There was a positive correlation (r = 0.60, P less than 0.001) between the age and the extent of these structures. Other aging products of the Descemet's membrane, ie, Hassall-Henle bodies, were partly surrounded by the "curly structures". The human "curly structures" consisted of collagen fibrils, halo structures in the collagen bundles, wide-spacing fibers, microfibrils, ground substances containing minute filaments and a structure resembling the Descemet's membrane. Components of "curly structures" of the monkey and rabbit were almost the same as those of the human except for the Descemet's membrane-like structure.

Inatomi T, Nakamura T, Koizumi N, Sotozono C, Yokoi N and Kinoshita S. Midterm results on ocular surface reconstruction using cultivated autologous oral mucosal epithelial transplantation. *Am J Ophthalmol* 2006;2:267-75

PURPOSE: To perform a midterm assessment of the integrity and reproducibility of cultivated autologous oral mucosal epithelial sheets, and to evaluate the clinical efficacy of their transplantation in ocular surface. DESIGN: Observational case series. METHODS: Cultivated autologous oral mucosal epithelial sheets were created using amniotic membrane and buccal mucosal epithelium from 12 patients with Stevens-Johnson syndrome, chemical and thermal injury, pseudo-ocular cicatricial pemphigoid, and idiopathic ocular surface disorder. They were transplanted onto 15 eyes from these patients who were then followed up for a mean of 20 months; with the longest follow-up being 34 months. We assessed their clinical outcomes with special reference to neovascularization. RESULTS: Cultivated autologous oral mucosal epithelial sheets could be generated from all patients. On the second postoperative day, 14 of 15 sheets transplanted demonstrated total re-epithelialization on the cornea. During the follow-up, the ocular surface was stable and transparent without any major complications in 10 of 15 eyes (67%), and the transplanted epithelium survived for at least 34 months. There were five eyes (33%) with small but long-standing epithelial

defects, three of these healed spontaneously, and two (13%) required reoperation. In 10 eyes, postoperative visual acuity was improved by more than 2 lines. All eyes manifested some peripheral corneal vascularization. CONCLUSIONS: We established a successful tissue-engineering technique to generate cultivated autologous oral mucosal epithelial sheets and succeeded in reconstructing the ocular surface. We suggest that this surgical modality may be both safe and useful, especially in younger patients with the most severe ocular surface disorders.
Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan.

Ishino Y, Sano Y, Nakamura T, Connon C J, Rigby H, Fullwood N J and Kinoshita S. Amniotic membrane as a carrier for cultivated human corneal endothelial cell transplantation. *Invest Ophthalmol Vis Sci* 2004;3:800-6

PURPOSE: It would be advantageous if cultivated human corneal endothelial cells (cHCECs) could be transplanted for the treatment of diseases caused by corneal endothelial disorders. To achieve this, a matrix that can serve as a carrier for cHCECs is needed. The present study was conducted to examine the feasibility of using amniotic membrane (AM) as a carrier for this application. METHODS: HCECs obtained from peripheral corneal tissue were cultivated, passaged, and transplanted onto denuded AM. The cell density and morphology of the resultant cHCECs on AM were examined by light, scanning electron, and transmission electron microscopy. To determine whether these cHCEC sheets on AM carrier were functional in vivo, the cHCEC sheets on AM were transplanted onto rabbit corneas whose Descemet's membrane and endothelial cells had been completely removed. After transplantation, the corneal appearance was examined by slit lamp biomicroscopy, and corneal thickness was measured daily by pachymetry. At 7 days after surgery, the grafts were examined by light, scanning electron, and transmission electron microscopy. RESULTS: The density of the cHCECs on AM was greater than 3000 cells/mm(2). Morphologically, the cHCEC sheets consisted of a fairly continuous layer of flat squamous polygonal endothelial cells that appeared uniform in size with tightly opposed cell junctions in vitro and in vivo after transplantation. The corneas that received transplanted cHCEC sheets had little edema and retained their thinness and transparency. CONCLUSIONS: The cell density and morphology of cHCECs on AM were similar to those of normal corneas, and cHCECs on AM were functional in vivo. These results indicate that AM maintains HCEC morphology and function and could serve as a carrier for cHCEC transplantation.
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Janzen V, Forkert R, Fleming H E, Saito Y, Waring M T, Dombkowski D M, Cheng T, DePinho R A, Sharpless N E and Scadden D T. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. *Nature* 2006;7110:421-6

Stem-cell ageing is thought to contribute to altered tissue maintenance and repair. Older humans experience increased bone marrow failure and poorer haematologic tolerance of cytotoxic injury. Haematopoietic stem cells (HSCs) in older mice have decreased per-cell repopulating activity, self-renewal and homing abilities, myeloid skewing of differentiation, and increased apoptosis with stress. Here we report that the cyclin-dependent kinase inhibitor p16INK4a, the level of which was previously noted to increase in other cell types with age, accumulates and modulates specific age-associated HSC functions. Notably, in the absence of p16INK4a, HSC repopulating defects and apoptosis were mitigated, improving the stress tolerance of cells and the survival of animals in successive transplants, a stem-cell-autonomous tissue regeneration model. Inhibition of p16INK4a may ameliorate the physiological impact of ageing on stem cells and thereby improve injury repair in aged tissue.
Center for Regenerative Medicine, Massachusetts General Hospital, Harvard Medical School, 185 Cambridge Street, Boston, Massachusetts 02114, USA.

Jenkins C, Tuft S, Liu C and Buckley R. Limbal transplantation in the management of chronic contact-lens-associated epitheliopathy. *Eye (Lond)* 1993;629-33

We describe the clinical management of 6 patients who developed a chronic corneal epitheliopathy 1-18 years after commencing soft contact lens wear. All had a history of exposure to thiomersal in contact lens fluids. The corneal changes were characterised by epithelial haze and superficial stromal vascularisation which extended from the limbus towards the visual axis. Five patients were observed for a minimum of 18 months after stopping contact lens wear before undergoing limbal transplantation. A good result was obtained in 1 patient who had worn a contact lens in one eye only. Recurrent epithelial changes were observed on the recipient eyes of the remaining patients who had previously worn contact lenses bilaterally, and in 1 patient epithelial haze also developed adjacent to the donor site in the previously clinically normal donor eye. All 5 patients experienced an improvement in symptoms post-operatively but in 2 patients the visual acuity later deteriorated because of epithelial irregularity. The sixth patient has not had surgery. We conclude that limbal stem cell dysfunction in chronic contact-lens-associated epitheliopathy may be subclinical and that autograft transplantation in bilaterally exposed patients may fail to restore the epithelial phenotype of the host eye whilst jeopardising the epithelial integrity of the donor eye by depleting its stem cell reserve.
Moorfields Eye Hospital, London, UK.

Jiang T S, Cai L, Ji W Y, Hui Y N, Wang Y S, Hu D and Zhu J. Reconstruction of the corneal epithelium with induced marrow mesenchymal stem cells in rats. *Mol Vis* 2010;1304-16

PURPOSE: To explore the feasibility of bone marrow mesenchymal stem cells (MSCs) transdifferentiating into corneal epithelial cells in a limbal stem cell deficiency (LSCD) model in rats. METHODS: Rat MSCs were isolated and purified using a gradient isolation procedure. The cells were induced by rat corneal stromal cells (CSCs) in a transwell co-culture system. The induced MSCs were identified by immunofluorescence staining, flow cytometry, and scanning electron microscopy (SEM). A corneal LSCD model was produced in the right eyes of 48 rats by alkali injury. The eyes of 12 rats without any transplant served as controls (Group 1). Amniotic membranes (AM; Group 2), uninduced MSCs (Group 3), or MSCs induced by CSCs (Group 4), were transplanted onto the cornea of the model (n=12 each). The therapeutic effects of the four groups were evaluated by slit lamp observation, hematoxylin and eosin staining, immunohistochemistry staining, and confocal laser corneal microscopy. RESULTS: Cultivated MSCs were positive for CD29, CD44, and CD90, but negative for CD34, CD45, CD133, and CK12, with typical MSCs characteristics revealed by SEM. After co-culture with CSCs, the induced MSCs expressed positive staining for CK12 with corneal epithelial cell characteristics confirmed by SEM; the induced MSCs were unchanged on the amnion. Compared with the other three groups, the corneal opacity, fluorescence staining, and neovascularization grades were significantly decreased in the induced MSCs group, both on postoperative week four and ten. CONCLUSION: MSCs induced by CSCs can transdifferentiate into corneal epithelial cells in vitro. The induced MSCs on an amniotic membrane have remarkable effects on the treatment of corneal alkali burn and the reconstruction of the corneal surface of rats.
Department of Ophthalmology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shannxi, China.

Jin E J, Burrus L W and Erickson C A. The expression patterns of Wnts and their antagonists during avian eye development. *Mech Dev* 2002;1-2:173-6

To determine the possible involvement of Wnt signaling in eye development, we analyzed the expression patterns of Wnts and Wnt inhibitors in the chicken eye at stage 25, when the first wave of neural crest migration into the cornea begins, and stage 27, just prior to the second wave of neural crest migration. Wnt expression is developmentally regulated in the chicken eye, and antagonists of Wnt signaling are generally expressed in patterns that are temporally distinct from the Wnts.
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Joo C K, Green W R, Pepose J S and Fleming T P. Repopulation of denuded murine Descemet's membrane with life-extended murine corneal endothelial cells as a model for corneal cell transplantation. *Graefes Arch Clin Exp Ophthalmol* 2000;2:174-80

BACKGROUND: Corneal endothelial cell transplantation has been an intriguing concept as an alternative to full-thickness penetrating keratoplasty. Using a murine corneal transplantation model, we sought to establish the optimal conditions to repopulate, ex vivo,

denuded murine Descemet's membrane with life-extended cell cultures of murine corneal endothelial cells. These ex vivo repopulated corneas were used as donor corneas in a murine orthotopic corneal transplantation model to assess, in vivo, the function of the transplanted, life-extended murine corneal endothelial cells (MCEC). METHODS: Mouse corneas were surgically trephined and the native corneal endothelium was removed mechanically using a sterile cotton swab. Cultured murine corneal endothelial cells (life extended by expression of the SV40 large T antigen) were added onto the denuded Descemet's membrane and allowed to attach in culture at 37 degree C. Evidence of corneal cell attachment to Descemet's membrane was determined between 1 and 8 h by scanning and transmission electron microscopy. Donor life-extended corneal endothelial cells were labeled with a fluorescent dye to allow tracking of the donor cells following seeding onto denuded Descemet's membrane. In four independent experiments, the Descemet's repopulated corneas were placed into syngeneic mice and evaluated for corneal clarity after 6 weeks. RESULTS: We could detect attachment of the life-extended murine CEC by scanning and transmission electron microscopy to denuded Descemet's membrane. The optimal time for adherence was 2 h and these repopulated corneas were used as donors in a murine model of penetrating keratoplasty. Of 20 mice evaluated after 6 weeks, 4 displayed corneal clarity, and fluorescent evaluation demonstrated that only the donor corneal endothelial cells were present. CONCLUSIONS: This experimental protocol establishes that "life-extended" MCEC can bind to Descemet's membrane ex vivo and form a distinct monolayer. The repopulated Descemet's membrane allowed us to directly test the hypothesis that cultured life-extended corneal endothelial cells are functional when reintroduced into an in vivo milieu and provides evidence that specific corneal endothelial cell transplantation may be a viable alternative to pentrating keratoplasty.
Department of Ophthalmology, Catholic University Medical College, Seoul, Korea.

Joyce N C, Harris D L and Mello D M. Mechanisms of mitotic inhibition in corneal endothelium: contact inhibition and TGF-beta2. *Invest Ophthalmol Vis Sci* 2002;7:2152-9

PURPOSE: Contact inhibition has been implicated as an important antiproliferative mechanism in developing and mature corneal endothelium. Although exogenous TGF-beta2 and TGF-beta2 in aqueous humor suppress S-phase entry in cultured rat corneal endothelial cells, it is not known whether TGF-beta2 contributes to the mitotic inhibition that occurs during in vivo endothelial development. TGF-beta receptors I, II, and III must be coexpressed for a TGF-beta2-induced intracellular signal to be transmitted. The current study was conducted to determine whether TGF-beta2 contributes to mitotic inhibition during endothelial development, by investigating when these receptors become coexpressed in the endothelium of neonatal rats. Cyclin-dependent kinase inhibitors (CKIs), such as p27kip1 and p15INK4b, help mediate mitotic inhibition in other cell types. The role of CKIs in inhibiting proliferation in corneal endothelium was examined by first determining the kinetics of p27kip1 expression in neonatal rat corneal endothelium. Studies were then extended to cultured cells to more directly compare the effects of TGF-beta2 and cell-cell contact on the relative protein and mRNA expression of the CKIs, p27kip1, and p15INK4b. METHODS: Immunocytochemistry (ICC) detected TGF-beta receptors I, II, and III (RI, RII, RIII, respectively) in the endothelium of rat corneas on postnatal days 1, 10, and 21, and in adult (3-month-old) rats. ICC for p27kip1 was conducted on postnatal days 1, 7, 14, and 21. Samples were taken for p27kip1 RT-PCR on postnatal days 7, 14, and 21 and from adult rats. The effect of TGF-beta2 on p27kip1 and p15INK4b expression was determined in G(0)-phase synchronized subconfluent rat corneal endothelial cells incubated for 24 hours in 10% serum +/- 5 ng/mL TGF-beta2. CKI expression was also examined in fully confluent cultures. RT-PCR and Western blot analysis detected p27kip1 and p15INK4b mRNA and protein expression, respectively. The effect of releasing cells from cell-cell contact on proliferation and p27kip1 protein expression was studied in confluent cultures treated for 1 hour with and without 2.0 mg/mL di-sodium EDTA and then maintained for 24 hours in 10% serum. Cultures were then either fixed for ICC of Ki67, a marker of actively cycling cells, or extracted for Western blot determination of p27kip1 protein. RESULTS: Positive staining for TGF-beta RIII was detected on postnatal day 10, and staining for RI and RII was detected on postnatal day 21. The endothelium stained positively for p27kip1 on postnatal day 1 and thereafter, and p27kip1 PCR product was detectable at the earliest time point tested (postnatal day 7). In cultured cells, TGF-beta2 and cell-cell contact had relatively little effect on expression of p27kip1 or p15INK4b mRNA. TGF-beta2 lowered the levels of both proteins, but p27kip1 remained at a higher level than p15INK4b. In confluent cultures, p15INK4b protein was reduced; however, p27kip1 protein levels increased 20-fold. Positive staining for Ki67 was detected, and p27kip1 protein levels substantially decreased in EDTA-treated confluent cells compared with the untreated control. CONCLUSIONS: Previous studies from this laboratory showed that corneal endothelial cell proliferation ceases in neonatal rat by postnatal day 13. This timing correlated with the formation of stable cell-cell contacts, implicating contact inhibition as an important mechanism of growth arrest during endothelial development. The current studies showed that coexpression of TGF-beta RI, RII, and RIII occurred too late for TGF-beta2 to have a significant role in inhibiting proliferation during endothelial development. Studies in cultured cells suggest that p27kip1 mediates inhibition of proliferation induced by TGF-beta2, although the response to this cytokine was relatively weak. ICC and RT-PCR of p27kip1 in neonatal endothelium and RT-PCR and Western blot studies in cultured cells indicate that contact inhibition is mediated, in large part, through the activity of p27kip1. These results, together with previous data from this laboratory, strongly suggest that contact inhibition is an important mechanism responsible for inducing cell cycle arrest during corneal endothelial development and for maintaining the mature monolayer in a nonproliferative state. In both cases, contact-induced inhibition is mediated, at least in part, by p27kip1. TGF-beta2 appears not to induce mitotic arrest in the developing endothelium, but may function to maintain the mature endothelium in a nonreplicative state should cell-cell contact be lost in the monolayer.
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Joyce N C, Meklir B, Joyce S J and Zieske J D. Cell cycle protein expression and proliferative status in human corneal cells. *Invest Ophthalmol Vis Sci* 1996a;4:645-55

PURPOSE: To determine the relative expression of cell cycle-association proteins in human corneal and limbal epithelium and corneal endothelium in situ, to correlate staining patterns of cell cycle-associated proteins with known proliferative status of corneal and limbal epithelial cells, and to determine the relative proliferative status of corneal endothelial cells in situ by comparing their staining patterns with those of corneal and limbal epithelial cells. METHODS: Corneas from donors 6 weeks and 17, 27, 37, 53, 66, and 67 years of age were preserved in Optisol, received on ice within 24 to 36 hours of death, and immediately fresh frozen. Transverse 6-micron corneal sections were prepared for indirect immunofluorescence localization using commercial antibodies that specifically recognize the following cell cycle-associated proteins: cyclins D, E, A, and B1; protein kinases p33cdk2 and p34cdc2; and Ki67, a marker of actively cycling cells. RESULTS: All cells of the corneal and limbal epithelium and corneal endothelium stained positively for protein kinases, p33cdk2 and p34cdc2, and for cyclin B1. Staining patterns for cyclins D, E, and A and for Ki67 differed depending on the relative proliferative status of the cells. Terminally differentiated, noncycling corneal epithelial subrabasal cells did not stain significantly for cyclins D, E, or A, or for Ki67. Some corneal epithelial basal cells showed nuclear staining, particularly for cyclin D and Ki67, indicating the presence of actively cycling cells in this regenerative cell layer. In peripheral corneal epithelium, the relative number of basal cells with positive cytoplasmic staining for cyclins D, E, and A increased with proximity to the limbus. Within this region, an occasional nucleus stained positively for Ki67. Limbal basal cells, which contain a slow-cycling stem cell population, stained positively for cyclins D, E, and A within the cytoplasm. Nuclear staining for cyclin D and Ki67 was observed in a few basal cells. Occasional cells within the suprabasal layers of the limbus stained positively for Ki67. The corneal endothelium, considered a nonrenewing population, exhibited staining patterns similar to those of limbal basal cells, except that in no specimen was Ki67 staining observed. CONCLUSIONS: All corneal and limbal epithelial and corneal endothelial cells express protein kinases, p33cdk2 and p34cdc2, and cyclin B1. Relative staining patterns of the cell cycle-dependent proteins, cyclins D, E, and A, and of Ki67 acted as markers to distinguish terminally differentiated epithelial suprabasal cells that have exited the cell cycle, actively cycling epithelial basal cells, and slowly-cycling limbal basal (stem) cells. Staining patterns of the corneal endothelium most closely corresponded to those of limbal basal cells, suggesting that endothelial cells are arrested in G1-phase and have not exited the cell cycle.
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Joyce N C, Navon S E, Roy S and Zieske J D. Expression of cell cycle-associated proteins in human and rabbit corneal endothelium in situ. *Invest Ophthalmol Vis Sci* 1996b;8:1566-75

PURPOSE: It is unknown why human corneal endothelium exhibits limited capacity to divide while the endothelia of other species, such as rabbit, divide in vivo at wounding and in culture. A potentially valuable source of information concerning why human endothelium has such a limited proliferative capacity lies in elucidating any differences in the molecular events governing the cell cycle of these two species. A recent study of the relative expression of cell cycle-associated proteins in donor corneas suggests that human corneal endothelial cells in vivo have not exited the cell cycle but are arrested in G1-phase. The purpose of the current study was to identify differences in cell cycle protein expression in human and rabbit endothelium that would explain the difference in their relative proliferative capacities. Specifically, the authors first ascertained the relative proliferative status of rabbit corneal endothelial cells in vivo. The expression and intracellular distribution of G1-phase regulatory proteins was then determined in both species, and the results were compared. METHODS: Corneas from New Zealand white rabbits (weight range, 2 to 3 kg) and from human donors (age range, 6 months to 67 years) were fresh frozen, cryostat sectioned, and prepared for indirect immunofluorescence microscopy using an established protocol. The following monoclonal antibodies were localized in rabbit corneal endothelium only: cyclins D, E, A, and B1; protein kinase p34cdc2; and Ki67, a marker of actively cycling cells. Localization patterns for the following G1-phase regulatory proteins were compared in both human and rabbit corneal endothelia: the tumor suppressors, pRb, p53, and p16INK4, and the transcription factor, E2F. Reverse transcription-polymerase chain reaction studies were conducted to detect mRNA for Ki67 in human and rabbit corneal cells. RESULTS: Cyclins D, E, and A were localized in the cytoplasm of rabbit corneal endothelium, whereas cyclins B1 and p34cdc2 were detected in the nucleus. No Ki67 protein or mRNA expression was detected in the endothelium of either species. In human and rabbit endothelia, p53 and p16INK4 were localized to the cytoplasm, whereas pRb was detected in the nucleus. E2F exhibited a nuclear and a cytoplasmic localization in each species. CONCLUSIONS: The corneal endothelium of rabbits stained positively for cyclins D, E, and A and did not stain for Ki67, suggesting that, as in humans, rabbit corneal endothelium in vivo is arrested in G1-phase upstream from Ki67 synthesis. Cyclin E was located in the cytoplasm of rabbit cells, whereas it was found in the nucleus in human endothelium. The apparent difference in cellular distribution of cyclin E in these two species may be significant because this cyclin is active during the G1-/S-phase transition. It is possible that in situ human and rabbit corneal endothelial cells are arrested at different points within G1-phase and/or that the difference in relative proliferative capacity exhibited by the corneal endothelium in these two species may be caused by differences in their relative ability to overcome G1-phase arrest.
Schepens Eye Research Institute, Boston, MA 02114, USA.

Joyce N C, Zhu C C and Harris D L. Relationship among oxidative stress, DNA damage, and proliferative capacity in human corneal endothelium. *Invest Ophthalmol Vis Sci* 2009;5:2116-22

PURPOSE: To determine whether human corneal endothelial cells (HCECs) exhibit signs of oxidative DNA damage and to test whether oxidative stress affects the proliferative capacity of HCECs. METHODS: Donor human corneas were divided into two age groups: young (<30 years) and older (>50 years). An 8-hydroxy-2'-deoxyguanosine (8-OHdG) ELISA assay was used to quantify oxidative DNA damage in HCECs freshly isolated from ex vivo corneas. 8-OHdG immunostaining localized the sites of oxidative DNA damage in corneal wholemounts and cultured HCECs. To test whether oxidative stress induces oxidative DNA damage, HCECs cultured from young donors were treated with increasing concentrations of hydrogen peroxide (H(2)O(2)) and immunostained for 8-OHdG. To test the effect of oxidative stress on proliferative capacity, HCECs cultured from young donors were treated with H(2)O(2) and cell numbers determined by WST-8 assay. RESULTS: 8-OHdG levels were significantly higher (P = 0.0031) in the central endothelium of older donors than of young donors. Intense nuclear staining for 8-OHdG was observed in central endothelium of older, but not young, donors. The relative intensity of 8-OHdG in the nuclei of cultured HCECs was similar to that observed in ex vivo corneas. Treatment of cultured HCECs from young donors with increasing concentrations of H(2)O(2) resulted in a dose-dependent increase in nuclear 8-OHdG staining and a decrease in proliferative capacity similar to that observed in untreated HCECs from older donors. CONCLUSIONS: Age-dependent and topographical decreases in proliferative capacity observed in HCECs resulted, at least in part, from nuclear oxidative DNA damage.
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Joyce N C and Zieske J D. Transforming growth factor-beta receptor expression in human cornea. *Invest Ophthalmol Vis Sci* 1997;10:1922-8

PURPOSE: Limbal basal cells and corneal endothelial cells appear to be inhibited in the G1 phase of the cell cycle. As a preliminary to determining whether transforming growth factor-beta (TGF-beta) might mediate this inhibition, investigation was made to determine whether human corneal and limbal cells express TGF-beta receptor types I (RI), II (RII), and III (RIII). METHODS: Corneas from eight human donors, aged stillborn to 85 years, were fresh frozen, cryostat sectioned, and prepared for indirect immunofluorescence localization of RI, RII, and RIII, using an established protocol. Corneas from donors 50 years of age or older were used to prepare RNA from the epithelium and endothelium. Reverse transcription-polymerase chain reaction was conducted using primers specific for each TGF-beta receptor type. RESULTS: Immunolocalization patterns for RI, RII, and RIII were similar, regardless of donor age. Binding of RI and RII antibodies was barely detectable in central corneal epithelium; however, most limbal basal cells stained positively for RI and RII. All layers of central corneal epithelium and the suprabasal layers of the limbus stained positively for RIII, whereas staining for this receptor was markedly decreased in limbal basal cells. Corneal endothelium bound the antibody for all three TGF-beta receptor types. In the same tissue sections, antibody staining for the RIII protein was more intense in corneal endothelial cells than in limbal basal cells. Polymerase chain reaction product for RI, RII, and RIII was detected in the epithelium and in the endothelium. CONCLUSIONS: Limbal basal cells and corneal endothelial cells expressed mRNA and protein for TGF-beta receptor types I, II, and III, suggesting that both cell types can transmit a TGF-beta-induced signal. These two cell types may differ in their relative response to those TGF-beta isoforms that require binding to RIII for signal transduction, in that staining intensity for RIII was relatively low in limbal basal cells compared with that in the endothelium. That limbal basal and corneal endothelial cells express receptors for TGF-beta suggests that this cytokine could mediate G1 phase arrest in these two cell types.
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Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, Baba S, Kato T, Kazuki Y, Toyokuni S, Toyoshima M, Niwa O, Oshimura M, Heike T, Nakahata T, Ishino F, Ogura A and Shinohara T. Generation of pluripotent stem cells from neonatal mouse testis. *Cell* 2004;7:1001-12

Although germline cells can form multipotential embryonic stem (ES)/embryonic germ (EG) cells, these cells can be derived only from embryonic tissues, and such multipotent cells have not been available from neonatal gonads. Here we report the successful establishment of ES-like cells from neonatal mouse testis. These ES-like cells were phenotypically similar to ES/EG cells except in their genomic imprinting pattern. They differentiated into various types of somatic cells in vitro under conditions used to induce the differentiation of ES cells and produced teratomas after inoculation into mice. Furthermore, these ES-like cells formed germline chimeras when injected into blastocysts. Thus, the capacity to form multipotent cells persists in neonatal testis. The ability to derive multipotential stem cells from the neonatal testis has important implications for germ cell biology and opens the possibility of using these cells for biotechnology and medicine.
Horizontal Medical Research Organization, Graduate School of Medicine, Kyoto University, Kyoto 606-8501, Japan.

Kawakita T, Shimmura S, Hornia A, Higa K and Tseng S C. Stratified epithelial sheets engineered from a single adult murine corneal/limbal progenitor cell. *J Cell Mol Med* 2008;4:1303-16

The limbal region of the adult cornea contains stem cells which are ultimately responsible for regeneration of the corneal epithelium during wound repair. However, primarily-isolated murine corneal/limbal epithelial cells rapidly senesce on plastic in a serum-free low [Ca(2+)] medium, suggesting only transit amplifying cells are promoted. We developed a novel expansion method by seeding at a low cell density (<500 cells/cm(2)) and prolonging each culture time beyond the lifespan of transit amplifying cells (4 weeks). Expanded cells were uniformly small, negative to K12 keratin, but positive for p63 nuclear staining, and could be subcultured beyond 100 passages. After limiting dilution, one clone (TKE2) was selected that exhibited single cell clonal expansion with a doubling time of 34.2 hrs, and had normal karyotyping, but no anchorage-independent growth. A single cell could be continually expanded to a confluent monolayer on denuded amniotic membrane

and became stratified by exposing to the air-medium interface. The resultant stratified epithelium expressed K14 keratin, involucrin, connexin 43 and p63, but not K12 keratin or Pax 6. However, expression of K12 could be up-regulated by increasing extracellular calcium concentration and addition of foetal bovine serum (FBS) at P12, but less so at P85. Therefore, this murine lim-bal/corneal epithelium-derived progenitor cell line still retained the plasticity for adopting corneal lineage differentiation, could be useful for investigating limbal niche cues that may promote corneal epithelial fate decision.
TissueTech, Inc., and Ocular Surface Center, Miami, FL 33173, USA.

Kawamoto H, Ohmura K and Katsura Y. Direct evidence for the commitment of hematopoietic stem cells to T, B and myeloid lineages in murine fetal liver. *Int Immunol* 1997;7:1011-9

We established an experimental system in vitro to examine the developmental capacity of individual hematopoietic progenitors to generate T, B and myeloid (M) cells. By using this system we analyzed the process of lineage commitment of hematopoietic progenitors in murine fetal liver (FL). It is known that small numbers of B and M cells, in addition to T cells, are generated in a co-culture of hematopoietic progenitors and a deoxyguanosine-treated fetal thymus (FT) lobe. We tried to enhance the growth of B and M cells by the addition of IL-7, IL-3 and stem cell factor into the co-culture. This cytokine-supplemented FT organ culture was used to examine the developmental capacity of individual hematopoietic progenitors in FL. Single cells of lineage marker (Lin)-c-kit+Sca-1+ (Sca-1+) and Lin-c-kit+Sca-1-(Sca-1-) populations from the FL harvested at day 12 of gestation were cultured for 10 days, and the phenotypes of cells generated in each lobe were analyzed with a flow cytometer. All progenitors in the Sca-1- population were shown to be committed to generate only T, B or M cells. On the other hand, multipotent progenitors, which are capable of generating T, B and M cells, as well as unipotent progenitors committed to the T, B or M lineage were found in the Sca-1+ population. Bipotent progenitors generating M and T cells and those generating M and B cells were also found in the Sca-1+ population, which probably represent progenitors in the process of commitment. However, no bipotent progenitors generating T and B cells were detected.
Department of Immunology, Chest Disease Research Institute, Kyoto University, Japan.

Kenyon K R and Tseng S C. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology* 1989;5:709-22; discussion 22-3

Limbal autograft transplantation is presented in 26 consecutive cases comprising both acute and chronic chemical injury (20 cases), thermal burns (2 cases), contact lens-induced keratopathy (3 cases), and ocular surface failure after multiple surgical procedures (1 case), with follow-up ranging from 2 to 45 months (mean, 18 months). The operative technique usually involved transfer of two free grafts of limbal tissue from the uninjured or less injured donor eye to the severely injured recipient eye, the latter having been prepared by limited conjunctival research and superficial dissection of fibrovascular pannus without keratectomy. Clinical results in 21 patients with follow-up of 6 months or more have consistently shown improved visual acuity (17 cases), rapid surface healing (19 cases), stable epithelial adhesion without recurrent erosion or persistent epithelial defect (20 cases), arrest or regression of corneal neovascularization (15 cases), and probable increased success for lamellar or penetrating keratoplasty (8 cases). No intraoperative complications were encountered, and no adverse reactions developed in donor eyes. Impression cytology in selected cases showed restoration of the corneal epithelial phenotype and regression of goblet cells from the recipient cornea. Therefore, limbal autograft transplantation is recommended for treatment of widespread ocular surface damage with loss of limbal epithelial stem cells and, specifically, for chemical or thermal burns, contact lens-induced keratopathy, and selected persistent corneal epithelial defects.
Massachusetts Eye & Ear Infirmary, Cornea Research Unit, Eye Research Institute, Boston, MA.

Kikuchi M, Hayashi R, Kanakubo S, Ogasawara A, Yamato M, Osumi N and Nishida K. Neural crest-derived multipotent cells in the adult mouse iris stroma. *Genes Cells* 2011;3:273-81

The purpose of this study was to characterize neural crest-derived cells within the adult murine iris. The iris was isolated from P0-Cre/Floxed-EGFP transgenic (TG) mice. The isolated iris cells formed EGFP-positive spheres on non-adhesive culture plates. Immunostaining showed that these EGFP-positive spheres expressed neural crest markers including Sox10 and p75NTR, and these cells showing in vitro sphere-forming ability were originally resided in the iris stroma (IS), in vivo. Real-time RT-PCR showed that the EGFP-positive spheres expressed significantly higher levels of the neural crest markers than EGFP-negative spheres and bone marrow-derived mesenchymal stem cells. Furthermore, the iris stromal sphere had capability to differentiate into various cell lineages including smooth muscle and cartilage. These data indicate that neural crest-derived multipotent cells can be isolated from the murine IS and expanded in sphere culture.
Department of Ophthalmology, Tohoku University School of Medicine, Sendai, Japan.

Kim M and Morshead C M. Distinct populations of forebrain neural stem and progenitor cells can be isolated using side-population analysis. *J Neurosci* 2003;33:10703-9

The absence of stem cell-specific markers has posed challenges to the identification and isolation of stem cells. We report the isolation of a discrete and highly enriched population of neural stem cells from clonally derived colonies of neural stem cell and progenitor cells (neurospheres) after exposure to the fluorescent DNA binding dye Hoeschst 33342 and subsequent analysis via dual wavelength flow cytometry. The low fluorescent side population comprised only 3.6% of all live cells sorted yet contained >99% of all the neural stem cells as assayed by the formation of neurospheres in culture. Most neurosphere-derived cells are progenitor cells, and these are found within the higher fluorescence (non-side population) fraction. The isolation of a highly enriched population of self-renewing, multipotential neural stem cells was seen from both adult- and embryonic-derived neurospheres; however, the relative percentage of cells comprising the side-population and the mechanism of dye efflux varied between adult and embryonic donor tissue. Combining the side-population analysis with markers recently shown to enrich for neural stem cells afforded no further enrichment in the case of peanut agglutinin expression and size criteria; however, when the side-population analysis was combined with Lewis X (LeX) expression, a slight enrichment was seen over side-population analysis alone.
Department of Surgery, University of Toronto, Toronto, Ontario, Canada M5S 1A8.

Kinoshita S. [Ocular surface reconstruction by tissue engineering]. *Nihon Ganka Gakkai Zasshi* 2002;12:837-68; discussion 69

Ocular surface reconstruction by tissue engineering using somatic stem cells is a second-generation modality. In order to treat bilaterally affected, severe ocular surface disorders, we investigated the transplantation of two types of cultivated mucosal epithelia: allogenic corneal epithelial stem cells, and autologous oral mucosal epithelial cells. For this, first, we summarized the clinical results of allogenic keratoepithelioplasty and limbal transplantation. In addition, we showed that the immunological shift from Th1 to Th2 by using keyhole limpet hemocyanin was effective in suppressing the incidence of immunological rejection. Second, we investigated the transplantation of cultivated human corneal epithelial stem cells onto amniotic membrane. The cultivated sheet was created by co-culture with 3T3 fibroblasts, using the air-lift method, in cultivating the corneal epithelial stem cell on the amniotic membrane. These cultivated cells demonstrated positive keratin 3 and 12 specific to in vivo corneal epithelium, tight junction related proteins, and telomerase activity. The transplanted allogenic human corneal epithelial sheet survived on the corneal surface in all cases, and was quite effective for achieving ocular surface stability in the acute phase of Stevens-Johnson syndrome, ocular cicatricial pemphigoid, or chemical injury. However, a few cases developed immunological rejection or opportunistic infection. Third, to establish the transplantation of the autologous cultivated oral mucosal epithelial sheet, we performed animal experiments using rabbits. In vitro oral mucosal epithelial sheet showed histology similar to that of in vivo corneal epithelial sheet. It expressed positive keratin 3 as well. Since the autologous transplantation of this sheet survived on the ocular surface with the recovery of corneal transparency, a cultivated oral mucosal epithelium may become a substitute for corneal epithelium. Fourth, we created a cultivated human corneal endothelial cell sheet on amniotic membrane using a similar technique, and transplanted it to a rabbit eye as a xenograft. The transplanted corneal endothelial cell density was over 3,000 cells/mm2, and it was actively functioning even after the transplantation. Lastly, to explore cell markers for corneal epithelial stem cells, we established a technique using laser micro-capture, and introduced amplified fragment length polymorphism (AFLP), identifying several candidate molecules as stem cell markers.

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Klenkler B and Sheardown H. Growth factors in the anterior segment: role in tissue maintenance, wound healing and ocular pathology. *Exp Eye Res* 2004;5:677-88

A number of growth factors and their associated receptors, including epidermal growth factor, transforming growth factor-beta, keratinocyte growth factor, hepatocyte growth factor, fibroblast growth factor and platelet-derived growth factor have been detected in the anterior segment of the eye. On binding to cellular receptors, these factors activate signalling cascades, which regulate functions including mitosis, differentiation, motility and apoptosis. Production of growth factors by corneal cells and their presence in the tear fluid and aqueous humour is essential for maintenance and renewal of normal tissue in the anterior eye and the prevention of undesirable immune or angiogenic reactions. Growth factors also play a vital role in corneal wound healing, mediating the proliferation of epithelial and stromal tissue and affecting the remodelling of the extracellular matrix (ECM). These functions depend on a complex interplay between growth factors of different types, the ECM, and regulatory mechanisms of the affected cells. Imbalances may lead to deficient wound healing and various ocular pathologies, including edema, neovascularization and glaucoma. Growth factors may be targeted in therapeutic ophthalmic applications, through exogenous application or selective inhibition, and may be used to elicit specific cellular responses to ophthalmic materials. A thorough understanding of the mechanism and function of growth factors and their actions in the complex environment of the anterior eye is required for these purposes. Growth factors, their function and mechanisms of action as well as the interplay between different growth factors based on recent in vitro and in vivo studies are presented.
Department of Chemical Engineering, McMaster University, 1280 Main St. W., Hamilton, Ont., Canada L8S 4L7.

Ko M K, Kim J G and Chi J G. Cell density of the corneal endothelium in human fetus by flat preparation. *Cornea* 2000;1:80-3

PURPOSE: To count the number of the corneal endothelial cells per unit of tissue area in 25 human fetal eyes ranging from 12-40 weeks of gestation with the histologic method. METHODS: The endothelium including Descemet's membrane was stained with hematoxylin-eosin by the flat preparation method. We photographed the endothelium using light microscopy. The number of nuclei was counted on each photograph. A calibrated micrometer was photographed with the light microscopy, and this was used to measure the number of corneal endothelial cells per square millimeter. RESULTS: The prenatal endothelial cell density of the human cornea decreases rapidly from 14,095 cells/mm2 (12 weeks of gestation) to 6,820 cells/mm2 (40 weeks of gestation). CONCLUSION: The estimate of the endothelial cell density at 12 weeks of gestation is twofold higher than the estimate at 40 weeks of gestation.
Department of Ophthalmology, Hanyang University, Seoul, Korea. Fovea@email.hanyang.ac.kr

Ko M K, Park W K, Lee J H and Chi J G. A histomorphometric study of corneal endothelial cells in normal human fetuses. *Exp Eye Res* 2001;4:403-9

The purpose of this study was to investigate the histomorphometric change in the normal development of human fetal corneal endothelial cells. Eighty one human fetal corneas, ranging from 12 to 40 weeks of gestation, were examined. For determination of gross parameters, corneal diameter and height were measured. Then the corneal endothelium including Descemet's membrane was stained with hematoxylin-eosin using a flat preparation method. In addition to histologic examination under the light microscope, computer-assisted image analysis was performed to determine the cell area, coefficient of variation in cell area and cell density, in both central and peripheral cornea, from each specimen. Total cell count per cornea was obtained by multiplying endothelial cell density by corneal surface area. Linear and nonlinear regression analysis of gestational age and each parameter were used to model corneal endothelial development during the prenatal period. Fetal cornea grows rapidly throughout the prenatal period. During the same period, mean cell area and total cell count also increases gradually, but there is a steep increase in the total cell count in the early period and of the cell area in the late period. The mean cell density decreases rapidly from 16 015 to 6167 cell x mm(-2). There was no significant difference in all parameters except cell density, between the central and peripheral cornea and the difference in cell density was only 2%. In the early prenatal period, there is a rapid increase of total cell count by mitosis, whereas in the late period enlarged endothelial cells cover the rapidly widening inner corneal surface without a significant change in the total cell count.
Department of Ophthalmology, Hanyang University School of Medicine, Seoul, Korea.

Koizumi N. [Cultivated corneal endothelial cell sheet transplantation in a primate model]. *Nihon Ganka Gakkai Zasshi* 2009;11:1050-9

Our new surgical treatment for corneal endothelial dysfunction involves replacing damaged the corneal endothelium with healthy corneal endothelial cells cultivated and multiplied in vitro. Monkey corneal endothelial cells (MCECs) were cultivated on collagen type- I carriers for four weeks. The corneal endothelium of the monkeys was mechanically scraped, and the cultivated MCEC sheet was inserted into the anterior chamber and fixed to the Descemet's membrane with air. As controls, a collagen sheet without MCECs was transplanted into one eye, and a suspension of cultivated MCECs was injected into the anterior chamber in another eye. In the early postoperative period MCEC sheets were attached to Descemet's membrane and corneal clarity recovered. This was accompanied by a decrease in corneal thickness. Fluorescein DiI labeled donor corneal endothelial cells were detected on the host cornea on postoperative day7. After transplantation, the MCEC-transplanted corneas remained clear for up to 4 years, and hexagonal cells of a density more than 1,500-2,000 cells/mm2 were observed by in vivo specular microscopy. Control eyes showed irreversible bullous keratopathy. We established a model of cultivated corneal endothelial transplantation for corneal endothelial dysfunction in primates whose corneal endothelial cells have less proliferative capacity in vivo. Our results suggest that this is a useful model for long-term observation in advance of future clinical application of cultivated corneal endothelial transplantation.
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Koizumi N, Inatomi T, Suzuki T, Sotozono C and Kinoshita S. Cultivated corneal epithelial stem cell transplantation in ocular surface disorders. *Ophthalmology* 2001a;9:1569-74

PURPOSE: To investigate the outcome of cultivated corneal epithelial transplantation for severe stem cell deficiencies using denuded amniotic membrane (AM) as a carrier. DESIGN: Retrospective, noncomparative case series. PARTICIPANTS: Thirteen eyes of 11 patients were studied. These consisted of five eyes with acute Stevens-Johnson syndrome (SJS), two with chronic SJS, one with an acute chemical injury, two with chronic chemical injuries, two with ocular cicatricial pemphigoid, and one with drug-induced pseudopemphigoid. All of these eyes had total stem cell deficiencies. MAIN OUTCOME MEASURES: Adaptation of the cultivated corneal epithelium onto the host corneal surface was confirmed 48 hours after surgery. The reconstruction of the ocular surface and visual acuity were measured. METHODS: Corneal limbal epithelium from donor corneas was cultivated for 4 weeks on a denuded AM carrier, with 3T3 fibroblast coculture and air lifting. The cultivated corneal epithelium showed four to five layers of stratification and was well differentiated. After conjunctival tissue removal from the cornea up to 3 mm outside the limbus and subconjunctival tissue treatment with 0.04% mitomycin C, cultivated allocorneal epithelium, including the AM carrier, was transplanted onto the corneal surface up to the limbus. Lamellar keratoplasty, using preserved donor graft without epithelium, was performed simultaneously for five chronic-phase patients showing corneal stromal scarring. Systemic immunosuppression was used to prevent allograft rejection. RESULTS: In all 13 eyes, the entire corneal surface, on which cultivated allocorneal epithelium had been placed, was free from epithelial defects 48 hours after surgery, indicating complete survival of the transplanted corneal epithelium. Visual acuity improved in all eyes after surgery, and 10 of the 13 eyes were restored to good vision (postoperative visual acuity improved two or more lines) 6 months after the operation. During the follow-up period (mean +/- standard deviation, 11.2 +/- 1.3 months), the corneal surfaces were clear, although three eyes experienced epithelial rejection. CONCLUSIONS: Cultivated corneal epithelial transplantation using denuded AM as a carrier can be used for severe stem cell deficiencies.
Department of Ophthalmology, Kyoto Prefectural University of Medicine, Kyoto, Japan.

Koizumi N, Inatomi T, Suzuki T, Sotozono C and Kinoshita S. Cultivated corneal epithelial transplantation for ocular surface reconstruction in acute phase of Stevens-Johnson syndrome. *Arch Ophthalmol* 2001b;2:298-300

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Koizumi N, Sakamoto Y, Okumura N, Okahara N, Tsuchiya H, Torii R, Cooper L J, Ban Y, Tanioka H and Kinoshita S. Cultivated corneal endothelial cell sheet transplantation in a primate model. *Invest Ophthalmol Vis Sci* 2007;10:4519-26

PURPOSE: To examine the feasibility of cultivated corneal endothelial cell transplantation in a primate model. METHODS: Monkey corneal endothelial cells (MCECs) obtained from three cynomolgus monkeys were cultivated, with subcultures grown on collagen type I carriers for 4 weeks. The corneal endothelium of the right eye of six monkeys was mechanically scraped, after which a cultivated MCEC sheet was brought into the anterior chamber of four eyes and fixed to Descemet's membrane by air. As the control, a collagen sheet without MCECs was transplanted into one eye of one monkey, and a suspension of cultivated MCECs was injected into the anterior chamber in one eye. RESULTS: Cultivated MCECs produced a confluent monolayer of closely attached hexagonal cells that showed both ZO-1 and Na(+)-K(+) ATPase expression. In the early postoperative period MCEC sheets were attached to Descemet's membrane and corneal clarity was recovered. The recovered clarity was accompanied by a decrease in corneal thickness. Fluorescein DiI labeled donor corneal endothelial cells were detected on the host cornea on postoperative day 7. Six months after transplantation MCEC-transplanted corneas remained clear, and hexagonal cells were observed by in vivo specular microscopy with a density of 1992 to 2475 cells/mm(2). Control eyes showed irreversible bullous keratopathy that precluded pachymetry and specular microscopy. CONCLUSIONS: A model of cultivated corneal endothelial transplantation for corneal endothelial dysfunction was established in primates whose corneal endothelial cells have less proliferative capacity in vivo. Our results suggest that this is a useful model for long-term observation in advance of the future clinical application of cultivated corneal endothelial transplantation.

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Koizumi N, Sakamoto Y, Okumura N, Tsuchiya H, Torii R, Cooper L J, Ban Y, Tanioka H and Kinoshita S. Cultivated corneal endothelial transplantation in a primate: possible future clinical application in corneal endothelial regenerative medicine. *Cornea* 2008;S48-55

PURPOSE: To review our attempt to devise a method of cultivated corneal endothelial transplantation using primates in which corneal endothelium, like that of humans, has low proliferative ability. METHODS: Monkey corneal endothelial cells (MCECs) were cultivated, with subcultures grown on collagen type I carriers. The corneal endothelia of 6 eyes of 6 monkeys were scraped intensively, after which cultivated MCEC sheets were inserted into the anterior chamber of 4 eyes. As controls, a collagen sheet without MCECs was transplanted in 1 eye of a monkey, and a suspension of cultivated MCECs was injected into the anterior chamber of 1 eye of another monkey. RESULTS: MCECs produced a confluent monolayer of closely attached hexagonal cells, which expressed both ZO-1 and Na-K adenosine triphosphatase. Early postoperative period MCEC sheets were attached to Descemet membrane, and corneal clarity was recovered. Six months after transplantation, MCEC-transplanted corneas remained clear, and closely attached hexagonal cells were observed. In 1 animal with longer observation, polygonal cells were observed by in vivo specular microscopy at a density of >2000 cells/mm2 and remained >1600 cells/mm2 for < or =2 years. Control eyes showed irreversible bullous keratopathy throughout the observation period. CONCLUSIONS: Cultivated MCECs become attached to the transplanted eye and maintain a clear cornea < or =2 years postoperatively, suggesting that corneal endothelial cells of primates might have proliferative ability in vivo once they have been cultured and proliferated in vitro. Our monkey model constitutes an important step forward for regenerative medicine with possible future application in patients with corneal endothelial dysfunction.

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Kondo M, Wagers A J, Manz M G, Prohaska S S, Scherer D C, Beilhack G F, Shizuru J A and Weissman I L. Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu Rev Immunol* 2003;759-806

Stem cell biology is scientifically, clinically, and politically a current topic. The hematopoietic stem cell, the common ancestor of all types of blood cells, is one of the best-characterized stem cells in the body and the only stem cell that is clinically applied in the treatment of diseases such as breast cancer, leukemias, and congenital immunodeficiencies. Multicolor cell sorting enables the purification not only of hematopoietic stem cells, but also of their downstream progenitors such as common lymphoid progenitors and common myeloid progenitors. Recent genetic approaches including gene chip technology have been used to elucidate the gene expression profile of hematopoietic stem cells and other progenitors. Although the mechanisms that control self-renewal and lineage commitment of hematopoietic stem cells are still ambiguous, recent rapid advances in understanding the biological nature of hematopoietic stem and progenitor cells have broadened the potential application of these cells in the treatment of diseases.

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Konomi K and Joyce N C. Age and topographical comparison of telomere lengths in human corneal endothelial cells. *Mol Vis* 2007;1251-8

PURPOSE: Human corneal endothelium exhibits both age-related and topographical differences in relative proliferative capacity and in senescence characteristics. The purpose of these studies was to compare telomere lengths in human corneal endothelial cells (HCEC) from the central and peripheral areas of corneas from young and older donors to determine whether these changes may be due to replicative senescence or to stress-induced premature senescence. METHODS: Pairs of corneas from five young (<30 years old) and six older donors (>65 years old) were separated into central and peripheral areas using a 9.5 mm diameter trephine to remove scleral tissue and a 6.0 mm diameter trephine to mark the central-peripheral boundary. One of the pair of corneas was cut into quarters and stained with a peptide nucleic acid (PNA)/fluorescein isothiocyanate (PNA/FITC) probe that specifically binds to telomere repeats. HCEC from the central (0-6.0 mm) and peripheral areas (6.0-9.5 mm) were isolated from the second cornea, mounted on slides by Cytospin, and stained with the PNA/FITC probe. Fluorescence confocal microscopy was used to obtain digital images. The average FITC intensity of nuclei was compared between the central and peripheral areas within and between the two age groups. Ccl185 and 1301 cells were analyzed as controls. Student's unpaired t-test was used to determine the statistical significance of the data. RESULTS: Average FITC intensity from the central endothelium was 205.8+/-4.2 (younger) and 194.2+/-10.5 (older) and from the peripheral endothelium was 208.1+/-9.3 (younger) and 195.9+/-10.8 (older). Average intensity of single cells isolated from central endothelium was 113.9+/-31.1 (younger) and 107.9+/-26.1 (older) and from the periphery was 109.9+/-12.0 (younger) and 106.9+/-32.4 (older). Average FITC intensity of Ccl185 cells and 1301 cells was 50.5+/-5.0 and 206.9+/-19.4, respectively. Comparison of the results indicates no statistically significant difference between the central and peripheral areas within each group or between the young and older age group. CONCLUSIONS: Results indicate that the age-related and topographical reduction in relative proliferative capacity and senescence characteristics observed in HCEC are not due to replicative senescence caused by critically short telomeres but implicate stress-induced premature senescence as a cause of these clinically important changes.

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Korbling M and Estrov Z. Adult stem cells for tissue repair - a new therapeutic concept? *N Engl J Med* 2003;6:570-82

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Korinek V, Barker N, Moerer P, van Donselaar E, Huls G, Peters P J and Clevers H. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat Genet* 1998;4:379-83

Mutations of the genes encoding APC or beta-catenin in colon carcinoma induce the constitutive formation of nuclear beta-catenin/Tcf-4 complexes, resulting in activated transcription of Tcf target genes. To study the physiological role of Tcf-4 (which is encoded by the Tcf7/2 gene), we disrupted Tcf7/2 by homologous recombination. Tcf7/2-/- mice die shortly after birth. A single histopathological abnormality was observed. An apparently normal transition of intestinal endoderm into epithelium occurred at approximately embryonic day (E) 14.5. However, no proliferative compartments were maintained in the prospective crypt regions between the villi. As a consequence, the neonatal epithelium was composed entirely of differentiated, non-dividing villus cells. We conclude that the genetic program controlled by Tcf-4 maintains the crypt stem cells of the small intestine. The constitutive activity of Tcf-4 in APC-deficient human epithelial cells may contribute to their malignant transformation by maintaining stem-cell characteristics.
Department of Immunology, University Hospital, Utrecht, The Netherlands.

Krishnan S, Sudha B and Krishnakumar S. Isoforms of p63 in corneal stem cells cultured on human amniotic membrane. *Biologicals* 2010;5:570-6

The p63 gene supports stem cell proliferation and regulation in epithelial cells. In this study, corneal epithelial cells were cultured on human amniotic membrane (HAM) and investigated for p63 and its isoform genes. Human limbal biopsies obtained from cadaveric donor eyes were cultivated on intact and denuded HAM. Transactivation (TA) specific domain was positive in the limbal cells cultured over denuded HAM and negative on others. TAp63alpha,beta,gamma isoforms are negative in all the limbal cells cultured on intact, denuded and limbal tissues but not in cornel epithelial tissue. p63alpha isoform is present in all except on denuded HAM. Alphabeta sharing region is not expressed only in cornel epithelial tissue. Gamma isoform is expressed in all the samples. DeltaNp63alpha region is present in cells cultured over the intact HAM whereas it is negative on the cells cultured over the denuded HAM. The other isoforms such as DeltaNp63beta and DeltaNp63gamma are negative in all samples. The limbal cells cultured over the intact HAM were able to maintain high proliferative potential when compared to denuded HAM. Thus, p63 isoforms plays a biological function to retain the proliferative capacity of corneal epithelial cells and maintains the stemness when cultured on intact HAM.
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Kruse F E. Stem cells and corneal epithelial regeneration. *Eye (Lond)* 1994a;170-83

Self-renewing tissues such as the corneal epithelium contain stem cells which represent the proliferative reserve. Studies of cellular differentiation and proliferation suggest that corneal epithelial stem cells are localised exclusively in the basal limbal epithelium. Although regulatory factors for the amplification of corneal stem cells are unknown, serum factors such as retinoic acid might induce differentiation of stem cells to transient amplifying cells which are responsible for cell amplification. These cells are regulated by various polypeptide growth factors and extracellular calcium. Loss or malfunction of stem cells does not permit maintenance or regeneration of the corneal epithelial mass but leads to conjunctivalisation of the corneal surface. Clinically, several ocular surface disorders such as chemical burns can cause limbal damage and consecutive limbal insufficiency. Treatment for these disorders is available only by transplantation of healthy stem cells, which can be performed as both autograft and allograft.
Augenklinik der Universitat Heidelberg, Germany.

Kruse F E and Tseng S C. A serum-free clonal growth assay for limbal, peripheral, and central corneal epithelium. *Invest Ophthalmol Vis Sci* 1991a;7:2086-95

The stem cells and transient amplifying cells of the corneal epithelium are thought to be localized in the limbal and corneal basal epithelium, respectively. To study the differential regulation of proliferation of these progenitor cells, a defined, serum-free, clonal growth assay was developed for central (CC) and peripheral (PC) corneal and limbal (L) epithelial cells. After incubation in Dispase II (1.2 U/ml; 1 hr for PC and CC and 3 hr for L) and subsequent brief trypsin-ethylenediaminetetraacetic acid digestion, 18 or 180 single cells/cm2 were seeded in MCDB 151 medium supplemented with insulin, transferrin, selenium, hydrocortisone, epidermal growth factor (EGF), phosphoethanolamine, ethanolamine, and calcium. During the first week of culture, the cells gradually developed an increasing number of colonies, and the mean colony-forming efficiency on day 6 for L was 4.2 +/- 2.4%, significantly lower than 11.4 +/- 5.9% for PC and 12.8 +/- 7.6% for CC (P less than 0.003). Colony morphology was identical in L, PC, and CC with small, elongated cells more cohesive in the center but more migratory in the periphery. There were no differences in immunofluorescent staining with monoclonal antibody AE-5, indicating the corneal derivation of all colonies. Cultures could be passaged on day 14 and grown for more than 3 weeks with increasing desquamation. Addition of a mixture of trace metals to yield MCDB 153 did not enhance growth; increased selenium concentrations were inhibitory. Elimination of EGF from the supplement abolished most of the clonal growth. The lower rate of L proliferation might be explained by the absence of serum and stromal mitogens. This culture system seems preferentially to support transient amplifying cells and allows investigation of the differentiation of isolated corneal stem cells to transient amplifying cells or the proliferation and differentiation of transient amplifying cells by various factors without the interaction of undefined serum components or paracrine influences from other cells.
Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami School of Medicine, FL 33101.

Kruse F E and Tseng S C. [The limbus epithelium in vitro]. *Fortschr Ophthalmol* 1991b;2:107-12

The limbal basal epithelium contains the stem cells of the corneal epithelium. In order to study the regulation of these cells we developed models allowing their investigation. While an explant model based on n-heptanol debridement did not allow outgrowth of corneal epithelium onto culture dishes, a serum-free single-cell clonal growth supported colony growth in limbal cells. MCDB 151 medium was supplemented with insulin, transferrin, selenium, hydrocortisone, phospho-/ethanolamine and calcium. Single cells (5,000) were seeded onto 60 mm plastic dishes following combined digestion with dispase and trypsin/EDTA. Increasing CFE was noted until day 6, allowing increasing recruitment into proliferation. This model will serve as a tool for the further investigation of factors that might govern the regulation of the differentiation and proliferation of limbal epithelial cells.
Bascom Palmer Eye Institute, Department of Ophthalmology, University of Miami School of Medicine, Florida 33101.

Kruse F E and Tseng S C. [Differing regulation of proliferation of limbus and corneal epithelium caused by serum factors]. *Ophthalmologe* 1993a;6:669-78

The basal limbal epithelium contains corneal stem cells, which are responsible for the maintenance of the corneal epithelial mass. In our study of the regulation of limbal progenitor cells, we investigated the proliferation of limbal and corneal epithelial cells in a defined single-cell clonal culture system. In a serum-free defined medium the limbal epithelium generated significantly fewer colonies than the cornea, which also proliferated more slowly. The addition of fetal calf serum resulted in the stimulation of a unique subpopulation of progenitor cells at the limbus. In contrast, proliferation of the peripheral corneal epithelium was inhibited by serum. These results further support the concept of a limbal location of corneal stem cells and suggest that limbal progenitor cells might be regulated preferentially by serum factors.
Universitats-Augenklinik Heidelberg.

Kruse F E and Tseng S C. Serum differentially modulates the clonal growth and differentiation of cultured limbal and corneal epithelium. *Invest Ophthalmol Vis Sci* 1993b;10:2976-89

PURPOSE: The stem cell-containing limbal epithelium is in proximity with highly vascularized tissue, as opposed to the transient amplifying cell-containing corneal basal epithelium, which resides on top of avascular corneal stroma. We therefore speculate that

limbal stem cells are preferentially under the modulation of serum-derived factors. METHODS: Using a previously reported serum-free, chemically defined culture system for ocular surface epithelium, a culture condition primarily supporting transient amplifying cells of both corneal and limbal epithelia, we compared the clonal growth measured by colony-forming efficiency (CFE), colony size, and BrdU labeling, as well as colony differentiation measured by colony morphology and immunofluorescence staining, with the monoclonal antibody AE-5 against keratin K3 when fetal bovine serum (FBS) was added at different concentrations. RESULTS: The addition of 1% FBS decreased CFE and colony size in peripheral corneal cultures but had no effect in limbal cultures. Both cultures showed no obvious difference in colony morphology or BrdU labeling and AE-5 staining. In contrast, at 10% or 20% FBS, CFE and colony size increased in limbal cultures, but dose dependently decreased in peripheral corneal cultures. The presence of a unique subpopulation of progenitor cells in limbal cultures different from transient amplifying cells in corneal cultures was further supported by the emergence of a higher proportion of a unique type (B) colonies in limbal cultures that had high BrdU labeling and heterogeneous or negative AE-5 staining, indicative of their being in a proliferating, undifferentiated state. These colonies showed continuous growth in late cultures and could be passaged into serum-free medium. CONCLUSION: These results indicate that serum contains factors responsible for stimulating limbal progenitor cells into clonal proliferation.
Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami School of Medicine, Miami.

Kruse F E and Tseng S C. Retinoic acid regulates clonal growth and differentiation of cultured limbal and peripheral corneal epithelium. *Invest Ophthalmol Vis Sci* 1994b;5:2405-20

PURPOSE: To determine if vitamin A could be one of the factors in serum responsible for the previously observed effect of 20% fetal bovine serum on stimulating clonal growth of an additional subpopulation in limbal cultures, possibly stem cells, but inhibiting that of transient amplifying cells in peripheral corneal cultures. METHODS: A reported serum-free clonal growth assay was used. The mitogenic response was measured by colony-forming efficiency (CFE), colony size, and BrdU labeling index; the differentiation was assessed by colony morphology, AE-5 monoclonal antibody staining, and cornified envelope formation. RESULTS: HPLC analyses revealed that added retinoic acid (RA) was rapidly taken up by cultured cells. As compared to the control without RA, low concentrations of RA (10(-9) M to 10(-7) M) stimulated the CFE of limbal cultures but did not change that of peripheral corneal cultures. Furthermore, 10(-8) M RA induced the emergence of two new types of colonies, one of which was almost exclusively present in limbal cultures, and allowed continuous clonal growth of some colonies in late limbal cultures. RA also dose dependently reduced colony size and BrdU labeling index in both limbal and peripheral corneal cultures. RA in concentrations above 10(-8) M stimulated normal differentiation of both limbal and peripheral corneal epithelial cells, as evidenced by increased AE-5 staining, but inhibited the formation of cornified envelopes, an index for abnormal, squamous metaplasia, in late cultures. CONCLUSION: These results suggest that RA has a differential dose-dependent effect on subpopulations of corneal and limbal epithelial cells. Although RA stimulates the conversion of limbal stem cells to transient amplifying cells, it inhibits the amplification of corneal and limbal transient amplifying cells and prevents abnormal terminal differentiation. These data further support the role of vitamin A as a physiological modulator of proliferation and differentiation of the ocular surface epithelium.
Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami School of Medicine, FL 33101.

Lagali N, Stenevi U, Claesson M, Fagerholm P, Hanson C, Weijdegard B and Strombeck A S. Donor and recipient endothelial cell population of the transplanted human cornea: a two-dimensional imaging study. *Invest Ophthalmol Vis Sci* 2010;4:1898-904

Purpose. To elucidate the pattern of donor and recipient endothelial cell populations in transplanted human corneas and determine the degree to which donor endothelial cells survive in the graft. Methods. Thirty-six corneal grafts were collected from recipients of opposite sex to the donor, at the time of retransplantation for various indications. Cells from the endothelial side of the grafts were harvested, preserving their relative location on the endothelium. Fluorescence in situ hybridization of the sex chromosomes enabled each cell to be identified as donor- or recipient-derived. Images of the graft endothelium were assembled, to depict the pattern of cell population of the graft, and the proportion of donor cells present was estimated. Results. Endothelial cells of donor origin were found in 26 of 36 grafts (72.2%)-in one case, up to 26 years after transplantation. The proportion of donor endothelium ranged from 2% to 99%; however, there was no significant correlation of this proportion with postoperative time (P = 0.19). The mean annual rate of donor cell loss correlated negatively with the time to graft failure by endothelial decompensation (P = 0.002). Endothelial images indicated a highly variable pattern of recipient cell repopulation of the graft. A tendency toward donor cell retention in transparent, successful grafts was noted; however, this feature alone was not a reliable indicator of long-term graft transparency. Conclusions. Two-dimensional imaging of the corneal graft endothelium revealed a variable pattern and extent of donor and recipient cell population, indicating the highly dynamic nature of the corneal endothelium after transplantation.
Department of Ophthalmology, Linkoping University Hospital, Linkoping, Sweden;

Lajtha L G. Stem cells and their properties. *Proc Can Cancer Conf* 1967;31-9

Lajtha L G. Stem cell concepts. *Differentiation* 1979;1-2:23-34

Lekhanont K, Choubtum L, Chuck R S, Sa-ngiampornpanit T, Chuckpaiwong V and Vongthongsri A. A serum- and feeder-free technique of culturing human corneal epithelial stem cells on amniotic membrane. *Mol Vis* 2009;1294-302

PURPOSE: To describe a simple technique of cultivating human corneal epithelial stem cells using an Epilife culture medium under serum- and feeder-free conditions. METHODS: Cadaveric donor limbal corneal epithelial cells were cultured on denuded amniotic membranes using an explant technique that was free of serum and feeder cells in the Epilife medium containing a growth supplement of defined composition. These cells were assessed by phase contrast microscope. The expressions of the proposed corneal epithelial stem cell markers (p63, ATP-binding cassette member 2 (ABCG2), and cytokeratin 15 and 19) and differentiation markers (cytokeratin 3, 12, connexin 43, and p75) were analyzed using reverse transcription polymerase chain reaction (RT-PCR) and immunocytochemical staining. RESULTS: Successful cultures were obtained, resulting in a monolayer to double layer cell sheets with a cobblestone-like morphology. RT-PCR and immunocytochemistry disclosed an expression of both putative limbal stem cell (LSC) markers and differentiation-associated markers in the cultured cells. Most of the cultured corneal epithelial cells that were immunopositive for putative LSC markers were smaller, more uniform, and closer to the limbal explant than cells positively stained with differentiation-associated markers. CONCLUSIONS: A serum- and feeder-free culture system using Epilife medium may grow human corneal epithelial equivalents, minimizing the risk of contamination during culture. The technique may also be useful for the clinical application of limbal stem cell culture.
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Levis H J, Brown R A and Daniels J T. Plastic compressed collagen as a biomimetic substrate for human limbal epithelial cell culture. *Biomaterials* 2010;30:7726-37

We describe, for the first time, the use of cellular plastic compressed collagen as a substrate for human limbal epithelial cell expansion and stratification. The characteristics of expanded limbal epithelial cells on either acellular collagen constructs or those containing human limbal fibroblasts were compared to a human central cornea control. After compression, human fibroblasts in collagen constructs remained viable and limbal epithelial cells were successfully expanded on the surface. After airlifting, a multilayered epithelium formed with epithelial cell morphology very similar to that of cells in the central cornea. Immunochemical staining revealed expression of basement membrane proteins and differentiated epithelial cell markers found in native central cornea. Ultrastructural analysis revealed cells on collagen

constructs had many features similar to central cornea, including polygonal, tightly opposed surface epithelial cells with microvilli and numerous desmosomes at cell-cell junctions. Taken together, these data demonstrate that plastic compressed collagen constructs can form the basis of a biomimetic tissue model for in vitro testing and could potentially provide a suitable alternative to amniotic membrane as a substrate for limbal epithelial cell transplantation.
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Levy V, Lindon C, Harfe B D and Morgan B A. Distinct stem cell populations regenerate the follicle and interfollicular epidermis. *Dev Cell* 2005;6:855-61

The regeneration of the skin and its appendages is thought to occur by the regulated activation of a dedicated stem cell population. A population of cells in the bulge region of the hair follicle has been identified as the putative stem cell of both the follicle and the interfollicular epidermis. While this assertion is supported by a variety of surrogate assays, there has been no direct confirmation of the normal contribution of these cells to the regeneration of structures other than the cycling portion of the hair follicle. Here, we report lineage analysis revealing that the follicular epithelium is derived from cells in the epidermal placode that express Sonic hedgehog. This analysis also demonstrates that the stem cells resident in the follicular bulge that regenerate the follicle are neither the stem cells of the epidermis nor the source of the stem cells of the epidermis in the absence of trauma.
Cutaneous Biology Research Center, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts 02129, USA.

Lewis J P and Trobaugh F E, Jr. Haematopoietic Stem Cells. *Nature* 1964;589-90

Li W, Hayashida Y, Chen Y T and Tseng S C. Niche regulation of corneal epithelial stem cells at the limbus. *Cell Res* 2007;1:26-36

Among all adult somatic stem cells, those of the corneal epithelium are unique in their exclusive location in a defined limbal structure termed Palisades of Vogt. As a result, surgical engraftment of limbal epithelial stem cells with or without ex vivo expansion has long been practiced to restore sights in patients inflicted with limbal stem cell deficiency. Nevertheless, compared to other stem cell examples, relatively little is known about the limbal niche, which is believed to play a pivotal role in regulating self-renewal and fate decision of limbal epithelial stem cells. This review summarizes relevant literature and formulates several key questions to guide future research into better understanding of the pathogenesis of limbal stem cell deficiency and further improvement of the tissue engineering of the corneal epithelium by focusing on the limbal niche.
Ocular Surface Center and TissueTech Inc. Miami, Florida, 33173, USA.

Ljubimov A V, Burgeson R E, Butkowski R J, Michael A F, Sun T T and Kenney M C. Human corneal basement membrane heterogeneity: topographical differences in the expression of type IV collagen and laminin isoforms. *Lab Invest* 1995;4:461-73

BACKGROUND: The corneal epithelium converges at the peripheral zone (limbus) with the conjunctival epithelium, forming a continuous sheet with phenotypically distinct regions--central, limbal, and conjunctival. The epithelial basement membrane (EBM) is important for corneal functions and cell adhesion, but its regional composition is poorly understood. Current literature is controversial as to the occurrence of type IV collagen in the cornea. The aim of this study was to investigate in detail corneal basement membrane (BM) composition and correlate it with the differentiation state of contributing cells. EXPERIMENTAL DESIGN: Adult human corneas (N = 8) were cryosectioned and analyzed by immunofluorescence with antibodies to 15 BM components and to keratin 3, a marker of corneal epithelial differentiation. RESULTS: A novel type of spatial heterogeneity ("horizontal") in the EBM composition was found between the central cornea, limbus, and conjunctiva. Central EBM had type IV collagen alpha 3-alpha 5 chains, whereas limbal and conjunctival EBM contained alpha 1-alpha 2 chains and also laminin alpha 2 and beta 2 chains. Limbal EBM in addition had alpha 5(IV) chain. Laminin-1 (alpha 1 beta 1 gamma 1), laminin-5 (alpha 3 beta 3 gamma 2), perlecan, fibronectin, entactin/nidogen, and type VII collagen were seen in the entire EBM. Another novel type of BM heterogeneity ("vertical") was typical for the corneal Descemet's membrane: its stromal face had alpha 1(IV) and alpha 2(IV) chains and fibronectin, whereas alpha 3(IV)-alpha 5(IV) chains, entactin/nidogen, laminin-1, and perlecan were present on the endothelial face. CONCLUSIONS: Type IV collagen controversy is the result of the shifts of isoforms in the limbus and conjunctiva. These shifts and the appearance of additional laminins in the limbus may be related to the differentiation state of corneal cells contributing to the EBM formation. Novel types of BM heterogeneity in the human cornea are described: regional (horizontal) in the EBM and vertical in the Descemet's membrane. The first one may be a common feature of converging complex epithelia, whereas the second one may be another unique property of the Descemet's membrane.
Ophthalmology Research Laboratories, Cedars-Sinai Medical Center, UCLA Medical School, USA.

Lorenzetti D W, Uotila M H, Parikh N and Kaufman H E. Central cornea guttata. Incidence in the general population. *Am J Ophthalmol* 1967;6:1155-8

Majo F, Rochat A, Nicolas M, Jaoude G A and Barrandon Y. Oligopotent stem cells are distributed throughout the mammalian ocular surface. *Nature* 2008;7219:250-4

The integrity of the cornea, the most anterior part of the eye, is indispensable for vision. Forty-five million individuals worldwide are bilaterally blind and another 135 million have severely impaired vision in both eyes because of loss of corneal transparency; treatments range from local medications to corneal transplants, and more recently to stem cell therapy. The corneal epithelium is a squamous epithelium that is constantly renewing, with a vertical turnover of 7 to 14 days in many mammals. Identification of slow cycling cells (label-retaining cells) in the limbus of the mouse has led to the notion that the limbus is the niche for the stem cells responsible for the long-term renewal of the cornea; hence, the corneal epithelium is supposedly renewed by cells generated at and migrating from the limbus, in marked opposition to other squamous epithelia in which each resident stem cell has in charge a limited area of epithelium. Here we show that the corneal epithelium of the mouse can be serially transplanted, is self-maintained and contains oligopotent stem cells with the capacity to generate goblet cells if provided with a conjunctival environment. Furthermore, the entire ocular surface of the pig, including the cornea, contains oligopotent stem cells (holoclones) with the capacity to generate individual colonies of corneal and conjunctival cells. Therefore, the limbus is not the only niche for corneal stem cells and corneal renewal is not different from other squamous epithelia. We propose a model that unifies our observations with the literature and explains why the limbal region is enriched in stem cells.
Laboratory of Stem Cell Dynamics, Ecole Polytechnique Federale de Lausanne (EPFL), 1015 Lausanne CH, Switzerland.

Marchini G, Pedrotti E, Pedrotti M, Barbaro V, Di Iorio E, Ferrari S, Bertolin M, Ferrari B, Passilongo M, Fasolo A and Ponzin D. Long-term effectiveness of autologous cultured limbal stem cell grafts in patients with limbal stem cell deficiency due to chemical burns. *Clin Experiment Ophthalmol* 2011;

Background: Chemical burns cause depletion of limbal stem cells and eventually lead to corneal opacity and visual loss. We investigated the long-term effectiveness of autologous cultured limbal stem cell grafts in patients with limbal stem cell deficiency. Design: Prospective , non-comparative interventional case series. Participants: Sixteen eyes from 16 patients with severe, unilateral limbal stem cell deficiency caused by chemical burns. Methods: Autologous ex vivo cultured limbal stem cells were grafted onto the recipient eye after superficial keratectomy. Main outcome measures: Clinical parameters of limbal stem cell deficiency (stability/transparency of the corneal epithelium, superficial corneal vascularization, and pain/photophobia), visual acuity, cytokeratin expression on impression cytology specimens

and histology on excised corneal buttons. Results: At 12 months post-surgery, evaluation of the 16 patients showed that 10 (62.6%) experienced complete restoration of a stable and clear epithelium and 3 (18.7%) had partially successful outcomes (re-appearance of conjunctiva in some sectors of the cornea and instable corneal surface). Graft failure (no change in corneal surface conditions) was seen in 3 (18.7%) patients. Penetrating keratoplasty was performed in 7 patients, with visual acuity improving up to 0.8 (best result). For 2 patients, regeneration of the corneal epithelium was confirmed by molecular marker (p63, cytokeratin 3, 12 and 19, mucin 1) analysis. Follow-up times ranged from 12 to 50 months. Conclusions: Grafts of autologous limbal stem cells cultured onto fibrin glue discs can successfully regenerate the corneal epithelium in patients with limbal stem cell deficiency, allowing to perform successful cornea transplantation and restore vision.
Ophthalmology Unit, Department of Neurological, Neuropsychological, Morphological and Movement Sciences, University of Verona, Verona, Italy Ophthalmology Unit, San Bortolo Hospital, Vicenza, Italy The Veneto Eye Bank Foundation, Zelarino-Venezia, Italy.

Marshman E, Booth C and Potten C S. The intestinal epithelial stem cell. *Bioessays* 2002;1:91-8

This article considers the role of the adult epithelial stem cell, with particular reference to the intestinal epithelial stem cell. Although the potential of adult stem cells has been revealed in a number of recent publications, the organization and control of the stem cell hierarchy in epithelial tissues is still not fully understood. The intestinal epithelium is an excellent model in which to study such hierarchies, having a distinctive polarity and high rate of cell proliferation and migration. Studies on the small intestinal crypt provide insight into the characteristics of the stem cells in normal and regenerating circumstances and demonstrate why a thorough understanding of these cells is an essential pre-requisite for stem cell based therapeutic approaches.
School of Biological Sciences, University of Manchester, Manchester, UK.

Martinez-Climent J A, Andreu E J and Prosper F. Somatic stem cells and the origin of cancer. *Clin Transl Oncol* 2006;9:647-63

Most human cancers derive from a single cell targeted by genetic and epigenetic alterations that initiate malignant transformation. Progressively, these early cancer cells give rise to different generations of daughter cells that accumulate additional mutations, acting in concert to drive the full neoplastic phenotype. As we have currently deciphered many of the gene pathways disrupted in cancer, our knowledge about the nature of the normal cells susceptible to transformation upon mutation has remained more elusive. Adult stem cells are those that show long-term replicative potential, together with the capacities of self-renewal and multi-lineage differentiation. These stem cell properties are tightly regulated in normal development, yet their alteration may be a critical issue for tumorigenesis. This concept has arisen from the striking degree of similarity noted between somatic stem cells and cancer cells, including the fundamental abilities to self-renew and differentiate. Given these shared attributes, it has been proposed that cancers are caused by transforming mutations occurring in tissue-specific stem cells. This hypothesis has been functionally supported by the observation that among all cancer cells within a particular tumor, only a minute cell fraction has the exclusive potential to regenerate the entire tumor cell population; these cells with stem-like properties have been termed cancer stem cells. Cancer stem cells can originate from mutation in normal somatic stem cells that deregulate their physiological programs. Alternatively, mutations may target more committed progenitor cells or even mature cells, which become reprogrammed to acquire stem-like functions. In any case, mutated genes should promote expansion of stem/progenitor cells, thus increasing their predisposition to cancer development by expanding self-renewal and pluripotency over their normal tendency towards relative quiescency and proper differentiation.
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Martino G and Pluchino S. The therapeutic potential of neural stem cells. *Nat Rev Neurosci* 2006;5:395-406

Recent evidence shows that transplantation of neural stem/precursor cells may protect the central nervous system from inflammatory damage through a 'bystander' mechanism that is alternative to cell replacement. This novel mechanism, which might improve the success of transplantation procedures, is exerted by undifferentiated neural stem cells, the functional characteristics of which are regulated by important stem cell regulators released by CNS-resident and blood-borne inflammatory cells. Here, we discuss this alternative bystander mechanism in the context of the atypical ectopic perivascular niche. We propose that it is the most challenging example of reciprocal therapeutic crosstalk between the inflamed CNS and systemically transplanted neural stem cells.
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Matic M, Petrov I N, Chen S, Wang C, Dimitrijevich S D and Wolosin J M. Stem cells of the corneal epithelium lack connexins and metabolite transfer capacity. *Differentiation* 1997;4:251-60

The stem cells of the corneal epithelial lineage are confined to the basal cell layer of the limbus, a vascularized outer corneal rim. These slow cycling cells of great proliferative potential maintain the corneal epithelial mass. Since cell-cell communication plays an important role in development and differentiation, we conducted a comparative examination of the expression of two corneal connexins, C x 43 and C x 50, and the tracer transfer capacity of the limbal and corneal epithelia using the scrape loading technique. C x 43 is abundantly expressed in the basal cell layer of the epithelium covering the cornea, but is essentially absent from the mouse, human, neonatal rabbit, and chicken limbal epithelium. In the adult rabbit the limbal epithelium displays an overall weak C x 43 immunoreactivity, but C x 43-free isolated basal cells can be distinguished. C x 50 is expressed throughout the corneal epithelium of the three mammalian corneas, but is not detectable in the limbus. Scrape loading experiments in the rabbit yielded results which were consistent with the immunohistological findings; limbal epithelium lacked tracer (lucifer yellow) transfer capacity, strongly suggesting the absence of functional gap junctions. Altogether, our results demonstrate the incompetence of stem cells for gap junction-mediated cell-to-cell communication. This property may reflect the need of these unique cells to maintain a distinct intracellular environment.
Department of Ophthalmology, Mount Sinai School of Medicine, New York, NY 10029-6574, USA.

McGowan S L, Edelhauser H F, Pfister R R and Whikehart D R. Stem cell markers in the human posterior limbus and corneal endothelium of unwounded and wounded corneas. *Mol Vis* 2007;1984-2000

PURPOSE: The corneal endothelium is a monolayer of cells in the posterior cornea that is responsible for maintaining a clear cornea. Corneal endothelial cells may be induced to divide, but it has been held that they do not divide in the normal cornea of an adult human. Some studies have suggested that a stem cell population for the corneal endothelium exists. This population could give rise to mature corneal endothelial cells and may reside either in the peripheral corneal endothelium or in the adjacent posterior limbus. This study was initiated to demonstrate the presence of such stem cells in the region of the posterior limbus and to show the response of these cells to corneal wounding. METHODS: Unwounded and wounded corneas with their attached limbal sections were analyzed by immunofluorescence for the presence of nestin, telomerase, Oct-3/4, Pax-6, Wnt-1, and Sox-2. Alkaline phosphatase activity was observed with an enzyme-based reaction that produced a fluorescent product. RESULTS: In the unwounded cornea, stem cell markers nestin, alkaline phosphatase, and telomerase were found in the trabecular meshwork (TM) and in the transition zone between the TM and the corneal endothelial periphery (including Schwalbe's line). Telomerase was also present in the peripheral corneal endothelium. When wounded corneas and their attached limbii were tested, the same markers were found. However, after wounding, additional stem cell markers, Oct-3/4 (in the TM) and Wnt-1 (in both the TM and the transition zone), appeared. Moreover, the differentiation markers Pax-6 and Sox-2 were seen. Pax-6 and Sox-2 were also manifest in the peripheral endothelium post-wounding. CONCLUSIONS: Well documented specific stem cell markers were found in the TM and the transition zone of the human posterior limbus. Wounding of the corneas activated the production of two additional stem cell markers (Oct-3/4, Wnt-1) as well as two differentiation markers (Pax-6, Sox-2), the latter of which also appeared in the corneal endothelial periphery. It is suggested that stem cells reside in the posterior limbus and respond to corneal wounding to initiate an endothelial repair process. The stem cells may also contribute to a normal, slow replacement of corneal endothelial cells.

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Menter J M. Temperature dependence of collagen fluorescence. *Photochem Photobiol Sci* 2006;4:403-10

Dermal collagens have several fluorescent moieties in the UV and visible spectral regions that may serve as molecular probes of collagen. We studied the temperature-dependence of a commercial calf skin collagen and acid-extracted Skh-1 hairless mouse collagen at temperatures from 9 degrees C to 60 degrees C for excitation/emission wavelengths 270/305 nm (tyrosine), 270/360 nm (excimer-like aggregated species), 325/400 nm (dityrosine) and 370/450 nm (glycation adduct). L-tyrosine (1 x 10(-5) M in 0.5 M HOAc) acted as a "reference compound" devoid of any collagen structural effects. In general, the fluorescence efficiency of these fluorophores decreases with increasing temperature. Assuming that rate constant for fluorescence deactivation has the form k(d)(T) = k(d) degrees exp (-DeltaE/RT), an Arrhenius plot of log[(1/Phi) - 1] vs. 1/T affords a straight line whose (negative) slope is proportional to the activation energy, DeltaE, of the radiationless process(es) that compete with fluorescence. Because it is difficult to accurately measure Phi(f) for collagen-bound fluorophores, we derived an approximate formula for an activation parameter, DeltaE*, evaluated from an Arrhenius-like plot of log 1/I(N)vs. 1/T, (1/I(N)vs. is the reciprocal normalized fluorescence intensity). Tyrosine in dilute solution affords a linear Arrhenius plot in both of the above cases. Using the known value of Phi(f) = 0.21 for free tyrosine at room temperature, we determined that DeltaE* is accurate to approximately 25% in the present instance. Collagen curves are non-linear, but they are quasi-linear below approximately 20 degrees C, where the helical form predominates. Values of DeltaE* determined from the data at T < 20 degrees C ranged from 6.2-8.4 kJ mol(-1) (1.5-2.0 kcal mol(-1)) for mouse collagen and 10.3-11.4 kJ mol(-1) (2.5-2.7 kcal mol(-1)) for calf skin collagen, consistent with collisional deactivation of the fluorescent state via thermally enhanced molecular vibrations and rotations. Above 20 degrees C, log 1/I(N)vs. 1/T plots from Skh-1 hairless mouse collagen are concave-downward, suggesting that fluorescence deactivation from the denatured coil has a significant temperature-independent component. For calf skin collagen, these plots are concave-upward, suggesting an increase in activation energy above Tm. These results suggest that collagen backbone and supramolecular structure can influence the temperature dependence of the bound fluorophores, indicating the future possibility of using activation data as a probe of supramolecular structure and conformation.
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Meyer-Blazejewska E A, Call M K, Yamanaka O, Liu H, Schlotzer-Schrehardt U, Kruse F E and Kao W W. From hair to cornea: toward the therapeutic use of hair follicle-derived stem cells in the treatment of limbal stem cell deficiency. *Stem Cells* 2011;1:57-66

Limbal stem cell deficiency (LSCD) leads to severe ocular surface abnormalities that can result in the loss of vision. The most successful therapy currently being used is transplantation of limbal epithelial cell sheets cultivated from a limbal biopsy obtained from the patient's healthy, contralateral eye or cadaveric tissue. In this study, we investigated the therapeutic potential of murine vibrissae hair follicle bulge-derived stem cells (HFSCs) as an autologous stem cell (SC) source for ocular surface reconstruction in patients bilaterally affected by LSCD. This study is an expansion of our previously published work showing transdifferentiation of HFSCs into cells of a corneal epithelial phenotype in an in vitro system. In this study, we used a transgenic mouse model, K12(rtTA/rtTA) /tetO-cre/ROSA(mTmG) , which allows for HFSCs to change color, from red to green, once differentiation to corneal epithelial cells occurs and Krt12, the corneal epithelial-specific differentiation marker, is expressed. HFSCs were isolated from transgenic mice, amplified by clonal expansion on a 3T3 feeder layer, and transplanted on a fibrin carrier to the eye of LSCD wild-type mice (n = 31). The HFSC transplant was able to reconstruct the ocular surface in 80% of the transplanted animals; differentiating into cells with a corneal epithelial phenotype, expressing Krt12, and repopulating the corneal SC pool while suppressing vascularization and conjunctival ingrowth. These data highlight the therapeutic properties of using HFSC to treat LSCD in a mouse model while demonstrating a strong translational potential and points to the niche as a key factor for determining stem cell differentiation.
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Meyer-Blazejewska E A, Kruse F E, Bitterer K, Meyer C, Hofmann-Rummelt C, Wunsch P H and Schlotzer-Schrehardt U. Preservation of the limbal stem cell phenotype by appropriate culture techniques. *Invest Ophthalmol Vis Sci* 2010;2:765-74

PURPOSE: To evaluate the effect of several culture variables on clonal growth and differentiation of limbal stem cells ex vivo and provide an improved culture technique that supports preferential expansion and preservation of stem cells for therapeutic applications. METHODS: Corneal epithelial stem cells were isolated from human limbal specimens and clonally expanded on a 3T3 feeder layer, followed by subcultivation of holoclones on fibrin gels. The effect of different limbal regions, enzymatic dissociation methods, and culture media supplemented with different calcium, serum, and growth factor concentrations on colony-forming efficiency, colony size, and colony density was compared. A panel of putative stem cell and differentiation markers was used to analyze the epithelial phenotype by morphologic and immunohistochemical methods. RESULTS: Limbal cells obtained from the superior limbus, isolated by a two-step enzymatic dissociation method (dispase II/trypsin-EDTA), and cultured in low to medium (0.03-0.4 mM) calcium concentrations with proper serum levels (10% FCS) and growth factor combinations (EGF, NGF) yielded the highest clonal growth capacity and an undifferentiated cellular phenotype. Subcultivation of holoclones supported the preservation of stem and progenitor cells in the basal layer of the fibrin-based epithelial sheets, as demonstrated by multiple molecular stem cell markers (p63alpha, Bmi-1, K15, and ABCG2), whereas increased calcium concentrations and air-lifting induced terminal differentiation and gradual loss of stem cells. CONCLUSIONS: The proposed culture system supports enrichment and survival of limbal stem and progenitor cells during the entire cultivation process and may be essential for long-term restoration of the damaged ocular surface.
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Mimura T and Joyce N C. Replication competence and senescence in central and peripheral human corneal endothelium. *Invest Ophthalmol Vis Sci* 2006;4:1387-96

PURPOSE: To compare replication competence and senescence in human corneal endothelial cells (HCECs) between the central and peripheral areas and between younger and older donors. METHODS: Human corneas were obtained from the eye bank and separated into two groups: young (younger than 30 years) and old (older than 50 years). Corneas were cut in quarters and a 2-mm scrape wound was created in the endothelium from the periphery to the center. Unwounded endothelium acted as a negative control. Corneal pieces were incubated for 24, 36, 48, 60, 72, 84, and 96 hours in medium containing 8% fetal bovine serum (FBS) plus additional growth factors. Tissue was fixed, immunostained for minichromosome maintenance (MCM)-2, a marker of replication competence, and mounted in medium containing propidium iodide (PI) to visualize all nuclei. Fluorescence microscope images were used to count PI-stained and MCM2-positive HCECs in three 100-microm2 areas within the central and peripheral wound area. Results are expressed as mean number of cells/100 microm2. Senescent HCECs in ex vivo corneas were identified by staining for senescence-associated beta-galactosidase activity (SA-beta-Gal). Whole corneas were cut in quarters and incubated in staining solution containing SA-beta-Gal at pH 6.0. The number of cells stained for SA-beta-Gal and the grade of SA-beta-Gal intensity in three 100-microm2 areas were averaged for the central and peripheral areas from each donor. For all studies, results were compared between central and peripheral cornea and between younger and older donors. RESULTS: In both age groups (n = 4/group), cells repopulated the wound area in a time-dependent manner. In corneas from older donors, significantly fewer HCECs migrated into the wound bed in the central cornea than in the periphery. At each time point, the density of cells in the central wound area was lower in corneas from older donors than from younger donors. In both age groups, the mean percentage of MCM2-positive cells increased with time until wound healing. In both age groups, more MCM2-positive cells were present in the wounded area of the peripheral than of the central cornea. At 36, 48, 60, and 72 hours after wounding, the percentage of MCM2-positive cells in the central or peripheral area of older corneas was significantly less than in the corresponding region in younger corneas. No MCM2-positive staining was observed in unwounded areas at any time point. HCECs in corneas from younger donors (n = 4) showed little to no SA-beta-Gal activity in either the central

or peripheral area. SA-beta-Gal activity was easily detectable in corneas from older donors (n = 4) and a significantly higher percentage of central HCECs showed strong SA-beta-Gal activity compared with HCECs in the periphery. CONCLUSIONS: In ex vivo corneas, HCECs from the peripheral area retain higher replication competence, regardless of donor age. HCECs in the central area of corneas from older donors retain replicative competence, but the relative percentage of cells that are competent to replicate is significantly lower than in the periphery or in the central area of corneas from younger donors. This reduction in replicative competence negatively correlates with the observed increase in the population of central HCECs exhibiting senescence-like characteristics.
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Mimura T, Yamagami S, Uchida S, Yokoo S, Ono K, Usui T and Amano S. Isolation of adult progenitor cells with neuronal potential from rabbit corneal epithelial cells in serum- and feeder layer-free culture conditions. *Mol Vis* 2010;1712-9

PURPOSE: To isolate progenitor cells from rabbit corneal epithelial cells (CEC) in serum- and feeder layer-free culture conditions and to compare the self-renewal capacity of corneal epithelial progenitor cells obtained from the central and limbal regions of the cornea. METHODS: Tissue samples of New Zealand white rabbit corneas were dissected from the limbal and central regions to obtain CEC for sphere-forming culture, in which the cells formed spheres in serum-free medium containing growth factors. The number of primary and secondary sphere colonies and the size of the primary spheres were compared between the limbal and central regions. To promote differentiation, isolated sphere colonies were plated in dishes coated with poly-L-lysine (PLL)/laminin. The expression of epithelial, neural, and mesenchymal mRNAs was examined in the sphere colonies and their progeny by immunocytochemistry and/or the reverse transcription-polymerase chain reaction (RT-PCR). Adherent differentiated cells from the sphere colonies were also examined morphologically. RESULTS: Primary spheres were isolated from both the limbal and central regions of the cornea. The rate of primary sphere formation by CEC from the limbal region (55.6+/-10.6/10,000 cells) was significantly higher than that by cells from the central cornea (43.1+/-7.2/10,000 cells, p=0.0028), but there was no significant difference in the size of primary spheres derived from both regions. The self-renewal capacity of cells from the limbal region was higher than that of cells from the central region, as evidenced by the significantly higher secondary sphere formation rate of limbal cells (38.7+/-8.5/10,000 cells) in comparison with that for central cells (31.3+/-5.7/10,000 cells, p=0.013). The primary sphere colonies expressed bromodeoxyuridine (BrdU), a 63-kDa protein (p63), p75 neurotrophin receptor (p75(NTR)), and nestin, whereas their progeny expressed cytokeratin 3, cytokeratin 12, vimentin, alpha-smooth muscle actin, microtubule-associated protein 2, and neuron-specific enolase on immunocytochemical analysis. These markers were confirmed by RT-PCR. CONCLUSIONS: Our findings indicate that limbal CEC contain more progenitor cells with a stronger self-renewal capacity than cells from the central region. These progenitor cells differentiate into the epithelial lineage, and can also produce neuronal protein.
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Mimura T, Yamagami S, Yokoo S, Araie M and Amano S. Comparison of rabbit corneal endothelial cell precursors in the central and peripheral cornea. *Invest Ophthalmol Vis Sci* 2005;10:3645-8

PURPOSE: To compare the distribution and self-renewal capacity of rabbit corneal endothelial cell precursors in the central and peripheral regions of the cornea. METHODS: The corneal endothelium (CE) and Descemet's membrane of New Zealand White rabbit corneas were divided into a peripheral region (6.0-10.0 mm in diameter) and a central region (6.0 mm in diameter). Then a sphere-forming assay was performed to isolate precursors from the CE of each region. Numbers of primary and secondary sphere colonies and sizes of primary spheres were compared between the central and peripheral regions. RESULTS: Primary spheres were isolated from the peripheral and the central regions of the CE. The rate of primary sphere formation in the peripheral region (34.4 +/- 10.4/10,000 cells) was significantly higher than in the central cornea (26.8 +/- 6.6/10,000 cells; P = 0.0042), but there was no significant difference in the size of primary spheres between the two regions. Self-renewal capacity was higher in the peripheral region than in the central region, as evidenced by a significantly higher secondary sphere formation rate for cells from the periphery (39.0 +/- 8.8/10,000 cells) compared with that for cells from the central region (25.4 +/- 4.2/10,000 cells; P = 0.00028). CONCLUSIONS: These findings demonstrate that peripheral and central rabbit corneal epithelia contain a significant number of precursors but that the peripheral endothelium contains more precursors and has a stronger self-renewal capacity than the central region.
Department of Ophthalmology, University of Tokyo Graduate School of Medicine, Japan.

Minami E, Laflamme M A, Saffitz J E and Murry C E. Extracardiac progenitor cells repopulate most major cell types in the transplanted human heart. *Circulation* 2005;19:2951-8

BACKGROUND: Extracardiac progenitor cells are capable of repopulating cardiomyocytes at very low levels in the human heart after injury. Here, we explored the extent of endothelial, smooth muscle, and Schwann cell chimerism in patients with sex-mismatched (female-to-male) heart transplants. METHODS AND RESULTS: Autopsy specimens from 5 patients and endomyocardial biopsies from 7 patients were used for this study. Endothelial, vascular smooth muscle, and Schwann cells were stained with antibodies against CD31 or Ulex europaeus lectin, smooth muscle alpha-actin, and S-100, respectively, and the Y chromosome was identified with in situ hybridization. Biopsy specimens from 1, 4, 6, and 12 months and 5 and 10 years after heart transplantation were evaluated. Y-positive cells were counted by conventional bright-field microscopy and confirmed by confocal microscopy. Endothelial cells showed the highest degree of chimerism, averaging 24.3+/-8.2% from extracardiac sources. Schwann cells showed the next highest chimerism, averaging 11.2+/-2.1%; vascular smooth muscle cells averaged 3.4+/-1.8%. All 3 cell types showed substantially higher chimerism than we previously observed for cardiomyocytes (0.04+/-0.05%). Endothelial chimerism was much higher in the microcirculation than in larger vessels. Analysis of serial endomyocardial biopsies revealed that high levels of endothelial chimerism occurred as early as 1 month after transplantation (22+/-6.6%) with no significant increases even up to 10 years after cardiac transplantation. CONCLUSIONS: Extracardiac progenitor cells are capable of repopulating most major cell types in the heart, but they do so with varying frequency. The signals for endothelial progenitor recruitment occur early and could relate to injury during allograft harvest or transplantation. The high degree of endothelial chimerism may have immune implications such as for myocardial rejection or graft vasculopathy.
Division of Cardiology, Department of Medicine, University of Washington School of Medicine, Seattle, WA, USA.

Monici M. Cell and tissue autofluorescence research and diagnostic applications. *Biotechnol Annu Rev* 2005;227-56

Cells contain molecules, which become fluorescent when excited by UV/Vis radiation of suitable wavelength. This fluorescence emission, arising from endogenous fluorophores, is an intrinsic property of cells and is called auto-fluorescence to be distinguished from fluorescent signals obtained by adding exogenous markers. The majority of cell auto-fluorescence originates from mitochondria and lysosomes. Together with aromatic amino acids and lipo-pigments, the most important endogenous fluorophores are pyridinic (NADPH) and flavin coenzymes. In tissues, the extracellular matrix often contributes to the auto-fluorescence emission more than the cellular component, because collagen and elastin have, among the endogenous fluorophores, a relatively high quantum yield. Changes occurring in the cell and tissue state during physiological and/or pathological processes result in modifications of the amount and distribution of endogenous fluorophores and chemical-physical properties of their microenvironment. Therefore, analytical techniques based on auto-fluorescence monitoring can be utilized in order to obtain information about morphological and physiological state of cells and tissues. Moreover, auto-fluorescence analysis can be performed in real time because it does not require any treatment of fixing or staining of the specimens. In the past few years spectroscopic and imaging techniques have been developed for many different applications both in basic research and diagnostics.
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Monteiro B G, Serafim R C, Melo G B, Silva M C, Lizier N F, Maranduba C M, Smith R L, Kerkis A, Cerruti H, Gomes J A and Kerkis I. Human immature dental pulp stem cells share key characteristic features with limbal stem cells. *Cell Prolif* 2009;5:587-94

OBJECTIVES: Limbal stem cells (LSC) are self-renewing, highly proliferative cells in vitro, which express a set of specific markers and in vivo have the capacity to reconstruct the entire corneal epithelium in cases of ocular surface injury. Currently, LSC transplantation is a commonly used procedure in patients with either uni- or bilateral total limbal stem cells deficiency (TLSCD). Although LSC transplantation holds great promise for patients, several problems need to be overcome. In order to find an alternative source of cells that can partially substitute LSC in cornea epithelium reconstruction, we aimed at investigating whether human immature dental pulp stem cells (hIDPSC) would present similar key characteristics as LSC and whether they could be used for corneal surface reconstruction in a rabbit TLSCD model. MATERIALS: We used hIDPSC, which co-express mesenchymal and embryonic stem cell markers and present the capacity to differentiate into derivative cells of the three germinal layers. TLSCD was induced by chemical burn in one eye of rabbits. After 30 days, the opaque tissue formed was removed by superficial keratectomy. Experimental group received undifferentiated hIDPSC, while control group only received amniotic membrane (AM). Both groups were sacrificed after 3 months. RESULTS AND CONCLUSIONS: We have demonstrated, using immunohistochemistry and reverse transcription-polymerase chain reaction, that hIDPSCs express markers in common with LSC, such as ABCG2, integrin beta1, vimentin, p63, connexin 43 and cytokeratins 3/12. They were also capable of reconstructing the eye surface after induction of unilateral TLSCD in rabbits, as shown by morphological and immunohistochemical analysis using human-specific antibodies against limbal and corneal epithelium. Our data suggest that hIDPSCs share similar characteristics with LSC and might be used as a potential alternative source of cells for corneal reconstruction.
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Moore K A and Lemischka I R. Stem cells and their niches. *Science* 2006;5769:1880-5

A constellation of intrinsic and extrinsic cellular mechanisms regulates the balance of self-renewal and differentiation in all stem cells. Stem cells, their progeny, and elements of their microenvironment make up an anatomical structure that coordinates normal homeostatic production of functional mature cells. Here we discuss the stem cell niche concept, highlight recent progress, and identify important unanswered questions. We focus on three mammalian stem cell systems where large numbers of mature cells must be continuously produced throughout adult life: intestinal epithelium, epidermal structures, and bone marrow.
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Moreira J E, Reese T S and Kachar B. Freeze-substitution as a preparative technique for immunoelectronmicroscopy: evaluation by atomic force microscopy. *Microsc Res Tech* 1996;3:251-61

Cryofixation followed by freeze substitution in osmium tetroxide was evaluated as a method for preparing biological specimens for immunoelectronmicroscopy. Samples were rapidly frozen by impact onto a sapphire block cooled with liquid nitrogen, substituted at -80 degrees C in acetone containing osmium tetroxide, and embedded in epoxy resin. With this protocol, excellent ultrastructure can be combined with localization of antigens that otherwise would be inactivated by the osmium, but labeling may need to be enhanced by chemically etching the sections prior to staining. The effects of etching on various structures in the sections were investigated by examining the sections with atomic force microscopy, an approach that yields three-dimensional views of the surface of the section. A considerable part of the section was removed or collapsed by the etching, and these effects occurred differentially in several components of the tissue and with different etching protocols. Nevertheless, the results suggest that the partial removal of the plastic by etching of freeze-substituted tissue can be explored as a method for exposing fine biological structures for observation with atomic force microscopy.
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Morrison S J and Kimble J. Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature* 2006;7097:1068-74

Much has been made of the idea that asymmetric cell division is a defining characteristic of stem cells that enables them to simultaneously perpetuate themselves (self-renew) and generate differentiated progeny. Yet many stem cells can divide symmetrically, particularly when they are expanding in number during development or after injury. Thus, asymmetric division is not necessary for stem-cell identity but rather is a tool that stem cells can use to maintain appropriate numbers of progeny. The facultative use of symmetric or asymmetric divisions by stem cells may be a key adaptation that is crucial for adult regenerative capacity.
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Murphy C, Alvarado J and Juster R. Prenatal and postnatal growth of the human Descemet's membrane. *Invest Ophthalmol Vis Sci* 1984a;12:1402-15

The origin, growth in thickness, and differentiation of Descemet's membrane was studied by light, electron microscopic, morphometric, and statistical methods in 67 specimens from 12 weeks of gestation to 98 years. Descemet's membrane is formed by three major processes: growth in thickness during the prenatal period, prenatal differentiation into a striated basement membrane, and growth in thickness during the postnatal period. The initial step is the synthesis of an ordinary basement membrane, which is very thin and quite different in appearance from the adult Descemet's membrane. Growth of the prenatal Descemet's membrane then proceeds by deposition of a series of similar "membrane units," which are stacked to form a lamellar structure consisting of at least 30 layers by the end of gestation. Second, during prenatal life, differentiation of the membrane leads to the formation of a striated structure through the gradual addition of short and thin cross-linking bridges separated by 110-nm intervals that are disposed in a plane perpendicular to the lamellae. The third process occurs in postnatal life when the membrane continues to grow in thickness by deposition of a nonstriated, nonlamellar material posterior to the striated prenatal layer. Regression analysis suggests that prenatal growth proceeds at a rapid but variable rate best described by a "sigmoid-like" function of age. Postnatal growth, in contrast, proceeds in a predominantly exponential manner but at a slower pace than in the prenatal period. The low variability and large size of our set of measurements make these data especially useful for comparisons with pathologic specimens.

Murphy C, Alvarado J, Juster R and Maglio M. Prenatal and postnatal cellularity of the human corneal endothelium. A quantitative histologic study. *Invest Ophthalmol Vis Sci* 1984b;3:312-22

The authors studied the cellularity of the normal corneal endothelium by histologic methods in 56 specimens from 16 weeks of gestation to 98 years of age. Ten step-serial sections were taken from each specimen through the central 6 mm of the cornea, in an area measuring 1.8 mm. The number of nuclei were counted on each section and a ratio of the number of nuclei per 100 micron of endothelial length was determined. This ratio provides a measure of cell density that they call cellularity. There is a decrease in cellularity that proceeds in a nonlinear manner and at a very rapid rate during the prenatal period and for the first few years of life. Cell death or necrosis, which might have contributed to this apparent loss of cells, was not observed. Instead, this rapid change in cellularity is correlated with a concomitant change in corneal size. The authors' calculations show that cell division may play a minor role in the formation of the endothelium after the second trimester of fetal life as most of the cells present by birth already exist by this time. After the first few years of life, the rate of change in endothelial cellularity decreases to proceed in a linear manner for the rest of the near 100 years of life examined. This latter age-related decline in cellularity is probably due to the loss of 0.56% cells per year from the endothelial layer, since the cornea does not appear to change in size during this time. Statistical analysis of the authors' data shows that these results are highly significant.

Nakamura T, Inatomi T, Sotozono C, Amemiya T, Kanamura N and Kinoshita S. Transplantation of cultivated autologous oral mucosal epithelial cells in patients with severe ocular surface disorders. *Br J Ophthalmol* 2004a;10:1280-4

BACKGROUND/AIMS: To determine outcomes of transplants of cultivated autologous oral epithelial cells in patients with severe ocular surface disorders. METHODS: The eyes (n = 6) of four patients with Stevens-Johnson syndrome (three eyes) or chemical burns (three eyes) were studied. Autologous oral epithelial cells, grown for 2-3 weeks on a denuded amniotic membrane carrier in the presence of 3T3 fibroblasts, were air lifted. The resultant sheet was transplanted onto the damaged eye, and acceptance of the sheet by the corneal surface was confirmed 48 hours after surgery. The success of ocular surface reconstruction, graft survival, changes in visual acuity, and postoperative complications were assessed and the quality of the cultivated oral epithelial sheet was evaluated histologically. RESULTS: At 48 hours after transplant, the entire corneal surface of all six eyes was free of epithelial defects indicating complete survival of the transplanted oral epithelium. Visual acuity was improved in all eyes. During follow up (mean 13.8 (SD 2.9) months), the corneal surface remained stable, although all eyes manifested mild peripheral neovascularisation. CONCLUSIONS: Autologous oral epithelial cells grown on denuded amniotic membrane can be transplanted to treat severe ocular surface disorders.
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Nakamura T, Inatomi T, Sotozono C, Ang L P, Koizumi N, Yokoi N and Kinoshita S. Transplantation of autologous serum-derived cultivated corneal epithelial equivalents for the treatment of severe ocular surface disease. *Ophthalmology* 2006;10:1765-72

PURPOSE: To evaluate the use of autologous serum (AS)-derived cultivated corneal epithelial transplantation for the treatment of severe ocular surface disease. DESIGN: Retrospective noncomparative case series. PARTICIPANTS: Nine eyes from 9 patients with total limbal stem cell deficiency were studied. These consisted of 2 eyes with Stevens-Johnson syndrome, 1 with chemical injury, 1 with ocular cicatricial pemphigoid, 1 with Salzmann corneal dystrophy, 1 with aniridia, 1 with graft-versus-host disease, and 2 with idiopathic ocular surface disease. METHODS: Autologous serum obtained from patients was used for cultivating corneal epithelial cells on an amniotic membrane substrate. These AS-derived corneal epithelial equivalents were compared with those derived from fetal bovine serum (FBS)-supplemented medium. At the time of surgery, complete removal of the corneal pannus and conjunctiva up to 3 mm from the limbus was performed. Allogeneic (7 cases) and autologous (2 cases) AS-derived cultivated corneal epithelial equivalents were transplanted onto the ocular surface. Postoperative follow-up included serial slit-lamp examinations with fluorescein staining, as well as photographic documentation. MAIN OUTCOME MEASURES: Ocular surface reconstruction with corneal epithelialization, graft integrity, visual acuity, and postoperative complications. RESULTS: The corneal epithelial sheets cultivated in AS- and FBS-supplemented media were morphologically similar, and demonstrated the normal expression of tissue-specific keratins and junctional specialization assembly proteins. After transplantation, complete corneal epithelialization was achieved within 2 to 5 days. All eyes demonstrated an improvement in visual acuity by > or =2 lines. During the follow-up period of 14.6+/-4.36 months, the corneal surface of all patients remained stable and transparent, without significant complications. CONCLUSIONS: Transplantation of AS-derived cultivated corneal epithelial equivalents was shown to be a feasible method of treating patients with severe ocular surface disease. The use of AS is of clinical importance in the development of autologous xenobiotic-free bioengineered ocular surface equivalents for clinical transplantation.
Department of Ophthalmology, Kyoto Prefectural University of Medicine, Graduate School of Medicine, Kyoto, Japan.

Nakamura T, Inatomi T, Sotozono C, Koizumi N and Kinoshita S. Successful primary culture and autologous transplantation of corneal limbal epithelial cells from minimal biopsy for unilateral severe ocular surface disease. *Acta Ophthalmol Scand* 2004b;4:468-71

BACKGROUND: Patients with severe unilateral ocular surface disease require reconstruction of the damaged ocular surface. We succeeded in culturing primary corneal limbal epithelial cells taken from minimal biopsy and, once grown, transplanting them on denuded amniotic membrane (AM). METHODS: Autologous corneal limbal epithelial cells from a 3 mm(2) biopsy of the uninjured eye were grown for 3 weeks on a denuded AM carrier. The resultant sheet was then transplanted onto the unilateral severely chemically injured eye. RESULTS: Minimal biopsy showed the autologous cultivated corneal epithelial cells to have 4-5 layers of sufficient stratification and to be well differentiated. At 19 months post-transplantation, the ocular surface epithelium was stable and there were no epithelial defects. CONCLUSION: We document that it is possible to produce sufficiently stratified, well differentiated, autologous cultivated corneal limbal epithelium on AM from a minimal biopsy of the donor eye and to transplant it onto the injured eye.
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Nakamura T and Kinoshita S. Ocular surface reconstruction using cultivated mucosal epithelial stem cells. *Cornea* 2003a;7 Suppl:S75-80

PURPOSE: To investigate the possibility of using cultivated oral epithelial cells in ocular surface reconstruction. METHODS: An ocular surface injury was created in adult albino rabbits by a lamellar keratectomy. Oral mucosal biopsy specimens taken from both adult albino rabbits and human volunteers were cultivated for 2-3 weeks on a denuded amniotic membrane (AM) carrier. The cultivated epithelium was examined by histologic and immunohistochemical analysis. At 3-4 weeks after the ocular surface injury, the rabbit conjunctivalized corneal surfaces were surgically reconstructed by transplanting both the rabbit and human cultivated oral epithelial cells on the AM carrier. RESULTS: Both the rabbit and human cultivated oral epithelial sheets had 5 or 6 layers of stratified, well-differentiated cells. Histologic examination revealed that the cultivated epithelial cells were similar in appearance to those of in vivo normal corneal epithelium. Immunohistochemistry confirmed the presence of the keratin pair 4 and 13 and keratin 3 in the cultivated oral epithelial cells. Corneas that were grafted with rabbit and human cultivated oral epithelial cells on an AM carrier were clear and were epithelialized 10 and 2 days after surgery, respectively. CONCLUSIONS: We have generated confluent cultures of both rabbit and human oral epithelial cells on AM expanded ex vivo from biopsy-derived oral mucosal tissues. We have successfully carried out xeno- and autologous transplantation of these cultivated oral epithelial cells onto the ocular surfaces of keratectomized rabbit eyes. We believe that xeno- and autologous transplantation of cultivated oral epithelium is a feasible method for ocular surface reconstruction.
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Nakamura T, Koizumi N, Tsuzuki M, Inoki K, Sano Y, Sotozono C and Kinoshita S. Successful regrafting of cultivated corneal epithelium using amniotic membrane as a carrier in severe ocular surface disease. *Cornea* 2003b;1:70-1

PURPOSE: Our group performed cultivated allogeneic corneal epithelial transplantation in 13 eyes from 11 patients with severe ocular surface disorders. After the clinical application of this new surgical treatment, some patients experienced epithelial and subepithelial opacities. We applied our procedure again in these patients to achieve successful ocular surface reconstruction. METHODS: The corneal limbal epithelial cells from donor corneas were cultivated for 4 weeks on denuded amniotic membrane (AM) carrier, with 3T3 fibroblast coculture and airlifting. The study subjects consisted of 3 patients. At 3 and 12 months after the first operation, the failed epithelial graft with AM was replaced with new allogeneic corneal epithelium cultivated on AM. RESULTS: At 48 hours after transplantation, the corneal surfaces of the 3 eyes were clear and smooth; the entire corneal surfaces were evenly covered with the transplanted cultivated corneal epithelium, which did not stain with fluorescein. The ocular surface epithelia of these patients are all stable without epithelial defects. CONCLUSIONS: We have shown that, in cases where the initially transplanted cultivated epithelium becomes opaque, it is possible to repeat the transplantation process with new cultivated epithelium on AM.
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Napoli C, William-Ignarro S, Byrns R, Balestrieri M L, Crimi E, Farzati B, Mancini F P, de Nigris F, Matarazzo A, D'Amora M, Abbondanza C, Fiorito C, Giovane A, Florio A, Varricchio E, Palagiano A, Minucci P B, Tecce M F, Giordano A, Pavan A and Ignarro L J. Therapeutic targeting of the stem cell niche in experimental hindlimb ischemia. *Nat Clin Pract Cardiovasc Med* 2008;9:571-9

BACKGROUND: The custom microenvironment 'vascular niche' is a potential therapeutic target for several pathophysiological conditions. Osteoblasts regulate the hematopoietic stem cell niche, and activation of the parathyroid hormone (PTH) receptor

can increase the number of cells mobilized into the bloodstream. METHODS: C57Bl/6 mice were randomly assigned treatment with granulocyte-colony stimulating factor (G-CSF), PTH, G-CSF plus PTH or saline. All mice underwent hindlimb ischemia. Blood flow was measured by laser Doppler imaging. Indices of capillary activity were determined by electron microscopy in muscle tissue. CD34(+) and Ki67(+) cells were detected and evaluated by immunofluorescence, apoptosis by TUNEL, surface antigen and endothelial progenitor cells by fluorescence-activated cell sorting analysis, and vascular endothelial growth factor-164 and angiopoietin-1 expression by reverse-transcriptase polymerase chain reaction. Frozen bone marrow sections were stained for antigen-specific B cells and fibronectin and analyzed by confocal laser scanning microscopy. RESULTS: Following mobilization induced by G-CSF treatment, mice also treated with PTH showed increases in blood flow, capillary density, nitrite/nitrate release, angiogenic factors and circulating progenitor cells, as well as reduced apoptosis, fibrosis, oxidative stress and inflammation in ischemic muscles. Furthermore, hematopoietic antigen-specific B cells in the bone marrow were also increased by G-CSF alone and in combination with PTH. CONCLUSIONS: PTH might increase the efficiency of hematopoietic stem-cell-based therapy in a recognized model of peripheral ischemia. Our translational experimental therapeutic targeting of the vascular niche points to novel clinical targets for the hematopoietic stem-cell treatment of ischemic vascular diseases.
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Narisawa Y, Kohda H and Tanaka T. Three-dimensional demonstration of melanocyte distribution of human hair follicles: special reference to the bulge area. *Acta Derm Venereol* 1997;2:97-101

Melanocytes of human hair follicles were histochemically and immunohistochemically examined in two and three dimensions. EDTA-treated extracted anagen vellus and intermediate hair follicles showed that monoclonal murine antibody (MoAb) NKI/ beteb-reactive melanocytes were distributed from the infundibulum to the bulb. Melanocytes of the infundibulum and bulb were larger and more strongly stained with MoAb NKI/beteb than those of the middle portion below the sebaceous gland. Latter melanocytes showed less dendricity. Dendritic melanocytes were exclusively observed in the bulb as well as the bulge area of vellus and intermediate hair follicles, where they were variably melanized. In longitudinal and transverse sections of adult human scalp some keratinocytes of the outer root sheath of the bulge area were melanized compared to other parts. This phenomenon was independent of the hair cycle. These findings may be characteristic of bulge melanocytes and/or keratinocytes.
Division of Dermatology, Department of Internal Medicine, Saga Medical School, Japan.

Nassiri N, Pandya H K and Djalilian A R. Limbal allograft transplantation using fibrin glue. *Arch Ophthalmol* 2011;2:218-22

Limbal transplantation is now widely accepted as the treatment of advanced limbal stem cell deficiency. Herein, we describe a technique for harvesting thin limbal grafts from cadaveric corneoscleral rims and a sutureless method to secure the grafts to the recipient eye using fibrin glue. We report the results of fibrin glue-assisted keratolimbal allograft in 19 eyes of 16 patients, with the outcome measures being ocular surface stability, visual acuity, and postoperative complications. The results indicate that limbal allograft transplantation can be performed safely and successfully using only fibrin glue to secure the grafts. This can potentially improve surgical efficiency and patient comfort postoperatively.
Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, 1855 W Taylor Street, Chicago, IL 60612, USA.

Nisonoff A. Enzymatic Digestion of Rabbit Gamma Globulin and Antibody and Chromatography of Digestion Products. *Methods Med Res* 1964;134-41

Notara M, Shortt A J, Galatowicz G, Calder V and Daniels J T. IL6 and the human limbal stem cell niche: a mediator of epithelial-stromal interaction. *Stem Cell Res* 2010;3:188-200

The corneal epithelium is maintained by the limbal epithelial stem cells (LESCs). In this study, an in vitro model is proposed for the investigation of cell-cell interactions involving LESC maintenance. Imaging of the limbal niche demonstrated close spatial arrangement between basal limbal epithelial cells within putative LESC niche structures and fibroblasts in the stroma. Interactions of the human limbal epithelial (HLE) cells and mitotically active human limbal fibroblasts (HLF) were studied for the first time in a serum-free in vitro model that simulated aspects of the limbal niche microenvironment. HLE cocultured in a ratio 3:1 with HLF exhibited enhanced expression of the putative stem cell markers ABCG2 and p63alpha and holoclones were preserved as shown by colony-forming efficiency assays, clonal analysis, and colony characterisation. Interleukin 6 (IL6) was found to be up-regulated in the 3.1SF system when compared to the HLE culture with growth-arrested fibroblasts and serum (gold standard system, GS). IL6 caused a time-dependent phosphorylation of STAT3 in HLE cells. STAT3 and IL6 inhibition in 3.1SF cultures significantly reduced HLE colony-forming efficiency, suggesting a previously undetected STAT3-mediated involvement of IL6 in the maintenance of HLE cells in a progenitor-like state.
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O'Brien C A, Pollett A, Gallinger S and Dick J E. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007;7123:106-10

Colon cancer is one of the best-understood neoplasms from a genetic perspective, yet it remains the second most common cause of cancer-related death, indicating that some of its cancer cells are not eradicated by current therapies. What has yet to be established is whether every colon cancer cell possesses the potential to initiate and sustain tumour growth, or whether the tumour is hierarchically organized so that only a subset of cells--cancer stem cells--possess such potential. Here we use renal capsule transplantation in immunodeficient NOD/SCID mice to identify a human colon cancer-initiating cell (CC-IC). Purification experiments established that all CC-ICs were CD133+; the CD133- cells that comprised the majority of the tumour were unable to initiate tumour growth. We calculated by limiting dilution analysis that there was one CC-IC in 5.7 x 10(4) unfractionated tumour cells, whereas there was one CC-IC in 262 CD133+ cells, representing >200-fold enrichment. CC-ICs within the CD133+ population were able to maintain themselves as well as differentiate and re-establish tumour heterogeneity upon serial transplantation. The identification of colon cancer stem cells that are distinct from the bulk tumour cells provides strong support for the hierarchical organization of human colon cancer, and their existence suggests that for therapeutic strategies to be effective, they must target the cancer stem cells.
Division of Cell and Molecular Biology, University Health Network, Toronto, Ontario, M5G 1L7, Canada.

Ogawa M, Porter P N and Nakahata T. Renewal and commitment to differentiation of hemopoietic stem cells (an interpretive review). *Blood* 1983;5:823-9

Omoto M, Miyashita H, Shimmura S, Higa K, Kawakita T, Yoshida S, McGrogan M, Shimazaki J and Tsubota K. The use of human mesenchymal stem cell-derived feeder cells for the cultivation of transplantable epithelial sheets. *Invest Ophthalmol Vis Sci* 2009;5:2109-15

PURPOSE: To report the efficacy of human bone marrow-derived mesenchymal stem cells as a source of feeder cells for the cultivation of transplantable corneal epithelial cell sheets. METHODS: Human mesenchymal stem cells (marrow adherent stem cells; MASCs) were cultured in alpha-modified Eagle's medium with 10% serum and were treated with mitomycin C. Expression of cytokines in MASCs was confirmed by reverse transcription-polymerase chain reaction. Human limbal epithelial cells were cocultured with MASCs or 3T3 feeder cells to compare colony-forming efficiency (CFE). Limbal epithelial cells were cultured on MASCs or 3T3 feeder cells at the air-liquid

interface to allow stratification, and stratified epithelial sheets were analyzed by immunohistochemistry against cytokeratin 3 (K3), K15, p63alpha, and ABCG2. Rabbit limbal epithelial cell sheets were cultivated with MASC feeder cells and transplanted to the ocular surface of the limbal-deficient rabbits. Epithelial grafts were observed by slit lamp microscopy for 4 weeks and then evaluated by histology and immunohistochemistry against K3 and K4. RESULTS: MASC feeder cells expressed keratinocyte growth factor, hepatocyte growth factor, and N-cadherin. The CFE of human limbal epithelial cells was similar in MASC and 3T3 feeder groups. Stratified cell sheets were successfully cultivated with MASC feeder cells expressing K3, K15, p63alpha, and ABCG2. Transplanted epithelial sheets regenerated the corneal phenotype in limbal-deficient rabbits. CONCLUSIONS: MASC-derived feeder cells are suitable for the engineering of epithelial sheets, avoiding the use of potentially hazardous xenologic feeder cells.
Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan.

Ono K, Yokoo S, Mimura T, Usui T, Miyata K, Araie M, Yamagami S and Amano S. Autologous transplantation of conjunctival epithelial cells cultured on amniotic membrane in a rabbit model. *Mol Vis* 2007;1138-43

PURPOSE: To evaluate the feasibility of autologous transplantation in a rabbit model of conjunctival epithelial cells cultured on amniotic membrane for ocular surface reconstruction. METHODS: Limbal stem cell deficiency was induced in the right eyes of 30 rabbits. This was done by performing a lamellar keratectomy of the entire cornea and a complete removal of the limbus and conjunctiva, extending 5 mm outside the limbus. Autologous conjunctival specimens were obtained from the left eyes of ten of those rabbits and cultured for four weeks on denuded amniotic membrane. Cultured epithelium was examined by transmission electron microscopy. Four weeks after lamellar keratectomy, conjunctivalized corneal surfaces were excised and autologous cultured conjunctival epithelial sheets transplanted (Conj-AM group, n=10). The controls were rabbits that underwent corneal surface removal but not transplantation (No Transplantation group, n=10) and those that underwent corneal surface removal but received only amniotic membrane (AM Alone group, n=10). A neovascularization and corneal opacity scoring system was used to evaluate each eye in the two months after surgery. RESULTS: Cultured conjunctival epithelium formed three to four layers on denuded amniotic membrane. Averaged scores of corneal neovascularization and corneal opacity two months after transplantation were significantly low in the Conj-AM group as compared with those in the AM and no transplantation groups. CONCLUSIONS: Transplantation of autologous conjunctival epithelial cells cultured on amniotic membrane should prove an effective strategy for treating total limbal stem cell deficiency.
Department of Ophthalmology, University of Tokyo Graduate School of Medicine, Tokyo, Japan.

Oshima K, Senn P and Heller S. Isolation of sphere-forming stem cells from the mouse inner ear. *Methods Mol Biol* 2009;141-62

The mammalian inner ear has very limited ability to regenerate lost sensory hair cells. This deficiency becomes apparent when hair cell loss leads to hearing loss as a result of either ototoxic insult or the aging process. Coincidently, with this inability to regenerate lost hair cells, the adult cochlea does not appear to harbor cells with a proliferative capacity that could serve as progenitor cells for lost cells. In contrast, adult mammalian vestibular sensory epithelia display a limited ability for hair cell regeneration, and sphere-forming cells with stem cell features can be isolated from the adult murine vestibular system. The neonatal inner ear, however, does harbor sphere-forming stem cells residing in cochlear and vestibular tissues. Here, we provide protocols to isolate sphere-forming stem cells from neonatal vestibular and cochlear sensory epithelia as well as from the spiral ganglion. We further describe procedures for sphere propagation, cell differentiation, and characterization of inner ear cell types derived from spheres. Sphere-forming stem cells from the mouse inner ear are an important tool for the development of cellular replacement strategies of damaged inner ears and are a bona fide progenitor cell source for transplantation studies.
Department of Otolaryngology-HNS, Stanford University School of Medicine, Stanford, CA, USA.

Pan J, Zhang Q, Wang Y and You M. 26S proteasome activity is down-regulated in lung cancer stem-like cells propagated in vitro. *PLoS One* 2010;10:e13298

Cancer stem cells (CSCs) are a small subset of cancer cells capable of self-renewal and tumor maintenance. Eradicating cancer stem cells, the root of tumor origin and recurrence, has emerged as one promising approach to improve lung cancer survival. Cancer stem cells are reported to reside in the side population (SP) of cultured lung cancer cells. We report here the coexistence of a distinct population of non-SP (NSP) cells that have equivalent self-renewal capacity compared to SP cells in a lung tumor sphere assay. Compared with the corresponding cells in monolayer cultures, lung tumor spheres, formed from human non-small cell lung carcinoma cell lines A549 or H1299, showed marked morphologic differences and increased expression of the stem cell markers CD133 and OCT3/4. Lung tumor spheres also exhibited increased tumorigenic potential as only 10,000 lung tumor sphere cells were required to produce xenografts tumors in nude mice, whereas the same number of monolayer cells failed to induce tumors. We also demonstrate that lung tumor spheres showed decreased 26S proteasome activity compared to monolayer. By using the ZsGreen-cODC (C-terminal sequence that directs degradation of Ornithine Decarboxylase) reporter assay in NSCLC cell lines, only less than 1% monolayer cultures were ZsGreen positive indicating low 26S proteasome, whereas lung tumor sphere showed increased numbers of ZsGreen-positive cells, suggesting the enrichment of CSCs in sphere cultures.
Department of Surgery, Siteman Cancer Center, Washington University School of Medicine, St. Louis, Missouri, United States of America.

Peh G S, Beuerman R W, Colman A, Tan D T and Mehta J S. Human corneal endothelial cell expansion for corneal endothelium transplantation: an overview. *Transplantation* 2011;8:811-9

The monolayer of cells forming the human corneal endothelium is critical to the maintenance of corneal transparency and is not known to regenerate in vivo. Thus, dysfunction of these cells constitutes the most often cited reasons for the 150,000 or so corneal transplants performed yearly. Although current corneal transplantation is more than 90% successful at 1 year, longer term results are not as encouraging with approximately 70% success at 5 years. Nonimmunologic graft failure and allograft endothelial rejection are the main problems. Furthermore, the global shortage of donor corneas greatly restricts several corneal transplantations performed. With advances in understanding corneal endothelial cell biology, it is now possible to cultivate human corneal endothelial cells (HCECs) in vitro, thus providing new opportunities to develop novel tissue-engineered human corneal endothelium. This review will provide an overview of (a) the characteristics of human corneal endothelium; (b) past and present HCECs isolation and culture protocols; (c) various potential carriers for the generation of tissue-engineered corneal endothelium, together with some of the functional studies reported in various animal models; and (d) the current rapid advancements in surgical techniques for keratoplasty. A successful combination of tissue-engineered human corneal endothelium coupled with innovative and groundbreaking surgical procedures will bridge basic research involving cultured HCECs, bringing it from bench to bedside.
Singapore Eye Research Institute, Singapore.

Pellegrini G, Golisano O, Paterna P, Lambiase A, Bonini S, Rama P and De Luca M. Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. *J Cell Biol* 1999;4:769-82

We have analyzed the proliferative and differentiation potential of human ocular keratinocytes. Holoclones, meroclones, and paraclones, previously identified in skin, constitute also the proliferative compartment of the ocular epithelium. Ocular holoclones have the expected properties of stem cells, while transient amplifying cells have variable proliferative potential. Corneal stem cells are segregated in the limbus, while conjunctival stem cells are uniformly distributed in bulbar and forniceal conjunctiva. Conjunctival keratinocytes and goblet cells derive from a common bipotent progenitor. Goblet cells were found in cultures of transient amplifying cells, suggesting that commitment for goblet cell differentiation can occur late in the life of a single conjunctival clone. We found that conjunctival keratinocytes with high proliferative capacity give rise to goblet cells at least twice in their life and, more importantly, at rather precise times of their life history, namely at 45-50 cell doublings and at approximately 15 cell doublings before senescence. Thus, the decision of conjunctival keratinocytes to differentiate into goblet cells appears to be dependent upon an intrinsic "cell doubling clock. " These data open new perspectives in the surgical treatment of severe defects of the anterior ocular surface with autologous cultured conjunctival epithelium.

Laboratory of Tissue Engineering, I.D.I.-IRCCS, Istituto Dermopatico dell'Immacolata, Rome, Italy.

Pellegrini G, Traverso C E, Franzi A T, Zingirian M, Cancedda R and De Luca M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet* 1997;9057:990-3

BACKGROUND: Complete loss of the corneal-limbal epithelium leads to re-epithelialisation by bulbar conjunctival cells. Since conjunctival and corneal-limbal epithelial cells represent two different cell lines, this conjunctival healing of the cornea is followed by stromal scarring, decreased visual acuity, and severe discomfort. Unilateral corneal-limbal epithelial defects can be resolved by the transplantation of limbal grafts taken from the uninjured eye. However, this procedure requires a large limbal graft to be taken from the healthy eye, and is not possible for bilateral lesions. We investigated the possibility of restoring the human corneal surface with autologous corneal epithelial sheets generated by serial cultivation of limbal cells. METHODS: Cells were cultivated from a 1 mm2 biopsy sample taken from the limbus of the healthy eye of two patients with severe alkali burns, and thus complete loss of the corneal-limbal surface, of one eye. Normal corneal differentiation was tested with a specific biochemical marker. Autologous cultured corneal sheets were then grafted onto the damaged eyes of the two patients. The patients were followed up at more than 2 years after grafting. FINDINGS: We have shown that corneal progenitor cells are localised in the limbus, that cultured limbal cells generate cohesive sheets of authentic corneal epithelium, and that autologous cultured corneal epithelium restored the corneal surface of two patients with complete loss of the corneal-limbus epithelium. Long-term follow-up showed the stability of regenerated corneal epithelium and the striking improvement in patients' comfort and visual acuity. INTERPRETATION: The cultivation of corneal epithelium might offer an alternative to patients with unilateral lesions and a therapeutic chance to patients with severe bilateral corneal-limbal epithelial defects. Our findings give a new perspective on the treatment of ocular disorders characterised by stem-cell deficiency.
Istituto Dermopatico dell'Immacolata, Roma, Italy.

Pera M F and Trounson A O. Human embryonic stem cells: prospects for development. *Development* 2004;22:5515-25

It is widely anticipated that human embryonic stem (ES) cells will serve as an experimental model for studying early development in our species, and, conversely, that studies of development in model systems, the mouse in particular, will inform our efforts to manipulate human stem cells in vitro. A comparison of primate and mouse ES cells suggests that a common underlying blueprint for the pluripotent state has undergone significant species-specific modification. As we discuss here, technical advances in the propagation and manipulation of human ES cells have improved our understanding of their growth and differentiation, providing the potential to investigate early human development and to develop new clinical therapies.
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Pileri S A, Roncador G, Ceccarelli C, Piccioli M, Briskomatis A, Sabattini E, Ascani S, Santini D, Piccaluga P P, Leone O, Damiani S, Ercolessi C, Sandri F, Pieri F, Leoncini L and Falini B. Antigen retrieval techniques in immunohistochemistry: comparison of different methods. *J Pathol* 1997;1:116-23

Routine sections of normal and pathological samples fixed in 10 per cent buffered formalin or B5, including EDTA-decalcified bone-marrow biopsies, were tested with 61 antibodies following heating in three different fluids: 0.01 M citrate buffer (pH 6.0), 0.1 M Tris-HCl (pH 8.0), and 1 mM EDTA-NaOH solution (pH 8.0). The sections underwent either three cycles of microwave treatment (5 min each) or pressure cooking for 1-2 min. The alkaline phosphatase/anti-alkaline phosphatase (APAAP) technique was used as the standard detection method; with 16 antibodies a slightly modified streptavidin-biotin complex (SABC)-immunoperoxidase technique was applied in parallel. The results obtained were compared with those observed without any antigen retrieval (AR), or following section digestion with 0.05 per cent protease XIV at 37 degrees C for 5 min. Chess-board titration tests showed that all antibodies but one profited by AR. Protease XIV digestion represented the gold standard for five antibodies, while 55 produced optimal results following the application of heat-based AR. By comparison with the other fluids, EDTA appeared to be superior in terms of both staining intensity and the number of marked cells. These results were independent of tissue processing, immunohistochemical approach, and heating device. Pressure cooking was found to be more convenient on practical grounds, as it allowed the simultaneous handling of a large number of slides and a time saving of 1 min 30 s, representing the proper time for the treatment.
Second Service of Pathologic Anatomy, Bologna University, Italy.

Porter R R. The hydrolysis of rabbit y-globulin and antibodies with crystalline papain. *Biochem J* 1959;119-26

Price F W, Jr., Whitson W E and Marks R G. Graft survival in four common groups of patients undergoing penetrating keratoplasty. *Ophthalmology* 1991a;3:322-8

Graft survival rates and causes of graft failure were evaluated for 1046 consecutive keratoplasties in four groups: keratoconus, Fuchs' dystrophy, pseudophakic bullous keratopathy with retained intraocular lenses (IOLs), and pseudophakic/aphakic bullous keratopathy with secondary lens implants. Patient follow-up averaged 23 months (range, 1-84 months). There was a significant difference in graft survival among the four groups, P less than 0.0001. The keratoconus and Fuchs' groups had the highest survival rates and pseudophakic bullous keratopathy with retained IOLs the lowest rate. Problems with the external surface of the graft caused the largest number of graft failures. Grafts for pseudophakic bullous keratopathy with retained posterior chamber lenses had a significantly higher failure rate from rejection and endothelial decompensation, 5.1%, compared with less than 1.0% in keratoconus, Fuchs' dystrophy, or bullous keratopathy with secondary implants (P less than 0.0005).
Corneal Consultants of Indiana, Indianapolis.

Price F W, Jr., Whitson W E and Marks R G. Progression of visual acuity after penetrating keratoplasty. *Ophthalmology* 1991b;8:1177-85

A consecutive series of 721 eyes was followed for visual acuity changes after keratoplasty in four groups: keratoconus, Fuchs' dystrophy, pseudophakic bullous keratopathy with retained intraocular lenses, and aphakic/pseudophakic bullous keratopathy with secondary implants during keratoplasty. Follow-up ranged from 12 to 84 months. Keratoconus eyes showed the quickest recovery of visual acuity: by 12 months, 91% attained a best-corrected vision of 20/40, and the mean lines of visual acuity for the group plateaued thereafter. The other three groups showed continuing improvement in vision through 24 months. From 3 months through 3 years after keratoplasty, the keratoconus and Fuchs' groups consistently showed better visual acuity levels than either the retained or the secondary implant groups (P less than 0.0001). Reporting changes in visual acuity over time offers multiple advantages compared with providing best-attained or last-recorded visual acuities after keratoplasty.
Corneal Consultants of Indiana, Indianapolis.

Puangsricharern V and Tseng S C. Cytologic evidence of corneal diseases with limbal stem cell deficiency. *Ophthalmology* 1995;10:1476-85

PURPOSE: To determine which human corneal diseases show similar abnormal corneal surfaces, characterized by conjunctival epithelial ingrowth (conjunctivalization), vascularization, and chronic keratitis (i.e., a constellation of signs termed limbal stem cell dysfunction [deficiency], which have been noted in experimental rabbit models). METHODS: A total of 134 impression cytology specimens of the perilimbal region collected from 1984 to 1994 were reviewed. Limbal deficiency was diagnosed if conjunctival goblet cells were found on the corneal surface. RESULTS: Ninety-four patients were found to have limbal deficiency. Category 1 comprised 53 patients with a clear history showing limbal stem cell destruction by chemical/thermal burns, Stevens-Johnson syndrome, multiple surgeries and cryotherapies, contact lens

wear, and severe microbial keratitis. Patients in category 2 (n = 41), did not have such a history, but gradual loss of stem cell functions over time was disclosed and included diverse causes such as aniridia, multiple endocrine deficiencies, neurotrophic keratopathy, peripheral inflammatory keratopathy or limbitis, and idiopathy. The 40 remaining patients with suspicious findings did not have limbal deficiency. CONCLUSIONS: Impression cytology can be used to diagnose and monitor corneal diseases with limbal deficiency, which manifest distinct clinical problems and are generally poor candidates for penetrating keratoplasty. The identification of category 1 diseases allows one to consider limbal (stem cell) transplantation for surface reconstruction. The presence of category 2 diseases indicates that limbal stem cell functions can be modulated by developmental, hormonal, neuronal, vascular, and inflammatory factors in the limbal stroma.
Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami School of Medicine, FL 33101, USA.

Puchtler H, Waldrop F S, Meloan S N, Terry M S and Conner H M. Methacarn (methanol-Carnoy) fixation. Practical and theoretical considerations. *Histochemie* 1970;2:97-116

Rajasekhar V K, Studer L, Gerald W, Socci N D and Scher H I. Tumour-initiating stem-like cells in human prostate cancer exhibit increased NF-kappaB signalling. *Nat Commun* 2011;162

Androgen depletion is a key strategy for treating human prostate cancer, but the presence of hormone-independent cells escaping treatment remains a major therapeutic challenge. Here, we identify a minor subset of stem-like human prostate tumour-initiating cells (TICs) that do not express prostate cancer markers, such as androgen receptor or prostate specific antigen. These TICs possess stem cell characteristics and multipotency as demonstrated by in vitro sphere-formation and in vivo tumour-initiation, respectively. The cells represent an undifferentiated subtype of basal cells and can be purified from prostate tumours based on coexpression of the human pluripotent stem cell marker TRA-1-60 with CD151 and CD166. Such triple-marker-positive TICs recapitulate the original parent tumour heterogeneity in serial xeno-transplantations indicating a tumour cell hierarchy in human prostate cancer development. These TICs exhibit increased nuclear factor-kappaB activity. These findings are important in understanding the molecular basis of human prostate cancer.
1] Stem Cell Center and Developmental Biology Program, Sloan-Kettering Institute, Memorial Sloan-Kettering Cancer Center, New York, New York 10065, USA. [2] Sidney Kimmel Center for Prostate and Urologic Cancers, Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, New York 10065, USA.

Rama P, Bonini S, Lambiase A, Golisano O, Paterna P, De Luca M and Pellegrini G. Autologous fibrin-cultured limbal stem cells permanently restore the corneal surface of patients with total limbal stem cell deficiency. *Transplantation* 2001;9:1478-85

BACKGROUND: Ocular burns cause depletion of limbal stem cells, which leads to corneal opacification and visual loss. Autologous cultured epithelial cells can restore damaged corneas, but this technology is still developing. We sought to establish a culture system that allows preservation of limbal stem cells and preparation of manageable epithelial sheets and to investigate whether such cultures can permanently restore total limbal stem cell deficiency. METHODS: We selected a homogeneous group of patients whose limbal cell deficiency was evaluated by scoring the gravity of the clinical picture and the keratin expression pattern. Stem cells, obtained from the limbus of the contralateral eye, were cultivated onto a fibrin substrate and their preservation was evaluated by clonal analysis. Fibrin cultures were grafted onto damaged corneas. RESULTS: Fibrin-cultured limbal stem cells were successful in 14 of 18 patients. Re-epithelialization occurred within the first week. Inflammation and vascularization regressed within the first 3-4 weeks. By the first month, the corneal surface was covered by a transparent, normal-looking epithelium. At 12-27 months follow-up, corneal surfaces were clinically and cytologically stable. Three patients had a penetrating keratoplasty approximately 1 year after restoration of their corneal surface. Their visual acuity improved from light perception or counting fingers to 0.8-1.0. CONCLUSIONS: Preservation of limbal stem cells in culture gives new perspectives on the treatment of ocular disorders characterized by complete limbal stem cell deficiency. The multicenter nature of this study and the handiness and ease of long-distance transportation of the fibrin-cultured epithelial sheets suggest that this technology can now be widely applied.
Division of Ophthalmology, Ospedale S.S. Giovanni e Paolo, Venice, Italy.

Reading M. A digestion technique for the reduction of background staining in the immunoperoxidase method. *J Clin Pathol* 1977;1:88-90

Reynolds B A and Weiss S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 1992;5052:1707-10

Neurogenesis in the mammalian central nervous system is believed to end in the period just after birth; in the mouse striatum no new neurons are produced after the first few days after birth. In this study, cells isolated from the striatum of the adult mouse brain were induced to proliferate in vitro by epidermal growth factor. The proliferating cells initially expressed nestin, an intermediate filament found in neuroepithelial stem cells, and subsequently developed the morphology and antigenic properties of neurons and astrocytes. Newly generated cells with neuronal morphology were immunoreactive for gamma-aminobutyric acid and substance P, two neurotransmitters of the adult striatum in vivo. Thus, cells of the adult mouse striatum have the capacity to divide and differentiate into neurons and astrocytes.
Department of Pathology, University of Calgary Faculty of Medicine, Alberta, Canada.

Rheinwald J G and Green H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 1975;3:331-43

Human diploid epidermis epidermal cells have been successfully grown in serial culture. To initiate colony formation, they require the presence of fibroblasts, but proliferation of fibroblasts must be controlled so that the epidermal cell population is not overgrown. Both conditions can be achieved by the use of lethally irradiated 3T3 cells at the correct density. When trypsinized human skin cells are plated together with the 3T3 cells, the growth of the human fibroblasts is largely suppressed, but epidermal cells grow from single cells into colonies. Each colony consists of keratinocytes ultimately forming a stratified squamous epithelium in which the dividing cells are confined to the lowest layer(s). Hydrocortisone is added to the medium, since in secondary and subsequent subcultures it makes the colony morphology more oderly and distinctive, and maintains proliferation at a slightly greater rate. Under these culture conditions, it is possible to isolate keratinocyte clones free of viable fibroblasts. Like human diploid fibroblasts, human diploid keratinocytes appear to have a finite culture lifetime. For 7 strains studied, the culture lifetime ranged from 20-50 cell generations. The plating efficiency of the epidermal cells taken directly from skin was usually 0.1-1.0%. On subsequent transfer of the cultures initiated from newborns, the plating efficiency rose to 10% or higher, but was most often in the range of 1-5% and dropped sharply toward the end of their culture life. The plating efficiency and culture lifetime were lower for keratinocytes of older persons.

Robinson J M and Vandre D D. Antigen retrieval in cells and tissues: enhancement with sodium dodecyl sulfate. *Histochem Cell Biol* 2001;2:119-30

Immunocytochemistry provides important information on the localization of antigens in cells and tissues. However, the procedures used to prepare cells and tissues for immunocytochemical labeling may have deleterious effects on the results achieved. That is, the antigen of interest may be difficult or impossible to detect following labeling. These sorts of observations have led to the concept of antigen masking in which the antigen (or specific epitope) is hidden from antibodies specific for that antigen (or epitope). Various procedures to

circumvent this problem have been developed. These different procedures generally fit under the term "antigen retrieval" (or epitope retrieval). The practice of antigen retrieval is widely employed with paraffin-embedded material. Antigen retrieval is less often applied to cells and tissues that are not embedded in paraffin. However, in the latter preparations there are situations in which the observed immunolabeling achieved falls short of expectations. This poor level of immunolabeling may, in some situations, be improved upon with antigen retrieval procedures. In this review, we describe experimental situations in which immunolabeling fell short of expectations. We also describe a procedure that has been useful in enhancing immunolabeling efficiency in these cases. The major feature of this procedure is the incorporation of a permeabilization/denaturation step using sodium dodecyl sulfate. This postfixation and prelabeling step dramatically improves immunolabeling for a number of antigens in both cells and cryosections of tissue.
Department of Physiology and Cell Biology, Ohio State University, 304 Hamilton Hall, 1645 Neil Avenue, Columbus, OH 43210, USA. Robinson.21@osu.edu

Rocha V, Garnier F, Ionescu I and Gluckman E. Hematopoietic stem-cell transplantation using umbilical-cord blood cells. *Rev Invest Clin* 2005;2:314-23

Eurocord Office-Bone and Marrow Transplant Hematology Department, Hospital Saint Louis, Paris, France.

Rodolfa K T. Inducing pluripotency. 2008;
The prospect of personalized regenerative medicine promises to provide treatments for a wide range of degenerative diseases and medical conditions. An important first step in attaining this goal is the production of pluripotent stem cells directly from individual patients, thereby providing autologous material which, after correcting intrinsic genetic defects and differentiation into required cell types or tissues, could be transplanted into the patient. This chapter reviews the current progress towards this first step, focusing on the techniques used to generate pluripotent cells, the advantages that each offers and the challenges that must be overcome.

Sato N, Meijer L, Skaltsounis L, Greengard P and Brivanlou A H. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med* 2004;1:55-63
Human and mouse embryonic stem cells (HESCs and MESCs, respectively) self-renew indefinitely while maintaining the ability to generate all three germ-layer derivatives. Despite the importance of ESCs in developmental biology and their potential impact on tissue replacement therapy, the molecular mechanism underlying ESC self-renewal is poorly understood. Here we show that activation of the canonical Wnt pathway is sufficient to maintain self-renewal of both HESCs and MESCs. Although Stat-3 signaling is involved in MESC self-renewal, stimulation of this pathway does not support self-renewal of HESCs. Instead we find that Wnt pathway activation by 6-bromoindirubin-3'-oxime (BIO), a specific pharmacological inhibitor of glycogen synthase kinase-3 (GSK-3), maintains the undifferentiated phenotype in both types of ESCs and sustains expression of the pluripotent state-specific transcription factors Oct-3/4, Rex-1 and Nanog. Wnt signaling is endogenously activated in undifferentiated MESCs and is downregulated upon differentiation. In addition, BIO-mediated Wnt activation is functionally reversible, as withdrawal of the compound leads to normal multidifferentiation programs in both HESCs and MESCs. These results suggest that the use of GSK-3-specific inhibitors such as BIO may have practical applications in regenerative medicine.
Laboratory of Molecular Vertebrate Embryology, The Rockefeller University, 1230 York Avenue, New York, NY 10021, USA.

Scadden D T. The stem-cell niche as an entity of action. *Nature* 2006;7097:1075-9
Stem-cell populations are established in 'niches'--specific anatomic locations that regulate how they participate in tissue generation, maintenance and repair. The niche saves stem cells from depletion, while protecting the host from over-exuberant stem-cell proliferation. It constitutes a basic unit of tissue physiology, integrating signals that mediate the balanced response of stem cells to the needs of organisms. Yet the niche may also induce pathologies by imposing aberrant function on stem cells or other targets. The interplay between stem cells and their niche creates the dynamic system necessary for sustaining tissues, and for the ultimate design of stem-cell therapeutics.
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Scharenberg C W, Harkey M A and Torok-Storb B. The ABCG2 transporter is an efficient Hoechst 33342 efflux pump and is preferentially expressed by immature human hematopoietic progenitors. *Blood* 2002;2:507-12
A promising and increasingly exploited property of hematopoietic stem cells is their ability to efflux the fluorescent dye Hoechst 33342. The Hoechst-negative cells are isolated by fluorescence-activated cell sorting as a so-called side "population" (SP) of bone marrow. This SP from bone marrow, as well as other tissues, is reported to contain immature stem cells with considerable plasticity. Some cell lines also efflux Hoechst and generate SP profiles. Reverse transcription-polymerase chain reaction (RT-PCR) and efflux inhibition studies with the lung carcinoma cell line, A549, implicated the ABCG2 transporter as a Hoechst efflux pump. Furthermore, it is shown that transient expression of ABCG2 generates a robust SP phenotype in human embryonic kidney (HEK293) cells. The results allow the conclusion that ABCG2 is a potent Hoechst efflux pump. Semiquantitative RT-PCR was used to characterize the developmental pattern of expression of ABCG2 in hematopoiesis. It is expressed at relatively high levels in putative hematopoietic stem cells (isolated as SP, 34+/38- or 34+/KDR+ populations) and drops sharply in committed progenitors (34+/38+, 34+/33+, or 34+/10+). Expression remains low in most maturing populations, but rises again in natural killer cells and erythroblasts. Comparison of messenger RNA (mRNA) levels for the 3 major multidrug-resistant efflux pumps, MDR1, MRP1, and ABCG2, in bone marrow SP cells reveals that ABCG2 is the predominant form in these cells. These data suggest that ABCG2 contributes significantly to the generation of the SP phenotype in hematopoietic stem cells. Furthermore, the sharp down-regulation of ABCG2 at the stage of lineage commitment suggests that this gene may play an important role in the unique physiology of the pluripotent stem cell.
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Schatton T, Murphy G F, Frank N Y, Yamaura K, Waaga-Gasser A M, Gasser M, Zhan Q, Jordan S, Duncan L M, Weishaupt C, Fuhlbrigge R C, Kupper T S, Sayegh M H and Frank M H. Identification of cells initiating human melanomas. *Nature* 2008;7176:345-9
Tumour-initiating cells capable of self-renewal and differentiation, which are responsible for tumour growth, have been identified in human haematological malignancies and solid cancers. If such minority populations are associated with tumour progression in human patients, specific targeting of tumour-initiating cells could be a strategy to eradicate cancers currently resistant to systemic therapy. Here we identify a subpopulation enriched for human malignant-melanoma-initiating cells (MMIC) defined by expression of the chemoresistance mediator ABCB5 (refs 7, 8) and show that specific targeting of this tumorigenic minority population inhibits tumour growth. ABCB5+ tumour cells detected in human melanoma patients show a primitive molecular phenotype and correlate with clinical melanoma progression. In serial human-to-mouse xenotransplantation experiments, ABCB5+ melanoma cells possess greater tumorigenic capacity than ABCB5- bulk populations and re-establish clinical tumour heterogeneity. In vivo genetic lineage tracking demonstrates a specific capacity of ABCB5+ subpopulations for self-renewal and differentiation, because ABCB5+ cancer cells generate both ABCB5+ and ABCB5- progeny, whereas ABCB5- tumour populations give rise, at lower rates, exclusively to ABCB5- cells. In an initial proof-of-principle analysis, designed to test the hypothesis that MMIC are also required for growth of established tumours, systemic administration of a monoclonal antibody directed at ABCB5, shown to be capable of inducing antibody-dependent cell-mediated cytotoxicity in ABCB5+ MMIC, exerted tumour-inhibitory effects. Identification of tumour-initiating cells with enhanced abundance in more advanced disease but susceptibility to specific targeting through a defining chemoresistance determinant has important implications for cancer therapy.

Transplantation Research Center, Children's Hospital Boston and Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, USA.

Schermer A, Galvin S and Sun T T. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 1986;1:49-62

In this paper we present keratin expression data that lend strong support to a model of corneal epithelial maturation in which the stem cells are located in the limbus, the transitional zone between cornea and conjunctiva. Using a new monoclonal antibody, AE5, which is highly specific for a 64,000-mol-wt corneal keratin, designated RK3, we demonstrate that this keratin is localized in all cell layers of rabbit corneal epithelium, but only in the suprabasal layers of the limbal epithelium. Analysis of cultured corneal keratinocytes showed that they express sequentially three major keratin pairs. Early cultures consisting of a monolayer of "basal" cells express mainly the 50/58K keratins, exponentially growing cells synthesize additional 48/56K keratins, and postconfluent, heavily stratified cultures begin to express the 55/64K corneal keratins. Cell separation experiments showed that basal cells isolated from postconfluent cultures contain predominantly the 50/58K pair, whereas suprabasal cells contain additional 55/64K and 48/56K pairs. Basal cells of the older, postconfluent cultures, however, can become AE5 positive, indicating that suprabasal location is not a prerequisite for the expression of the 64K keratin. Taken together, these results suggest that the acidic 55K and basic 64K keratins represent markers for an advanced stage of corneal epithelial differentiation. The fact that epithelial basal cells of central cornea but not those of the limbus possess the 64K keratin therefore indicates that corneal basal cells are in a more differentiated state than limbal basal cells. These findings, coupled with the known centripetal migration of corneal epithelial cells, strongly suggest that corneal epithelial stem cells are located in the limbus, and that corneal basal cells correspond to "transient amplifying cells" in the scheme of "stem cells----transient amplifying cells----terminally differentiated cells."

Schimmelpfennig B H. Direct and indirect determination of nonuniform cell density distribution in human corneal endothelium. *Invest Ophthalmol Vis Sci* 1984;2:223-9

The density distribution of endothelial cells was determined, directly and indirectly, by counting cells and cell nuclei in two separate groups of unpaired human corneas. Four areas, measuring 1 square mm each, were counted in the corneal center as well as in the periphery close to Schwalbe's line. In 19 Orcein-stained corneas, the peripheral density of nuclei was 3632/mm2 +/- 592 (SD) as compared with central counts of 2778 mm2 +/- 284 (SD). The other group of 22 corneas, stained supravitally with Alizarin-red revealed a peripheral cell density of 3696/mm2 +/- 721 (SD), in contrast with a central density of 2811/mm2 +/- 425 (SD). There was also an uneven density distribution in the central endothelium. The average difference between the highest and lowest central square millimeter counts in the two groups was 8.0 +/- 7.7% (SD) and 9.0 +/- 3.6% (SD), respectively. The data indicate a nonuniform endothelial cell density distribution in the human cornea that may have clinical implications.

Schlotzer-Schrehardt U, Dietrich T, Saito K, Sorokin L, Sasaki T, Paulsson M and Kruse F E. Characterization of extracellular matrix components in the limbal epithelial stem cell compartment. *Exp Eye Res* 2007;6:845-60

A specialized microenvironment or niche, which regulates maintenance, self-renewal, activation, and proliferation of stem cells by external signals, is one of the key prerequisites for stem cell function. However, the parameters determining the limbal stem cell niche are not yet defined. In order to characterize the role of basement membrane (BM) and extracellular matrix components in the generation of a microenvironmental niche for limbal stem and progenitor cells, we extensively analyzed the topographical variations of the BM zone of human ocular surface epithelia using immunohistochemistry and a large panel of antibodies to most of the presently described intrinsic and associated BM components. Apart from BM components uniformly expressed throughout all ocular surface epithelia (e.g. type IV collagen alpha5 and alpha6 chains, collagen types VII, XV, XVII, and XVIII, laminin-111, laminin-332, laminin chains alpha3, beta3,and gamma2, fibronectin, matrilin-2 and -4, and perlecan), the BM of the limbal epithelium shared many similarities with that of the conjunctival epithelium, including positive labelling for type IV collagen alpha1 and alpha2 chains, laminin alpha5, beta2, and gamma1 chains, nidogen-1 and -2, and thrombospondin-4, whereas type IV collagen alpha3, type V collagen, fibrillin-1 and -2, thrombospondin-1, and endostatin were present in the corneal BM, but lacking or more weakly expressed in the limbal and conjunctival BMs. As compared to both the corneal and conjunctival BMs, the limbal BM showed a markedly increased immunoreactivity for laminin alpha1, alpha2, beta1 chains, and agrin, and a specific but patchy immunoreactivity for laminin gamma3 chain, BM40/SPARC, and tenascin-C, which co-localized with ABCG2/p63/K19-positive and K3/Cx43/desmoglein/integrin-alpha2-negative cell clusters comprising putative stem and early progenitor cells in the basal epithelium of the limbal palisades. Components that were particularly expressed in the corneal-limbal transition zone included type XVI collagen, fibulin-2, tenascin-C/R, vitronectin, bamacan, chondroitin sulfate, and versican, all of which co-localized with vimentin-positive cell clusters comprising putative late progenitor cells in the basal epithelium. This pronounced heterogeneity of the BM in the limbal area, both in the region of limbal palisades and the corneal-limbal transition zone, appears to be involved in providing unique microenvironments for corneal epithelial stem and late progenitor cells. Identification of specific niche parameters might not only help to understand limbal stem cell regulation, but also to improve their selective enrichment and in vitro expansion for therapeutic strategies.
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Schlotzer-Schrehardt U and Kruse F E. Identification and characterization of limbal stem cells. *Exp Eye Res* 2005;3:247-64

The maintenance of a healthy corneal epithelium under both normal and wound healing conditions is achieved by a population of stem cells (SC) located in the basal epithelium at the corneoscleral limbus. In the light of the development of strategies for reconstruction of the ocular surface in patients with limbal stem cell deficiency, a major challenge in corneal SC biology remains the ability to identify stem cells in situ and in vitro. Until recently, the identification of limbal stem cells mainly has been based on general properties of stem cells, e.g. lack of differentiation, prolonged label-retaining, indefinite capacity of proliferation exemplified by the clonogenic assay as well as their special role in corneal wound healing. During the last years, a number of molecular markers for the limbal SC compartment has been proposed, however, their role in distinguishing limbal SC from their early progeny is still under debate. Data reported from the literature combined with our own recent observations suggest, that the basal epithelial cells of the human limbus contain ABCG2, K19, vimentin, KGF-R, metallothionein, and integrin alpha9, but do not stain for K3/K12, Cx43, involucrin, P-cadherin, integrins alpha2, alpha6, and beta4, and nestin, when compared to the basal cells of the corneal epithelium. A relatively higher expression level in basal limbal cells was observed for p63, alpha-enolase, K5/14, and HGF-R, whereas there were no significant differences in staining intensity for beta-catenin, integrins alphav, beta1, beta2, and beta5, CD71, EGF-R, TGF-beta-RI, TGF-beta-RII, and TrkA between limbal and corneal basal epithelial cells. Therefore, a combination of differentiation-associated markers (e.g. K3/K12, Cx43, or involucrin) and putative SC-associated markers (e.g. ABCG2, K19, vimentin, or integrin alpha9) may provide a suitable tool for identification of human limbal SC. While most putative SC markers label the majority of limbal basal cells and, therefore, may not distinguish SC from progenitor cells, only ABCG2 was strictly confined to small clusters of basal cells in the limbal epithelium. At present, ABCG2 therefore appears to be the most useful cell surface marker for the identification and isolation of corneal epithelial SC. Moreover, the characteristics of the specific microenvironment of corneal SC, as provided by growth factor activity and basement membrane heterogeneity in the limbal area, could serve as additional tools for their selective enrichment and in vitro expansion for the purpose of ocular surface reconstruction.
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Schofield R. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* 1978;1-2:7-25

Several experimental findings that are inconsistent with the view that the spleen colony-forming cell (CFU-S) is the primary haemopoietic stem cell are reviewed. Recovery of CFU-S, both quantitatively and qualitatively, can proceed differently depending upon the cytotoxic agent or regime used to bring about the depletion. The virtual immortality of the stem cell population is at variance with evidence that the CFU-S population has an 'age-structure' which has been invoked by several workers to explain experimental and clinical observations. To account for these inconsistencies, a hypothesis is proposed in which the stem cell is seen in association with other cells which determine its behaviour. It becomes essentially a fixed tissue cell. Its maturation is prevented and, as a result, its continued proliferation as a stem cell is assured. Its progeny, unless they can occupy a similar stem cell 'niche', are first generation colony-forming cells, which proliferate and mature to acquire a high probability of differentiation, i.e., they have an age-structure. Some of the experimental situations reviewed are discussed in relation to the proposed hypothesis.

Schofield R. The stem cell system. *Biomed Pharmacother* 1983;8:375-80

The stem cell is defined as that cell in a tissue which, under normal circumstances, maintains its own population, undiminished in function and size, and furnishes daughters to provide new functional cells of that tissue. The daughters may, or may not, have to undergo further differentiation and/or maturation in order to achieve their functional stage. The fundamental characteristic of a stem cell, therefore, is self-renewal. Evidence is presented which implicates the microenvironment as a major component of the stem cell system, without which stem cells cannot be maintained. Furthermore, it is suggested that stem cell properties do not reside in one specific cell type in the population but, when necessary, cells other than those normally playing the stem cell role, can have stem cell function imposed upon them by the appropriate microenvironment. The stem cell "niche" hypothesis is presented to explain the dependence of stem cells upon their microenvironment. The postulate is offered that there are no cells which are intrinsically stem cells but that a range of cells in a tissue possess stem cell potential to a greater or lesser extent.

Secker G A and Daniels J T. Corneal epithelial stem cells: deficiency and regulation. *Stem Cell Rev* 2008;3:159-68

The corneal epithelium is continuously renewed by a population of stem cells that reside in the corneoscleral junction, otherwise known as the limbus. These limbal epithelial stem cells (LESC) are imperative for corneal maintenance with deficiencies leading to in-growth of conjunctival cells, neovascularisation of the corneal stroma and eventual corneal opacity and visual loss. One such disease that has traditionally been thought to be due to LESC deficiency is aniridia, a pan-ocular congenital eye disease due to mutations in the PAX6 gene. Corneal changes or aniridia related keratopathy (ARK) seen in aniridia are typical of LESC deficiency. However, the pathophysiology behind ARK is still ill defined, with current theories suggesting it may be caused by a deficiency in the stem cell niche and adjacent corneal stroma, with altered wound healing responses also playing a role (Ramaesh et al, International Journal of Biochemistry & Cell Biology 37:547-557, 2005) or abnormal epidermal differentiation of LESC (Li et al., The Journal of Pathology 214:9, 2008). PAX6 is considered the master control gene for the eye and is required for normal eye development with expression continuing in the adult cornea, thus inferring a role for corneal repair and regeneration (Sivak et al., Developments in Biologicals 222:41-54, 2000). Studies of models of Pax6 deficiency, such as the small eyed (sey) mouse, should help to reveal the intrinsic and extrinsic mechanisms involved in normal LESC function.
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Sell S. On the stem cell origin of cancer. *Am J Pathol* 2010;6:2584-494

In each major theory of the origin of cancer-field theory, chemical carcinogenesis, infection, mutation, or epigenetic change-the tissue stem cell is involved in the generation of cancer. Although the cancer type is identified by the more highly differentiated cells in the cancer cell lineage or hierarchy (transit-amplifying cells), the property of malignancy and the molecular lesion of the cancer exist in the cancer stem cell. In the case of teratocarcinomas, normal germinal stem cells have the potential to become cancers if placed in an environment that allows expression of the cancer phenotype (field theory). In cancers due to chemically induced mutations, viral infections, somatic and inherited mutations, or epigenetic changes, the molecular lesion or infection usually first occurs in the tissue stem cells. Cancer stem cells then give rise to transit-amplifying cells and terminally differentiated cells, similar to what happens in normal tissue renewal. However, the major difference between cancer growth and normal tissue renewal is that whereas normal transit amplifying cells usually differentiate and die, at various levels of differentiation, the cancer transit-amplifying cells fail to differentiate normally and instead accumulate (ie, they undergo maturation arrest), resulting in cancer growth.
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Serafini M and Verfaillie C M. Pluripotency in adult stem cells: state of the art. *Semin Reprod Med* 2006;5:379-88

For many years, it has long been known that stem cells derived from adult tissues maintain the capacity for self-renewal and differentiation into multiple cell types that are characteristic of the tissue of origin. Recent studies have shown new evidence that several tissues may contain cells capable of generating differentiated cells beyond their own tissue boundaries, defining a process termed stem cell plasticity. The pluripotency of adult stem cells have evoked significant excitement over the possibility of novel functional uses of stem cells, with the final purpose to develop new and more effective treatment strategies. However, despite the number of promising studies describing the plasticity of adult stem cells, many questions remain to be answered. In this article, we critically review the current state of the art in the field of adult stem cells, focussing on the present understanding of the concepts of stem cell pluripotency and plasticity.
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Shanmuganathan V A, Foster T, Kulkarni B B, Hopkinson A, Gray T, Powe D G, Lowe J and Dua H S. Morphological characteristics of the limbal epithelial crypt. *Br J Ophthalmol* 2007;4:514-9

AIM: In 2005 we reported the discovery of a novel anatomical structure at the limbus, which we termed the limbal epithelial crypt (LEC). The purpose of this study was to further evaluate the distribution, immunophenotypical, and ultra structural characteristics of the LEC as a putative niche of stem cells. METHODS: Sequential histological sections of human corneo-scleral limbal rims were examined for the presence and distribution of the LEC. Immunophenotypical characterisation of the LEC cells using a panel of antibodies of interest was undertaken. Transmission electron microscopy of the LEC was used to examine the ultra structural and morphometric features of cells within the LEC and adjacent limbus. RESULTS: A total of 74 LECs were identified in eight corneo-scleral rims. These varied in number, size and distribution within rims. Cells within the crypt demonstrated the following phenotype: CK3-/CK19+/CD 34-/Vimentin+/p63+/Connexin 43+/MIB1 (Ki67)-. Presence of Cx43 was also demonstrated in the rete pegs adjacent to the LEC. Basal cells of the LEC were significantly smaller than basal cells found in adjacent rete pegs and also smaller than suprabasal limbal and central corneal epithelial cells ($p<0.05$). Morphologically they had a high nuclear:cytoplasmic ratio and were adherent to the underlying basement membrane by means of complex convolutions of cytoplasmic processes. CONCLUSIONS: LECs are sparse but a consistent finding in the human corneo-scleral limbus. The LEC contains a unique sub-population of cells expressing several characteristics that are consistent with it representing a putative stem cell niche.
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Shao C, Fu Y, Lu W and Fan X. Bone marrow-derived endothelial progenitor cells: a promising therapeutic alternative for corneal endothelial dysfunction. *Cells Tissues Organs* 2011;4:253-63

BACKGROUND: The global shortage of donor corneas has motivated the development of bioengineered corneas. Although corneal endothelium has been reconstituted using corneal endothelial cells (CEC) and precursor cells with various carrier materials, all of the current options require corneal tissue and are also limited by the scarcity of donor corneas. Here, we explored the feasibility of inducing bone marrow-derived endothelial progenitor cells (BEPC) to differentiate into CEC for the repair of corneal endothelial defects. MATERIALS AND METHODS: BEPC were isolated from human fetal bone marrow, and identified using several antigen markers. BEPC were cocultured with CEC for 10 days in a transwell system with conditioned medium from CEC, and cell transdifferentiation was then examined. With a porcine corneal acellular matrix (PCACM) as the carrier, the induced BEPC were transplanted onto a cat's cornea from which Descemet's membrane and the endothelium had been stripped. RESULTS: The induced BEPC resembled CEC in polygonal shape, expressing aquaporin-1, tightly opposed cell junctions, and neurone-specific enolase. Twenty-eight days after surgery, the transparency gradually returned to the corneas transplanted with the induced BEPC on PCACM. CONCLUSIONS: Human fetal BEPC transdifferentiate into corneal endothelial-like cells in vitro. Features of the induced BEPC indicated that they may be useful for the repair of corneal endothelial dysfunction.
Department of Ophthalmology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China.

Shi S R, Cote R J, Young L, Imam S A and Taylor C R. Use of pH 9.5 Tris-HCl buffer containing 5% urea for antigen retrieval immunohistochemistry. *Biotech Histochem* 1996;4:190-6

Successful antigen retrieval (AR) immunohistochemistry is dependent on the temperature, heating time, and pH value of the AR solutions. There is no single standardized AR solution, however, that is suitable for all antibodies "routinely" used in surgical pathology for immunostaining archival tissue sections. We tested a variety of AR solutions varying in pH value, chemical composition, and molarity. Based upon preliminary results, we compared three AR solutions: 0.1 M Tris-HCl buffer, pH 9.5, containing 5% urea, 0.1 M Tris-HCl buffer pH 9.5 without urea, and citrate buffer, pH 6.0. Each AR solution was tested with a panel of 34 antibodies using microwave heating for antigen retrieval. The heating conditions were standardized at 10 min and an automated stainer was used to standardize the immunostaining method. The Tris-HCl containing urea was superior to pH 6.0 citrate buffer for 22 antibodies. In 12 cases, Tris-HCl with urea was also superior to Tris-HCl alone. In 12 cases, the intensity was similar for all three retrieval solutions. The staining obtained with Tris-HCl with urea was equal to or better than with pH 6.0 citrate buffer in all cases. The Tris-HCl with urea solution is satisfactory for AR of most antibodies employed in routine surgical pathology.
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Shortt A J, Secker G A, Lomas R J, Wilshaw S P, Kearney J N, Tuft S J and Daniels J T. The effect of amniotic membrane preparation method on its ability to serve as a substrate for the ex-vivo expansion of limbal epithelial cells. *Biomaterials* 2009;6:1056-65

Human amniotic membrane (HAM) is employed as a substrate for the ex-vivo expansion of limbal epithelial cells (LECs) used to treat corneal epithelial stem cell deficiency in humans. The optimal method of HAM preparation for this purpose is unknown. This study evaluated the ability of different preparations of stored HAM to serve as substrates for LEC expansion ex-vivo. The effect of removing the amniotic epithelial cells (decellularisation) from HAM prior to seeding of LECs, the effect of glycerol cryopreservation and the effect of peracetic acid (PAA) sterilization and antibiotic disinfection were evaluated using different HAM test groups. Human LECs were cultured on each preparation and the following outcomes were assessed: confluence of growth, cell density, cell morphology and expression of the putative LESC markers deltaN-p63alpha and ABCG2. Removing amniotic epithelial cells prior to seeding of LECs resulted in a higher percentage of confluence but a lower cell density than intact HAM suggesting that decellularisation does not increase proliferation, but rather that it facilitates migration of LECs resulting in larger cells. Decellularisation did not affect the percentage of cells expressing the putative LESC markers deltaN-p63alpha (< or =4% in both intact and acellular groups) and ABCG2 (< or =3% in both intact and acellular groups). Glycerol cryopreservation of HAM resulted in poor morphology and a low proportion of cells expressing deltaN-p63alpha (< or =6%) and ABCG2 (< or =8%). HAM frozen at -80 degrees C in Hank's Balanced Salt Solution (HBSS) was superior, demonstrating excellent morphology of cultured LECs and high levels of deltaN-p63alpha (< or =68%) and ABCG2 (< or =62%) expression (p<0.001). The use of PAA or antibiotics to decontaminate HAM does not appear to affect this function. The variables affecting the ability of HAM to serve as a substrate for LEC expansion ex-vivo are poorly understood. The use of glycerol as a cryoprotectant impairs this ability whereas simple frozen HAM appears to work extremely well for this purpose.
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Shortt A J, Secker G A, Notara M D, Limb G A, Khaw P T, Tuft S J and Daniels J T. Transplantation of ex vivo cultured limbal epithelial stem cells: a review of techniques and clinical results. *Surv Ophthalmol* 2007;5:483-502

Ex vivo cultured limbal epithelial stem cells have been used successfully to treat corneal limbal stem cell deficiency. We identified 17 reports of the application of this novel cell-based therapy in humans. In addition we identified four reports of the use of culture oral mucosal epithelial cells to treat limbal stem cell deficiency. We examined these reports to discern the success rate, complication rate, visual outcome, whether there is an optimal technique and which patients are the most likely to benefit. We also discuss the different culture methods employed and the regulations governing cell banks that are providing this service. We found that the techniques used to cultivate and transplant cells varied, but that no individual method was clearly superior. The reported success rate is similar across all studies for both allografts and autografts. The clinical indications for this treatment are not clearly defined as indicated by the variety of disorders treated. Follow-up is limited and the long-term success rate is yet to be established. Nonetheless, we conclude that there is sufficient evidence to support the continued use and refinement of this procedure as a treatment for corneal stem cell deficiency.
Ocular Repair and Regeneration Biology Unit, Institute of Ophthalmology, London.

Shortt A J, Secker G A, Rajan M S, Meligonis G, Dart J K, Tuft S J and Daniels J T. Ex vivo expansion and transplantation of limbal epithelial stem cells. *Ophthalmology* 2008;11:1989-97

OBJECTIVE: To determine, using objective measures, the outcome of ex vivo cultured limbal epithelial stem cell (LESC) transplantation performed in compliance with good manufacturing practice using a novel culture system without 3T3 feeder cells. DESIGN: Prospective, noncomparative, interventional case series. PARTICIPANTS: Ten eyes of 10 patients with profound LESC deficiency arising from chemical injury (4 eyes), aniridia (3 eyes), ectodermal dysplasia (1 eye), Reiger's anomaly with Pax6 haploinsufficiency (1 eye), and unknown cause (1 eye). METHODS: Allogeneic (7 eyes) or autologous (3 eyes) corneal LESCs were cultured on human amniotic membrane. Tissue was transplanted to the recipient eye after superficial keratectomy. Impression cytology and confocal microscopy were performed 6 months after surgery with clinical follow-up to 13 months. Success was defined as an improvement in the defined clinical parameters of LESC deficiency, an improvement in visual acuity, the restoration of a more normal corneal phenotype on impression cytology, and the appearance of a regular hexagonal basal layer of cells on corneal confocal microscopy. MAIN OUTCOME MEASURES: Clinical parameters of LESC deficiency (loss of epithelial transparency, superficial corneal vascularization, epithelial irregularity, and epithelial breakdown), visual acuity, impression cytology and cytokeratin expression profiles, and in vivo confocal corneal confocal microscopy. RESULTS: The success rate using this technique was 60% (autografts 33%, allografts 71%). All patients with a successful outcome experienced an improvement in visual acuity of >/=2 lines Snellen acuity. Preoperatively, CK3+ and CK19+ cells accounted for 12+/-2.4% (mean +/- standard error of the mean) and 80+/-2.15% of cells, respectively, whereas postoperatively these accounted for 69+/-6.43% (P<0.0001) and 30+/-6.34% (P<0.0001) of cells, respectively. Goblet cells accounted for 8+/-1.19% of cells preoperatively and 1+/-0.35% of cells postoperatively (P<0.0001). CONCLUSIONS: These data demonstrate that it is possible to culture LESCs ex vivo in compliance with good manufacturing practice regulations. A set of objective outcome measures that confirm the efficiency of this technique in treating LESC deficiency is described. The widespread use of such standardized and objective outcome measures would facilitate a comparison between the different culture methods in use.
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Shortt A J, Tuft S J and Daniels J T. Ex vivo cultured limbal epithelial transplantation. A clinical perspective. *Ocul Surf* 2010;2:80-90

The term ex vivo cultured limbal epithelial transplantation (CLET) refers to the process of culturing a sheet of human limbal epithelium in the laboratory and transplanting this sheet back onto the limbal stem cell-deficient cornea of the same patient or another recipient. This emerging technology represents one of the earliest successes in regenerative medicine. CLET is, at present, best suited to patients who have unilateral total limbal stem cell deficiency arising from chemical injury and who are suitable for autologous cell culture and transplantation. Although the results of allogeneic cell transplantation are encouraging and superior to conventional stem cell transplantation techniques, insufficient follow-up precludes conclusions regarding the long-term outcomes. Other tissues, such as oral mucosal epithelium, are emerging as viable alternative sources of cells, especially for patients with bilateral disease.

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Singh S K, Clarke I D, Terasaki M, Bonn V E, Hawkins C, Squire J and Dirks P B. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003;18:5821-8

Most current research on human brain tumors is focused on the molecular and cellular analysis of the bulk tumor mass. However, there is overwhelming evidence in some malignancies that the tumor clone is heterogeneous with respect to proliferation and differentiation. In human leukemia, the tumor clone is organized as a hierarchy that originates from rare leukemic stem cells that possess extensive proliferative and self-renewal potential, and are responsible for maintaining the tumor clone. We report here the identification and purification of a cancer stem cell from human brain tumors of different phenotypes that possesses a marked capacity for proliferation, self-renewal, and differentiation. The increased self-renewal capacity of the brain tumor stem cell (BTSC) was highest from the most aggressive clinical samples of medulloblastoma compared with low-grade gliomas. The BTSC was exclusively isolated with the cell fraction expressing the neural stem cell surface marker CD133. These CD133+ cells could differentiate in culture into tumor cells that phenotypically resembled the tumor from the patient. The identification of a BTSC provides a powerful tool to investigate the tumorigenic process in the central nervous system and to develop therapies targeted to the BTSC.

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Sonmez B and Beden U. Fibrin glue-assisted sutureless limbal stem cell transplantation surgery for the treatment of severe ocular chemical injury. *Cornea* 2011;3:296-300

PURPOSE: To report the use of fibrin tissue glue in securing the keratolimbal allograft (KLAL) and living-related conjunctival limbal allograft to the ocular surface in patients with severe ocular chemical injury. DESIGN: A retrospective review of interventional case series. METHODS: Conjunctival limbal allografts were harvested from the first-degree living-related relatives under topical anesthesia and fixated to the superior and inferior limbal quadrants in the recipient eye. The KLALs were fixated mainly to the nasal and temporal limbus with the help of fibrin tissue glue after being cut into 2 crescents and manually dissected to near one-third thicknesses in a lamellar fashion. RESULTS: Five eyes of 4 patients were included in the study. The sources of the chemical injuries were: CaOH2 (3 eyes), NaOH (1 eye), and mitomycin C (1 eye). The limbal stem cell deficiency was 360 degrees in 4 eyes and 300 degrees in 1 eye. Corneas were covered with conjunctiva or fibrovascular tissue adjacent to the areas with limbal stem cell deficiency. The fibrin tissue glue was effective in securing both the keratolimbal and the conjunctivolimbal grafts at the surgery. Postoperatively, the corneal epithelium healed within 1 week in all of the eyes. Neither graft dislocation nor graft rejection occurred after a mean of 18.2 months of follow-up. CONCLUSIONS: The use of fibrin glue to fixate the KLAL and the living-related conjunctival limbal allograft in patients with severe chemical trauma is practical and effective. This technique may also be beneficial in terms of decreasing the risk of rejection in this patient group.

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Stepp M A, Zhu L, Sheppard D and Cranfill R L. Localized distribution of alpha 9 integrin in the cornea and changes in expression during corneal epithelial cell differentiation. *J Histochem Cytochem* 1995;4:353-62

A recently characterized integrin alpha-chain, alpha 9, forms heterodimers with the integrin beta 1-chain and is present in the skin with a distribution similar to that of alpha 2 and alpha 3, other beta 1 integrins. To determine whether alpha 9 is expressed in the stratified squamous epithelium of the cornea, we used immunohistochemical techniques to compare the distribution of alpha 9 in the adult mouse cornea with that of alpha 3. Abundant alpha 9 was expressed in the lateral and basal membranes of the basal cells of the conjunctiva and corneal limbus, but very little alpha 9 was present in the basal cells of the central corneal epithelium. In contrast, alpha 3 was present in the membranes of basal cells of the conjunctiva, limbus, and central cornea. To determine when during postnatal maturation of the corneal epithelium alpha 9 becomes restricted to the limbus, we looked at the distribution of alpha 9 and alpha 3 in the developing mouse eye from birth to eyelid opening. At birth, the basal cells of the cornea and developing limbal region did not express alpha 9, but there was abundant alpha 9 expressed in suprabasal cells between the fused lids and in the basal cells of the skin and conjunctiva. In contrast, alpha 3, integrin was expressed uniformly in the basal cells across the surface of the conjunctiva, limbus, and cornea and was present only in the basal cells of the epithelium between the fused eyelids. In the central cornea, alpha 9 expression increased in basal cells up until Day 10 after birth. After Day 10, alpha 9 expression in the central cornea began to decrease; after the lids were open, alpha 9 expression in the central cornea became restricted to the limbus. In the basal and suprabasal cells between the fused eyelids expression of alpha 9 became increasingly restricted over time to the basal cells. Recent data suggest that alpha 9 beta 1 can interact with tenascin. Our dual labeling confocal microscopy studies indicate that localization of alpha 9 and tenascin are not coordinated in the developing mouse cornea. Many recent studies have shown an important role for beta 1 integrins in mediating epithelial cell differentiation in vitro; in vivo, changes in integrin expression have been found in wound healing, psoriasis, and in basal and squamous cell carcinomas.(ABSTRACT TRUNCATED AT 400 WORDS)

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Stojkovic M, Lako M, Stojkovic P, Stewart R, Przyborski S, Armstrong L, Evans J, Herbert M, Hyslop L, Ahmad S, Murdoch A and Strachan T. Derivation of human embryonic stem cells from day-8 blastocysts recovered after three-step in vitro culture. *Stem Cells* 2004;5:790-7

Human embryonic stem cells (hESCs) have been derived from the inner cell mass (ICM) of day 5-7 blastocysts and hold great promise for research into human developmental biology and the development of cell therapies for the treatment of human diseases. We report here that our novel three-step culture conditions successfully support the development of day-8 human blastocysts, which possess significantly ($p < .01$) more ICM cells than day-6 blastocysts. Plating of ICMs isolated from day-8 blastocysts resulted in the formation of a colony with hESC morphology from which a new hESC line (hES-NCL1) was derived. Our stem cell line is characterized by the expression of specific cell surface and gene markers: GTCM-2, TG343, TRA1-60, SSEA-4, alkaline phosphatase, OCT-4, NANOG, and REX-1. Cytogenetic analysis of the hESCs revealed that hES-NCL1 line has a normal female (46, XX) karyotype. The pluripotency of the cell line was confirmed by the formation of teratomas after injection into severely combined immunodeficient mice and spontaneous differentiation under in vitro conditions.

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Sudha B, Sitalakshmi G, Iyer G K and Krishnakumar S. Putative stem cell markers in limbal epithelial cells cultured on intact & denuded human amniotic membrane. *Indian J Med Res* 2008;2:149-56

BACKGROUND & OBJECTIVES: The ocular surface is an ideal region to study the epithelial stem cell (SC) biology because of the unique spatial arrangement of stem cells and transient amplifying cells. A major challenge in corneal SC biology is the ability to identify SC in vitro and in situ, and one of the major controversies in the field relates to reliable SC markers. This study was carried out to evaluate and compare the expression of the stem cell associated marker: ABCG2, keratinocyte stem cell marker: p63 and corneal differentiation markers: Cnx43 and K3/K12 on limbal explants cultured on human amniotic membrane (HAM) with intact epithelium and HAM denuded of its epithelium. METHODS: Human limbal biopsies obtained from the cadaveric donor eyes were used in this study. The cells were cultured over the

HAM with intact and denuded epithelium. Reverse transcriptase PCR, immunohistochemistry, Western blotting for ABCG2, P63, Cnx43 and K3/K12 were done. RESULTS: The limbal epithelial cells cultured over intact HAM expressed the stem cell associated markers (ABCG2, p63) and showed reduced expression of the differentiation markers (Cnx43 and K3/K12) when compared to limbal epithelial cells cultured over denuded HAM, which expressed more differentiation markers at the end of three weeks. BrdU label retaining cells were observed in the limbal epithelial cells cultured over HAM with epihelium only. INTERPRETATION & CONCLUSIONS: Our results showed that the intact HAM supported the growth of limbal epithelial cells expressing stem cell associated markers, and allowing little differentiation of the limbal cells to cornea phenotype. Further studies are needed to understand the properties of the amniotic epithelium that retains the stemness in the cultured limbal stem cells.
L&T Department of Ocular Pathology, Vision Research Foundation, Chennai, India.

Sugar A, Meyer R F, Heidemann D, Kaplan S, Berka T, Maguire K and Martonyi C. Specular microscopic follow-up of corneal grafts for pseudophakic bullous keratopathy. *Ophthalmology* 1985;3:325-30

Pseudophakic bullous keratopathy (PBK) has become the leading indication for penetrating keratoplasty. In our initial fifty patients having keratoplasty for PBK there was gradual loss of clear grafts in patients with retained intraocular lenses (IOLs). Specular microscopy was performed on donor tissue, and periodically postoperatively, for 130 grafts for PBK. The highest cell loss at one year was in eyes with retained iris support (39.2%) or anterior chamber (37.2%) IOLs. Cell loss in grafts with removed iris support IOLs (21.3%) was significantly less. When iris support IOLs were exchanged for anterior chamber (AC) IOLs, the cell loss at one year (27.1%) was intermediate. We recommend that iris support IOLs be removed at keratoplasty. Exchange for an AC IOL should be considered depending on the visual needs of each patient. Removal of AC IOLs should be based on consideration of prior tolerance and position of the implant.

Sukach A N and Ivanov E N. [Formation of spherical colonies as a property of stem cells]. *Tsitologiia* 2007;11:916-22

The report deals with the ability of embryonic cells harvested from the nervous, hematopoietic and epithelial-muscular human tissues to form spheres during in vitro culturing. Judging from their own data and those reported elsewhere, the authors have hypothesized that formation of spherical colonies in vitro is a common feature of stem cells of various origin and varying degree of maturation. Probably, the formation of spheres provides for development of stem cells according to the locations and their inner temporal programs, which are specific and particular for every type of cells.

Sumide T, Nishida K, Yamato M, Ide T, Hayashida Y, Watanabe K, Yang J, Kohno C, Kikuchi A, Maeda N, Watanabe H, Okano T and Tano Y. Functional human corneal endothelial cell sheets harvested from temperature-responsive culture surfaces. *FASEB J* 2006;2:392-4

This study reports a new method for fabricating bioengineered human corneal endothelial cell sheets suitable for ocular surgery and repair. We have initially cultured human corneal endothelial cells on type IV collagen-coated dishes and, after several passages, expanded cells were then seeded onto novel temperature-responsive culture dishes. Four weeks after reaching confluence, these cultured endothelial cells were harvested as intact monolayer cell sheets by simple temperature reduction without enzymatic treatment. Scanning electron microscopy indicated that these cells were primarily hexagonal with numerous microvilli and cilia, similar to the native corneal endothelium. The Na+, K+-ATPase pump sites were located at the cell borders as in vivo. Moreover, cell densities and numbers of pump sites were identical to those of in vivo human corneal endothelium under optimized conditions. A 3H-ouabain binding analysis demonstrated a linear proportionality for cell pump density between confluent cell densities of 575 cells/mm2 and 3070 cells/mm2. We also confirmed Na+, K+-ATPase activity in the sheets in vitro. Xenograft transplantation results showed that the fabricated sheets retain their function of maintaining proper stromal hydration in vivo. We have established a regimen to culture and proliferate human corneal endothelial cells and fabricate endothelial sheets ex vivo morphologically and functionally similar to the native corneal endothelium. Our results support the value of harvested cell sheets for clinical applications in ocular reconstructive surgery in patients with ocular endothelial decompensation.
Department of Ophthalmology, Osaka University Medical School, Suita, Japan.

Summer R, Kotton D N, Sun X, Ma B, Fitzsimmons K and Fine A. Side population cells and Bcrp1 expression in lung. *Am J Physiol Lung Cell Mol Physiol* 2003;1:L97-104

Side population (SP) cells are a rare subset of cells found in various tissues that are highly enriched for stem cell activity. SP cells can be isolated by dual-wavelength flow cytometry because of their capacity to efflux Hoechst dye, a process mediated by the ATP-binding cassette transporter breast cancer resistance protein (Bcrp) 1. By performing flow cytometry of enzymedigested mouse lung stained with Hoechst dye, we found that SP cells comprise 0.03-0.07% of total lung cells and are evenly distributed in proximal and distal lung regions. By RT-PCR, we found that lung SP cells express hepatocyte nuclear factor-3beta, but not thyroid transcription factor-1. Surface marker analysis revealed lung SP cells to be stem cell antigen 1 positive, Bcrp1 positive, lineage marker negative, and heterogeneous at the CD45 locus. As expected, we did not detect lung SP cells in Bcrp1-deficient animals. We, therefore, employed nonisotopic in situ hybridization and immunostaining for Bcrp1 as a strategy to localize these cells in vivo. Expression was observed in distinct lung cell types: bronchial and vascular smooth muscle cells and round cells within the distal air space. We confirmed the expression of Bcrp1 in primary bronchial smooth muscle cell cultures (BSMC) and in lavaged distal airway cells, but neither possessed the capacity to efflux Hoechst dye. In BSMC, Bcrp1 was localized to an intracellular compartment, suggesting that the molecular site of Bcrp1 expression regulates SP phenotype.
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Sun L, Sun T T and Lavker R M. CLED: a calcium-linked protein associated with early epithelial differentiation. *Exp Cell Res* 2000;1:96-106

Although it has been well established that Ca(2+) plays a key role in triggering keratinocyte differentiation, relatively little is known about the molecules that mediate this signaling process. By analyzing a bovine corneal epithelial subtraction cDNA library, we have identified a novel gene that we named CLED (calcium-linked epithelial differentiation), which encodes a messenger RNA present in all stratified squamous epithelia, hair follicle, the bladder transitional epithelium, and small intestinal epithelium. The deduced amino acid sequence of CLED, based on a bovine partial cDNA and its full-length, human and mouse homologues that have been described only as ESTs, contains 2 EF-hand Ca(2+)-binding domains, a myristoylation motif, and several potential protein kinase phosphorylation sites; the CLED protein is therefore related to the S100 protein family. In all stratified squamous epithelia, the CLED message is associated with the intermediate cell layers. Similar CLED association with cells that are above the proliferative compartment but below the terminally differentiated compartment is seen in hair follicle, bladder, and small intestinal epithelia. The only exception is corneal epithelium, where CLED is expressed in both basal and intermediate cells. The presence of CLED in corneal epithelial basal cells, but not in the adjacent limbal basal (stem) cells, provides additional, strong evidence for the unique lateral heterogeneity of the limbal/corneal epithelium. These results suggest that CLED, via Ca(2+)-related mechanisms, may play a role in the epithelial cell's commitment to undergo early differentiation, and that its down-regulation is required before the cells can undergo the final stages of terminal differentiation.
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Sun T T, Tseng S C and Lavker R M. Location of corneal epithelial stem cells. *Nature* 2010;7284:E10-1; discussion E1

The longstanding concept that corneal epithelial stem cells reside mainly in the limbus is supported by the absence of major corneal epithelial differentiation markers, that is, K3 and K12 keratins, in limbal basal cells (these markers are expressed, however, in corneal basal cells, thus distinguishing the mode of keratin expression in corneal epithelium from that of all other stratified epithelia), the centripetal migration of corneal epithelial cells, the exclusive location of slow-cycling cells in the limbal basal layer, the superior in vitro proliferative potential

of limbal epithelial cells, and the transplanted limbal cells' ability to reconstitute corneal epithelium in vivo (reviewed in refs 1-4). Moreover, previous data indicate that corneal and conjunctival epithelia represent two separate cell lineages (reviewed in refs 1-4). Majo et al. suggested, however, that corneal and conjunctival epithelia are equipotent, and that identical oligopotent stem cells are present throughout the corneal, limbal and conjunctival epithelia. We point out here that these suggestions are inconsistent with many known growth, differentiation and cell migration properties of the anterior ocular epithelia.
Department of Cell Biology, New York University School of Medicine, New York, New York 10016, USA. sunt01@nyumc.org

Takahashi K and Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006;4:663-76

Differentiated cells can be reprogrammed to an embryonic-like state by transfer of nuclear contents into oocytes or by fusion with embryonic stem (ES) cells. Little is known about factors that induce this reprogramming. Here, we demonstrate induction of pluripotent stem cells from mouse embryonic or adult fibroblasts by introducing four factors, Oct3/4, Sox2, c-Myc, and Klf4, under ES cell culture conditions. Unexpectedly, Nanog was dispensable. These cells, which we designated iPS (induced pluripotent stem) cells, exhibit the morphology and growth properties of ES cells and express ES cell marker genes. Subcutaneous transplantation of iPS cells into nude mice resulted in tumors containing a variety of tissues from all three germ layers. Following injection into blastocysts, iPS cells contributed to mouse embryonic development. These data demonstrate that pluripotent stem cells can be directly generated from fibroblast cultures by the addition of only a few defined factors.
Department of Stem Cell Biology, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507, Japan.

Takeuchi J K and Bruneau B G. Directed transdifferentiation of mouse mesoderm to heart tissue by defined factors. *Nature* 2009;7247:708-11

Heart disease is the leading cause of mortality and morbidity in the western world. The heart has little regenerative capacity after damage, leading to much interest in understanding the factors required to produce new cardiac myocytes. Despite a robust understanding of the molecular networks regulating cardiac differentiation, no single transcription factor or combination of factors has been shown to activate the cardiac gene program de novo in mammalian cells or tissues. Here we define the minimal requirements for transdifferentiation of mouse mesoderm to cardiac myocytes. We show that two cardiac transcription factors, Gata4 and Tbx5, and a cardiac-specific subunit of BAF chromatin-remodelling complexes, Baf60c (also called Smarcd3), can direct ectopic differentiation of mouse mesoderm into beating cardiomyocytes, including the normally non-cardiogenic posterior mesoderm and the extraembryonic mesoderm of the amnion. Gata4 with Baf60c initiated ectopic cardiac gene expression. Addition of Tbx5 allowed differentiation into contracting cardiomyocytes and repression of non-cardiac mesodermal genes. Baf60c was essential for the ectopic cardiogenic activity of Gata4 and Tbx5, partly by permitting binding of Gata4 to cardiac genes, indicating a novel instructive role for BAF complexes in tissue-specific regulation. The combined function of these factors establishes a robust mechanism for controlling cellular differentiation, and may allow reprogramming of new cardiomyocytes for regenerative purposes.
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Thepot A, Morel A P, Justin V, Desanlis A, Thivillier L, Hoffman E, Till M, Accardi R, Tommasino M, Breton P, Hainaut P and Damour O. Evaluation of tumorigenic risk of tissue-engineered oral mucosal epithelial cells by using combinational examinations. *Cell Transplant* 2010;8:999-1006

Recently, oral mucosal epithelial cells were proposed as a cell source of the autologous cell transplant therapy for corneal trauma or disease. The question addressed is to know if the biological conditions of grafting could induce certain cellular, molecular, and genetic alterations that might increase the risk of mutations and possibly of cellular transformation. Recent progress in cancer research enables us to depict the generation mechanisms and basic characteristics of human cancer cells from molecular, cytological, and biological aspects. The aim of this study is to evaluate the risk of tumorigenicity of the oral mucosal epithelial culture process in order to mitigate that risk, if any, before clinical application. Oral mucosal epithelial cells from three different human donors were investigated by combinational examinations to detect possible tumorigenic transformation. We investigated (i) clonogenic and karyology types, (ii) the validation of proliferation rate, (iii) the epithelial-mesenchymal transition, (iv) anchorage-independent growth potential, and (v) tumorigenicity on nude mice. Results show that the culture process used in this study presents no risk of tumorigenicity.
Banque de Tissus et Cellules, Hopital E. Herriot, Lyon Cedex 03, France.

Thoft R A and Friend J. The X, Y, Z hypothesis of corneal epithelial maintenance. *Invest Ophthalmol Vis Sci* 1983;10:1442-3

Thoft R A and Sugar J. Graft failure in keratoepithelioplasty. *Cornea* 1993;4:362-5

We report three patients undergoing keratoepithelioplasty using donor limbal tissue. All three had significant vascularization of the recipient bed and all three had inflammatory reactions with apparent partial replacement of the donor epithelium with host epithelium. Limbal transplantation, which is likely to include significant numbers of donor Langerhans' cells, may well increase the risk of graft failure in keratoepithelioplasty.
Eye and Ear Institute of Pittsburgh, University of Pittsburgh, Pennsylvania.

Thompson R W, Jr., Price M O, Bowers P J and Price F W, Jr. Long-term graft survival after penetrating keratoplasty. *Ophthalmology* 2003;7:1396-402

PURPOSE: To determine long-term graft survival rates and causes of secondary graft failures for a large series of penetrating keratoplasties (PKPs). DESIGN: Retrospective, noncomparative case series. PARTICIPANTS: Longitudinal review of 3992 consecutive eyes that underwent PKP at a large tertiary care referral center from 1982 through 1996. Data were collected retrospectively from August 1982 through December 1988 and prospectively thereafter. INTERVENTION: Three thousand six hundred forty primary grafts and 352 regrafts. MAIN OUTCOME MEASURES: Corneal graft survival and etiology of graft failures. Patients were evaluated preoperatively and at 1, 3, 6, 9, 12, 18, and 24 months after transplant, then at yearly intervals. RESULTS: Mean recipient age was 67 years (range, 1-98 years). The predominant indications for PKP were pseudophakic bullous keratopathy (32%) and Fuchs' dystrophy (23%). Graft failure occurred in 10% (385) of the eyes. The most common causes of secondary graft failure were endothelial failure (29%) or immunologic endothelial rejection (27%). Survival of first time grafts was 90% at 5 years and 82% at 10 years. Initial regrafts had significantly lower 5-year and 10-year survival rates, 53% and 41%, respectively. The highest 5-year and 10-year survival rates were noted in primary grafts for eyes with a preoperative diagnosis of keratoconus (97% and 92%, respectively), or Fuchs' dystrophy (97% and 90%, respectively). Primary grafts for aphakic bullous keratopathy without intraocular lens placement had the lowest 5-year survival rate, 70%. CONCLUSIONS: The 5-year and 10-year survival rates in this series demonstrate that PKP is a safe and effective treatment for the corneal diseases commonly transplanted in the United States. However, endothelial failure and immunologic graft rejection were persistent risks over the long term, supporting the need for continued patient follow-up. Regrafts, aphakic eyes without intraocular lens placement at the time of transplant, and corneas with deep stromal vascularization had reduced graft survival rates. Pseudophakic bullous keratopathy grafts with a retained posterior chamber intraocular lens were at increased risk of endothelial failure compared with primary grafts done for other causes or compared with pseudophakic bullous keratopathy grafts done with intraocular lens exchange.
Price Vision Group, Indianapolis, Indiana, USA.

Thomson J A, Itskovitz-Eldor J, Shapiro S S, Waknitz M A, Swiergiel J J, Marshall V S and Jones J M. Embryonic stem cell lines derived from human blastocysts. *Science* 1998;5391:1145-7

Human blastocyst-derived, pluripotent cell lines are described that have normal karyotypes, express high levels of telomerase activity, and express cell surface markers that characterize primate embryonic stem cells but do not characterize other early lineages. After undifferentiated proliferation in vitro for 4 to 5 months, these cells still maintained the developmental potential to form trophoblast and derivatives of all three embryonic germ layers, including gut epithelium (endoderm); cartilage, bone, smooth muscle, and striated muscle (mesoderm); and neural epithelium, embryonic ganglia, and stratified squamous epithelium (ectoderm). These cell lines should be useful in human developmental biology, drug discovery, and transplantation medicine.
Wisconsin Regional Primate Research Center, University of Wisconsin, Madison, WI 53715, USA.

Toma J G, Akhavan M, Fernandes K J, Barnabe-Heider F, Sadikot A, Kaplan D R and Miller F D. Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 2001;9:778-84

We describe here the isolation of stem cells from juvenile and adult rodent skin. These cells derive from the dermis, and clones of individual cells can proliferate and differentiate in culture to produce neurons, glia, smooth muscle cells and adipocytes. Similar precursors that produce neuron-specific proteins upon differentiation can be isolated from adult human scalp. Because these cells (termed SKPs for skin-derived precursors) generate both neural and mesodermal progeny, we propose that they represent a novel multipotent adult stem cell and suggest that skin may provide an accessible, autologous source of stem cells for transplantation.
Center for Neuronal Survival, Montreal Neurological Institute, McGill University, 3801 rue University, Montreal, Quebec, Canada H3A 2B4.

Townsend W M. The limbal palisades of Vogt. *Trans Am Ophthalmol Soc* 1991;721-56

Trounson A O. The derivation and potential use of human embryonic stem cells. *Reprod Fertil Dev* 2001;7-8:523-32

Human embryonic stem cells lines can be derived from human blastocysts at high efficiency (>50%) by immunosurgical isolation of the inner cell mass and culture on embryonic fibroblast cell lines. These cells will spontaneously differentiate into all the primary embryonic lineages in vitro and in vivo, but they are unable to form an integrated embryo or body plan by themselves or when combined with trophectoderm cells. They may be directed into a number of specific cell types and this enrichment process requires specific growth factors, cell-surface molecules, matrix molecules and secreted products of other cell types. Embryonic stem (ES) cells are immortal and represent a major potential for cell therapies for regenerative medicine. Their use in transplantation may depend on the formation of a large bank of suitable human leucocyte antigen (HLA) types or the genetic erasure of their HLA expression. Successful transplantation may also require induction of tolerance in recipients and ongoing immune suppression. Although it is possible to customize ES cells by therapeutic cloning or cytoplasmic transfer, it would appear unlikely that these strategies will be used extensively for producing ES cells compatible for transplantation. Embryonic stem cell research may deliver a new pathway for regenerative medicine.
Institute of Reproduction and Development, Monash University, Clayton, Victoria, Australia.

Tsai R J, Li L M and Chen J K. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N Engl J Med* 2000;2:86-93

BACKGROUND: Stevens-Johnson syndrome, ocular pemphigoid, and thermal or chemical burns can cause scarring and opacification of the cornea and loss of vision. Transplantation of epithelial cells from the limbus of the contralateral cornea can restore useful vision. However, this procedure requires a large limbal graft from the healthy eye and is not possible in patients who have bilateral lesions. METHODS: We took specimens of limbal epithelial cells from the healthy contralateral eyes of six patients with severe unilateral corneal disease. The epithelial cells were cultured and expanded on amniotic membrane. The amniotic membrane, together with the sheet of limbal epithelial cells, was transplanted to the denuded corneal surface of the damaged eye after superficial keratectomy to remove fibrovascular ingrowth. The mean (+/-SD) follow-up period was 15+/-2 months. RESULTS: Complete reepithelialization of the corneal surface occurred within two to four days of transplantation in all six eyes receiving transplants. By one month, the ocular surface was covered with corneal epithelium, and the clarity of the cornea was improved. In five of the six eyes receiving transplants (83 percent), the mean visual acuity improved from 20/112 to 20/45. In one patient with a chemical burn who had total opacification of the cornea, the acuity improved from the ability to count fingers at 40 cm to 20/200. No patient had recurrent neovascularization or inflammation in the transplanted area during the follow-up period. CONCLUSIONS: Transplantation of autologous limbal epithelial cells cultured on amniotic membrane is a simple and effective method of reconstructing the corneal surface and restoring useful vision in patients with unilateral deficiency of limbal epithelial cells.
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Tseng S C. Concept and application of limbal stem cells. *Eye (Lond)* 1989;141-57

Cumulative reported evidence indicates that some fraction of limbal basal epithelial cells are the stem cells for corneal epithelial cell proliferation and differentiation. Limbal epithelium is therefore crucial in maintaining the cell mass of corneal epithelium under normal conditions and plays an important role in corneal epithelial wound healing. Deficiency or absence of limbal stem cells explains well the pathogenesis of several ocular surface disorders characterised by defective conjunctival transdifferentiation or conjunctivalisation of cornea. This paper reviews and updates the basic concept of stem cells, the reported findings of limbal stem cells for corneal epithelium, and their therapeutic applications. Through this review, one hopes to gain a more complete understanding and increase proficiency in treating these diseases.
Department of Ophthalmology, Bascom Palmer Eye Institute, University of Miami School of Medicine, Florida 33101.

Tseng S C. Move stem cells from the mouth to the eye. *Am J Ophthalmol* 2006;2:356-7

Tseng S C, Kruse F E, Merritt J and Li D Q. Comparison between serum-free and fibroblast-cocultured single-cell clonal culture systems: evidence showing that epithelial anti-apoptotic activity is present in 3T3 fibroblast-conditioned media. *Curr Eye Res* 1996;9:973-84

PURPOSE: To compare the supporting mechanism between the serum-free and the fibroblast-cocultured single-cell clonal culture systems. METHODS: Clonal growth, measured by colony forming efficiency (CFE) and size, was compared between rabbit corneal and limbal epithelial cells in a previously-established serum-free MCDB medium supplemented with growth factors, and in a coculture system with a feeder layer of mitomycin C-treated mouse 3T3 fibroblasts grown in the MCDB or DMEM medium plus 20% fetal bovine serum (FBS). RESULTS: Limbal epithelial cells in the serum-free MCDB medium had a significantly lower CFE than corneal epithelial cells ($p < 0.001$), suggesting that this system promoted more clonal growth of corneal progenitor cells. In contrast, with cocultured 3T3 fibroblasts limbal CFE was significantly increased ($p < 0.001$), while corneal CFE was not changed, indicating that the 3T3 system promoted more clonal growth of limbal progenitor cells. Addition of 20% FBS in the MCDB medium cocultured with 3T3 fibroblasts significantly promoted both limbal and corneal CFEs ($p < 0.001$). For both cultures, switching the serum-containing MCDB medium to the serum-containing DMEM medium produced clonal growth only with cocultured fibroblasts. This epithelial growth-promoting activity was not present on the cell surface or in the extracellular matrix, but present in pre-centrifuged and prefiltered 3T3 fibroblast-conditioned media. Both growth-promoting and anti-apoptotic activities were present in fibroblast-derived serum-free conditioned media. In the presence of this anti-apoptotic activity, serum addition promoted clonal growth, and the

expression of cornea-type K3 keratin in limbal colonies was negative using AE-5 monoclonal antibody. CONCLUSIONS: Further purification and characterization of this fibroblast-derived anti-apoptotic survival factor will facilitate understanding of the mechanism by which epithelial stem cells are regulated via epithelial-mesenchymal interactions.
Department of Ophthalmology, Bascom Palmer Eye Institute, William L. McKnight Vision Research Center, Miami, FL 33136, USA.

Tseng S C, Li D Q and Ma X. Suppression of transforming growth factor-beta isoforms, TGF-beta receptor type II, and myofibroblast differentiation in cultured human corneal and limbal fibroblasts by amniotic membrane matrix. *J Cell Physiol* 1999;3:325-35

Down-regulation of the transforming growth factor-beta (TGF-beta) signaling system is a strategy for preventing scarring during wound healing. Human corneal and limbal fibroblasts were cultured on the stromal matrix side of preserved human amniotic membrane. The levels of TGF-beta1, beta2, and beta3 and TGF-beta type II receptor transcripts and TGF-beta1 and beta2 proteins were suppressed as early as 8 hr and more dramatically at 24 hr after contact with an amniotic membrane. This suppressive effect was accompanied by down-regulation of alpha-smooth muscle actin, EDA spliced form of fibronectin, and integrin alpha5. It persisted even when challenged by 10 ng/ml TGF-beta1. In contrast with their counterparts grown on plastic or in collagen gel, such suppression in amniotic membrane cultures remained complete after 1 week of culturing. Cells cultured on amniotic membrane showed significantly reduced [3H]-thymidine incorporation compared to cells cultured on plastic and displayed no DNA fragmentation. These results reveal a novel mechanism by which the TGF-beta signaling system, DNA synthesis, and subsequent myofibroblast differentiation can be suppressed by an amnionic membrane matrix. This action explains in part the antiscarring results of amniotic membrane transplantation used for ocular surface reconstruction, a surgical technique applicable to other subspecialties. It may also explain in part why fetal wound healing is scarless.
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Tseng S C, Prabhasawat P, Barton K, Gray T and Meller D. Amniotic membrane transplantation with or without limbal allografts for corneal surface reconstruction in patients with limbal stem cell deficiency. *Arch Ophthalmol* 1998;4:431-41

OBJECTIVE: To examine whether amniotic membrane transplantation (AMT), in preparing the perilimbal stroma, enhances the success of allograft limbal transplantation (ALT). METHODS: Thirty-one eyes of 26 consecutive patients had cytologically proven limbal deficiency resulting from chemical burns (14 eyes); Stevens-Johnson syndrome, toxic epidermal necrolysis, or pseudopemphigoid (5 eyes); contact lens-induced keratopathy (3 eyes); aniridia (3 eyes); multiple surgical procedures (2 eyes); atopy (2 eyes); or an unknown cause (2 eyes). Based on the severity of limbal deficiency, group A (mild), comprising 10 eyes, received AMT alone; group B (moderate), comprising 7 eyes, received AMT and ALT; and group C (severe), comprising 14 eyes, received AMT, ALT, and penetrating keratoplasty. All patients except those in group A received continuous oral cyclosporine. RESULTS: Except for the 2 eyes with atopy, all amniotic membrane-covered surfaces showed rapid epithelialization (in 2 to 4 weeks) and reduced inflammation, vascularization, and scarring, and the surfaces became smooth and wettable. For the mean follow-up period of 15.4 months, 25 (83%) of 30 eyes showed visual improvement, consisting of 6 or more lines (13 eyes), 4 to 5 lines (6 eyes), or 1 to 3 lines (6 eyes). Visual improvement decreased with the severity of limbal deficiency from 8 (100%) of 8 eyes in group A to 5 (71%) of 7 eyes in group B and 11 (79%) of 14 eyes in group C. In group C, corneal graft rejection occurred in 9 (64%) of 14 eyes, and reversible early limbal allograft rejection was noted in 3 (14%) of 21 eyes of groups B and C. CONCLUSIONS: For partial limbal deficiency with superficial involvement, AMT alone is sufficient and hence superior to ALT because there is no need to administer cyclosporine. For total limbal deficiency, additional ALT is needed, and AMT helps reconstruct the perilimbal stroma, with reduced inflammation and vascularization, which collectively may enhance the success of ALT.
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Tsubota K, Toda I, Saito H, Shinozaki N and Shimazaki J. Reconstruction of the corneal epithelium by limbal allograft transplantation for severe ocular surface disorders. *Ophthalmology* 1995;10:1486-96

PURPOSE: Although penetrating keratoplasty is an established surgical procedure, it often is ineffective for severe ocular surface diseases such as alkali burns or limbal deficiency. The authors have performed limbal allograft transplantation for the reconstruction of the corneal epithelium. METHODS: A total of nine patients (3 with chemical injury, 3 with limbal deficiency with unknown etiology, 2 with moderate ocular pemphigoid [OCP], and 1 with traumatic limbal deficiency) were treated by limbal allograft transplantation. Penetrating keratoplasties were performed in all patients with the exception of one with OCP. Patients received cyclosporine both systemically (10 mg/kg) and topically (0.05%) as well as high-dose intravenous dexamethasone (8 mg). RESULTS: The corneal epithelium was reconstructed in all patients, although two showed partial increased fluorescein permeability and two others required a second surgery. The other five epithelia remained clear at mean follow-up of 12.3 months, with two episodes of graft rejection which were controlled successfully by medication. CONCLUSIONS: Limbal allograft transplantation with intensive immunosuppression by cyclosporine and high-dose steroids appears to be a promising surgical intervention for the reconstruction of corneas affected by severe ocular surface disease.
Department of Ophthalmology, Tokyo Dental College, Chiba, Japan.

Turgeon P W, Nauheim R C, Roat M I, Stopak S S and Thoft R A. Indications for keratoepithelioplasty. *Arch Ophthalmol* 1990;2:233-6

Thirteen patients with ocular surface failure were treated by keratoepithelioplasty using allografts of corneal limbal epithelial cells from donor eyes. The ocular surface was stabilized with long-term healing of persistent epithelial defects in five of eight eyes followed up for 4 to 19 months. The procedure was performed on an additional 5 patients with superficial keratopathies. Three of those five procedures resulted in a stable and clear optical surface. These results suggest that epithelial transplantation may be a useful option in the care of chronic ocular surface failure unresponsive to conventional medical management.
Eye and Ear Institute, University of Pittsburgh, PA 15213.

Wagers A J and Weissman I L. Plasticity of adult stem cells. *Cell* 2004;5:639-48

Recent years have seen much excitement over the possibility that adult mammalian stem cells may be capable of differentiating across tissue lineage boundaries, and as such may represent novel, accessible, and very versatile effectors of therapeutic tissue regeneration. Yet studies proposing such "plasticity" of adult somatic stem cells remain controversial, and in general, existing evidence suggests that in vivo such unexpected transformations are exceedingly rare and in some cases can be accounted for by equally unexpected alternative explanations.
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Walker M R, Patel K K and Stappenbeck T S. The stem cell niche. *J Pathol* 2009;2:169-80

Virtually every tissue of the adult organism maintains a population of putatively slowly-cycling stem cells that maintain homeostasis of the tissue and respond to injury when challenged. These cells are regulated and supported by the surrounding microenvironment, referred to as the stem cell 'niche'. The niche includes all cellular and non-cellular components that interact in order to control the adult stem cell, and these interactions can often be broken down into one of two major mechanistic categories--physical contact and diffusible factors. The niche has been studied directly and indirectly in a number of adult stem cell systems. Herein, we will first focus on the most well-understood niches supporting the germline stem cells in the lower organisms Caenorhabditis elegans and Drosophila melanogaster before concentrating on the more complex, less well-understood mammalian niches supporting the neural, epidermal, haematopoietic and intestinal stem cells.
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Warrington A E and Pfeiffer S E. Proliferation and differentiation of O4+ oligodendrocytes in postnatal rat cerebellum: analysis in unfixed tissue slices using anti-glycolipid antibodies. *J Neurosci Res* 1992;2:338-53

We report the study of the in vivo morphology, differentiation, and proliferation of oligodendrocytes (OLs) and their progenitors identified by the antiglycolipid antibodies O4, R-mAb, and O1 in postnatal rat cerebellum, using a novel immunocytochemical staining protocol which allows the analysis of the expression of OL-specific glycolipids in live, unfixed brain slices. An analysis of the individual cells identified in double label immunocytochemistry indicated that the order of antigen expression in OLs during in vivo development is, first, antigens recognized by O4, second, antigens recognized R-mAb, and third, antigens recognized by O1. This order of antigen expression is correlated with increasing morphological complexity and is a pattern mimicked in many culture systems. In vivo O4 identified 3 distinct stages of the OL lineage: (1) morphologically simple proligodendrocyte antigen+ (POA+) R-mAb- blast cells localized at the leading edge of myelinogenesis; (2) morphologically more complex R-mAb+O1- cells; and (3) actively myelinating O1+ [i.e., galactocerebroside+ (GalC)] OLs residing within the white matter. Only the POA+R-mAb- cells incorporated BrdU in animals that were prelabeled 3 hr before immunocytochemistry. We have demonstrated in vivo the subdivision of pre-GalC+ OL progenitors into shorter, biologically noteworthy, stages of maturation. A spatial comparison of the cell populations identified by O4, R-mAb, and O1 demonstrated a progressive wave of OL maturation from the base of the cerebellum toward the folia. The data are consistent with the hypothesis that multiprocessed O4+GalC- progenitors are the most mature stage of the OL lineage with significant proliferative capacity and the first postmigratory stage in normal development.
Department of Microbiology, University of Connecticut School of Medicine, Farmington 06030.

Wencan W, Mao Y, Wentao Y, Fan L, Jia Q, Qinmei W and Xiangtian Z. Using basement membrane of human amniotic membrane as a cell carrier for cultivated cat corneal endothelial cell transplantation. *Curr Eye Res* 2007;3:199-215

PURPOSE: To study the feasibility of using basement membrane of human amniotic membrane (BMHAM) as a carrier for transplantation of cultivated cat corneal endothelial cells (cCCECs). METHODS: BHMAM was obtained by enzymic digestion. cCCECs were seeded on the BMHAM and cultivated traditionally. The resulting continuous monolayer of cCCECs was transplanted onto the cat corneal graft stripped of the Descemet membrane with endothelium. To determine whether the transplanted cCCECs were vital and functional in vivo, the corneal grafts were examined by slit-lamp microscope every day for 6 weeks, and corneal thickness was measured by ultrasonic pachymetry. Either in vivo or in vitro, the cCCEC sheets on BMHAMs were examined morphologically by light and electron microscope, and the cell density was measured. RESULTS: Seven to 10 days after seeding on the BMHAM, the cCCECs were confluent and formed a continuous monolayer with 3486 +/- 53 cells/mm(2) cell density. Like normal corneal endothelial cells, the cCCECs were almost hexagonal, squamous, and uniform in size. After transplantation, most cells were vital and functional nearly enough to maintain corneal graft thickness and transparency without rejection for at least 6 weeks. Six weeks after operation, the average thickness of the transplanted corneal grafts was only slightly greater than that before operation. Compared with that in vitro, after transplantation there was 5% to 8% reduction per week in cell density, which lasted for almost 3 weeks. After that, the average cCCEC density of corneal grafts was 2837 +/- 57 cells/mm(2) and quite stable maintained. CONCLUSIONS: This study demonstrated that BMHAM would be an ideal alternative for corneal Descemet membrane and a cell carrier for cCCEC transplantation.
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Whikehart D R, Parikh C H, Vaughn A V, Mishler K and Edelhauser H F. Evidence suggesting the existence of stem cells for the human corneal endothelium. *Mol Vis* 2005;816-24

PURPOSE: The well-known reluctance of human corneal endothelial cells (HCECs) to divide has continually intrigued investigators. Related to this, the discovery of an increased endothelial cell population in the periphery of the human cornea has prompted an investigation for evidence of the existence of stem-like cells in the endothelial periphery. Showing that stem cells or transient amplifying cells may exist in the periphery might explain the origin of HCECs and indicate a source for these cells in wound repair. In addition, these cells might be of value in culturing or as a source for the synthesis of artificial corneas. METHODS: Human corneas with attached scleral rims were obtained from eye banks and were assayed for telomerase activity and BrdU (bromodeoxyridine) incorporation to determine, respectively, the presence of a stem-like cell marker and replicative activity. In the case of telomerase activity, the tissues were divided into central, intermediate and peripheral areas by the use of trephines. BrdU staining (using alkaline phosphatase bound secondary antibody) was performed on whole corneas plus scleral rims exposed to BrdU antibodies on the endothelial side whereas BrdU fluorescence (using fluorescein bound secondary antibody) was obtained from transverse sections of the these tissues by the same procedure. Some corneas were wounded to determine whether the wounded areas stimulated BrdU (by staining or fluorescence) followed by the synthesis of transforming growth factor beta (TGFbeta). The latter was determined by quantitative ELISA. Rabbit corneas were also assayed for BrdU incorporation to compare their evidence of cell division with that of humans. RESULTS: After dividing corneas into central, intermediate, and peripheral sections, the dissected endothelial tissues exhibited positive telomerase activity in the peripheral and intermediate sections. No activity was observed in the central endothelial tissues or the limbus between the trabecular meshwork and Schwalbe's line. BrdU staining with alkaline phosphatase was occasionally observed in the wounded area's human corneal endothelial cells after wounding. When BrdU fluorescence assays were made on corneal transverse sections with fluorescein, fluorescence occurred in an area just at and adjacent to the trabecular meshwork, but was not seen at the corneal endothelium. After wounding, BrdU fluorescence extended into the corneal endothelium. TGF-beta levels were increased in fluids bathing the endothelium following wounding, but the increases lagged behind the wounding event. CONCLUSIONS: It is suggested stem-like cells may be sequestered in a niche at the junctional region where the corneal endothelial cells and the trabecular meshwork come together. These putative stem cells may supply new cells for both the corneal endothelium and the trabeculae. Evidence suggests that cells from this area migrate (perhaps as transient amplifying cells) to the endothelial periphery and, perhaps, to wounded areas of the corneal endothelium when needed. The migration may not be constant and may be age dependent.
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Williams M A, Gawley S D, Jackson A J and Frazer D G. Traumatic graft dehiscence after penetrating keratoplasty. *Ophthalmology* 2008;2:276-8 e1

OBJECTIVE: To determine the incidence and to explore the risk factors for traumatic graft dehiscence after penetrating keratoplasty. DESIGN: Retrospective case note review. PARTICIPANTS: Five hundred seventy-two consecutive cases were included. INTERVENTION: All subjects who underwent penetrating keratoplasty in 1 regional center between 1992 and 2004 inclusive. MAIN OUTCOME MEASURES: Cases that experienced postoperative traumatic graft dehiscence were identified. Results from 12 other similar studies were pooled for comparison. RESULTS: Fifteen eyes (2.6%) were treated for traumatic wound dehiscence after penetrating keratoplasty. The most striking feature of this series was the bimodal relationship of age and cause of graft dehiscence, with older patients involved in falls and younger patients in accidental or deliberate trauma. Factors that may influence the risk of traumatic graft dehiscence are discussed, in the light of the present findings and pooled data from previous series. CONCLUSIONS: This case series indicates that there is long-term risk of traumatic wound dehiscence after penetrating keratoplasty. Younger patients, especially males, should be made aware that their eye, after keratoplasty, will always be vulnerable to injury. High-risk situations should be avoided if possible. Older patients at particular risk should have adequate risk reduction strategies, social support, and supervision, in particular to minimize the risk of falls.
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Wilmut I, Schnieke A E, McWhir J, Kind A J and Campbell K H. Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997;6619:810-3

Fertilization of mammalian eggs is followed by successive cell divisions and progressive differentiation, first into the early embryo and subsequently into all of the cell types that make up the adult animal. Transfer of a single nucleus at a specific stage of development, to an enucleated unfertilized egg, provided an opportunity to investigate whether cellular differentiation to that stage involved irreversible genetic modification. The first offspring to develop from a differentiated cell were born after nuclear transfer from an embryo-derived cell line that had been induced to become quiescent. Using the same procedure, we now report the birth of live lambs from three new cell populations established from adult mammary gland, fetus and embryo. The fact that a lamb was derived from an adult cell confirms that differentiation of that cell did not involve the irreversible modification of genetic material required for development to term. The birth of lambs from differentiated fetal and adult cells also reinforces previous speculation that by inducing donor cells to become quiescent it will be possible to obtain normal development from a wide variety of differentiated cells.
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Wilson S E, Weng J, Blair S, He Y G and Lloyd S. Expression of E6/E7 or SV40 large T antigen-coding oncogenes in human corneal endothelial cells indicates regulated high-proliferative capacity. *Invest Ophthalmol Vis Sci* 1995;1:32-40

PURPOSE: Human corneal endothelial cells are thought to have limited capacity for proliferation. Little is known about the mechanisms that regulate the proliferation of these cells. The authors introduced oncogenes into human corneal endothelial cells to modulate proliferation. In addition, they sought to establish cell lines to facilitate study of human corneal endothelial cells. METHODS: Early-passage human corneal endothelial cells were transduced with disabled retrovirus (pLXSN16E6/E7) coding for the human papilloma virus type 16 transforming oncoproteins E6 and E7. Early-passage cells were also stably transfected by electroporation with the pMTV-D305 plasmid vector, in which SV40 large T antigen (SV40 LTAg) mRNA expression is positively regulated by the mouse mammary tumor virus promoter. Expression of E6/E7 mRNA or SV40 LTAg mRNA in cell lines was monitored with the polymerase chain reaction. SV40 LTAg protein expression was detected by immunocytology and Western blot analysis. RESULTS: Human corneal endothelial cells were efficiently infected with disabled retrovirus coding for E6/E7, and seven strains of cells have continued active proliferation for more than 50 population doublings (PD) (< 8 control PD). E6/E7 mRNA was expressed by each cell strain. E6/E7 transformed cells proliferate rapidly and form a monolayer of cells with a high degree of contact inhibition. Transfection with pMTV-D305 is less efficient, and only a single strain was developed. pMTV-D305-transfected endothelial cells (dexamethasone induced) proliferated at a lower rate than E6/E7-transduced cells or cells transfected with a vector (pSV3neo) in which SV40 LTAg is constitutively regulated. In the absence of dexamethasone, the proliferation of pMTV-D305-transfected cells was even slower, but cells continued to produce SV40 LTAg mRNA and protein. The latter results indicated that SV40 LTAg mRNA continued to be synthesized at significant levels in pMTV-D305-transfected cells in the absence of the inducer dexamethasone. CONCLUSIONS: This study suggests that human corneal endothelial cells have a high capacity for proliferation. Thus, cell division is normally controlled in human corneal endothelial cells by poorly characterized, but efficient, mechanisms. Because the E6 and E7 proteins, as well as the SV40 large T antigen, specifically bind to and interfere with the activity of the retinoblastoma (RB) and p53 tumor suppressor proteins, our results suggest that these proteins have critical roles in regulating the proliferation of human corneal endothelial cells.
Department of Ophthalmology, University of Texas Southwestern Medical Center at Dallas 75235.

Wolf X A, Rasmussen M A, Schauser K, Jensen A T, Schmidt M and Hyttel P. OCT4 expression in outgrowth colonies derived from porcine inner cell masses and epiblasts. *Reprod Domest Anim* 2011;3:385-92

The present study was conducted to test different methods for porcine inner cell mass (ICM) and epiblast isolation and to evaluate the morphology and expression of pluripotency genes in ICM- and epiblast-derived outgrowth colonies (OCs) and passages thereof with particular attention on the relationship between OCT4 expression and embryonic stem cell (ESC)-like morphology. A total of 104 zona pellucida-enclosed and 101 hatched blastocysts were subjected to four different methods of ICM and epiblast isolation, respectively: Manual isolation, immunosurgery, immunosurgery with manual cleaning, or whole blastocyst culture. OCs were established on mouse embryonic fibroblast (MEF) cells and categorized according to morphology and OCT4 staining. Although all isolation methods resulted in ESC-like OCs, immunosurgery with manual cleaning yielded significantly higher rates of ICM/epiblast attachment and subsequent ESC-like morphology, whereas no significant difference was found between ICM and epiblasts with respect to these characteristics. All ESC-like OCs showed nuclear OCT4 staining and expression of OCT4, NANOG and SOX2 as evaluated by RT-PCR. Upon initial passages, the expression of pluripotency markers was, however, gradually lost in spite of maintained ESC-like morphology. In conclusion, we have established a robust system for derivation of ESC-like OCs from porcine ICM and epiblasts and we have shown that localization of OCT4 is associated with an ESC-like morphology although this relationship is lost during early passages.
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Wollensak G and Green W R. Analysis of sex-mismatched human corneal transplants by fluorescence in situ hybridization of the sex-chromosomes. *Exp Eye Res* 1999;3:341-6

The fate of the cells of corneal transplants has been controversial from the early days of keratoplasty. Various methods such as histological evaluation, radiolabeling of donor cells or Barr-body analysis have been applied to clarify the issue. However, the question whether the transplanted cells are replaced or survive, remains unsolved. In this study, we applied fluorescence in situ hybridization (FISH) of the X- and Y-chromosomes in paraffin sections of explanted sex-mismatched corneal transplants to distinguish between host and donor cells. Fourteen sex-mismatched cases with various reasons for explantation and different postoperative time intervals ranging from 11 months to 30 years were analysed. We found that all cell types, including epithelium, keratocytes and endothelial donor cells were replaced in most cases as early as 1 year after transplantation. In three cases, however, up to 26% of donor keratocytes were still detected up to 4.5 years after transplantation, demonstrating a certain individual variability in the process of replacement. Further studies must show if the extent and timing of donor cell replacement in clinically successful, totally clear transplants is different. Our results are in keeping with the phenomenon of recurrences of corneal dystrophies in the graft, the significant postoperative decline of the endothelial cell density, the fact that typical graft rejections usually take place within 1-2 years postoperatively and that relatively late rejections can occur in rare cases probably due to some surviving stromal keratocytes. Donor cell replacement is a special feature of corneal transplants when compared with other kinds of organ transplants and might be due to the presence of the same tissue type in the immediate neighbourhood of the graft.
Eye Pathology Lab, Wilmer Eye Institute, Johns Hopkins Hospital, Baltimore, MD 21287-9248, USA.

Wu X Z. Origin of cancer stem cells: the role of self-renewal and differentiation. *Ann Surg Oncol* 2008;2:407-14

BACKGROUND: Self-renewal and differentiation potential is the feature of stem cells. Differentiation is usually considered to be a one-way process of specialization as cells develop the functions of their ultimate fate and lose their immature characteristics, such as self-renewal. Progenitor cells, the products of stem cells losing the activity of self-renewal, could differentiate to mature cells, which have the feature of differentiation and lose the activity of self-renewal. The roles for cancer stem cells have been demonstrated for some cancers. However, the origin of the cancer stem cells remains elusive. METHODS: This review focuses on current scientific controversies related to the establishment of the cancer stem cells--in particular, how self-renewal and differentiation block might contribute to the evolution of cancer. RESULTS: Cancer stem cells may be caused by transforming mutations occurring in multi-potential stem cells, tissue-specific stem cells, progenitor cells, mature cells and cancer cells. Progenitor cells obtain the self-renewal activity by activating the self-renewal-associated genes rather than dedifferentiate to tissue special stem cells. The transform multi-potential stem cells gain the differentiation feature of special tissue by differentiating to cancer cells. Mature cells and cancer cells may dedifferentiate or reprogram to cancer stem cells by genetic and / or epigenetic events to gain the self-renewal activity and lose some features of differentiation. The cancer-derived stem cells are not the "cause", but the "consequence" of carcinogenesis. The genetic program controlling self-renewal and differentiation is a key unresolved issue. CONCLUSION: Cancer stem cells

may be caused by disturbance of self-renewal and differentiation occurring in multi-potential stem cells, tissue-specific stem cells, progenitor cells, mature cells and cancer cells.
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Yamagami S, Mimura T, Yokoo S, Takato T and Amano S. Isolation of human corneal endothelial cell precursors and construction of cell sheets by precursors. *Cornea* 2006;10 Suppl 1:S90-2

PURPOSE: To review recently published experimental data on human corneal endothelium (CE) precursors. METHODS: A sphere-forming assay was used for the isolation of human CE precursors from human donor corneas and cultured human CE. CE morphology derived from precursors was compared with that from cultured human CE. RESULTS: Human CE from donor corneas formed primary and secondary sphere colonies and expressed neural and mesenchymal proteins. The progeny of these colonies had a human-CE-like hexagonal shape and showed transport activity, suggesting that the isolated spheres were indeed precursors of human CE. Similar precursors were isolated from cultured human CE, which provided further evidence that the sphere-forming assay facilitates the mass production of human CE precursors. Cultured human CE obtained in this manner had a regular hexagonal morphology in contrast to passaged, cultured human CE. CONCLUSION: The sphere-forming assay may become a powerful tool for the regeneration of human CE by precursor injection and the construction of cultured CE sheets.
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Yamagami S, Yokoo S, Mimura T, Takato T, Araie M and Amano S. Distribution of precursors in human corneal stromal cells and endothelial cells. *Ophthalmology* 2007;3:433-9

PURPOSE: We identified original tissue-committed precursors with limited self-renewal capacity from human corneal stromal (HCS) cells and human corneal endothelial (HCE) cells, then tried to determine the distribution and proliferative capacity of the precursors. DESIGN: Experimental study. PARTICIPANTS: Eighteen human corneas from donors 56 to 68 years old. METHODS: Human corneal stromal cells were divided into groups based on distance from the center of the cornea: <6 mm (central), 6 to 8 mm (paracentral), and 8 to 10 mm (peripheral). Human corneal endothelial cells were separated into 2 groups: <7.5 mm (central) and 7.5 to 10 mm (peripheral) from the center. Each group was subjected to the sphere-forming assay using serum-free medium containing growth factors in floating culture. Sphere numbers and the proliferative capacity of spheres in adherent culture were compared among the groups. MAIN OUTCOME MEASURES: Density and proliferative capacity of precursors from each area of HCS and HCE cells. RESULTS: Primary spheres were isolated from all groups of HCS and HCE cells. The rate of primary sphere formation from peripheral HCS cells was higher than those of the other 2 groups, being 1.5-fold greater than in the paracentral cornea and 4-fold greater than in the central cornea. The rate of primary sphere formation by peripheral HCE cells was significantly higher than that by central HCE cells, being 4-fold greater than in the central cornea. There were no differences in the proliferative capacity of HCS and HCE cell spheres from the different areas after adherent culture. CONCLUSIONS: All HCS and HCE cells contain a significant number of precursors, but the peripheral cells have a density of precursors higher than that of the central cells. Precursors from each area do not show differences of proliferative capacity. Our findings may in part explain changes after excimer laser treatment and may have implications for corneal transplantation procedures.
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Yang X, Moldovan N I, Zhao Q, Mi S, Zhou Z, Chen D, Gao Z, Tong D and Dou Z. Reconstruction of damaged cornea by autologous transplantation of epidermal adult stem cells. *Mol Vis* 2008;1064-70

PURPOSE: It is crucial for the treatment of severe ocular surface diseases such as Stevens-Johnson syndrome (SJS) and ocular cicatricial pemphigoid (OCP) to find strategies that avoid the risks of allograft rejection and immunosuppression. Here, we report a new strategy for reconstructing the damaged corneal surface in a goat model of total limbal stem cell deficiency (LSCD) by autologous transplantation of epidermal adult stem cells (EpiASC). METHODS: EpiASC derived from adult goat ear skin by explant culture were purified by selecting single cell-derived clones. These EpiASC were cultivated on denuded human amniotic membrane (HAM) and transplanted onto goat eyes with total LSCD. The characteristics of both EpiASC and reconstructed corneal epithelium were identified by histology and immunohistochemistry. The clinical characteristic of reconstructed corneal surface was observed by digital camera. RESULTS: Ten LSCD goats (10 eyes) were treated with EpiASC transplantation, leading to the restoration of corneal transparency and improvement of postoperative visual acuity to varying degrees in 80.00% (8/10) of the experimental eyes. The corneal epithelium of control groups either with HAM transplantation only or without any transplantation showed irregular surfaces, diffuse vascularization, and pannus on the entire cornea. The reconstructed corneal epithelium (RCE) expressed CK3, CK12, and PAX-6 and had the function of secreting glycocalyx-like material (AB-PAS positive). During the follow-up period, all corneal surfaces remained transparent and there were no serious complications. We also observed that the REC expressed CK1/10 weakly at six months after operation but not at 12 months after operation, suggesting that the REC was derived from grafted EpiASC. CONCLUSIONS: Our results showed that EpiASC repaired the damaged cornea of goats with total LSCD and demonstrated that EpiASC can be induced to differentiate into corneal epithelial cell types in vivo, which at least in part correlated with down-regulation of CK1/10 and upregulation of PAX-6.
Department of Life Sciences, Luoyang Normal University, Luoyang, China.

Ye J, Yao K and Kim J C. Mesenchymal stem cell transplantation in a rabbit corneal alkali burn model: engraftment and involvement in wound healing. *Eye (Lond)* 2006;4:482-90

PURPOSE: To investigate whether systemically transplanted mesenchymal stem cells (MSCs) can home and engraft in tissue to promote cornea wound healing after alkali burn, as a new source for treatment. METHODS: Corneal alkali burn was created in four group rabbits: Group I, normal bone marrow function, without MSCs transplantation; Group II, normal bone marrow function, with MSCs transplantation; Group III, bone marrow suppressed by cyclophosphamide, without MSCs; Group IV, bone marrow suppressed by cyclophosphamide, with MSCs. Clinical outcome was evaluated by cornea re-epithelization, cornea opacity, and neovascularization. Cell engraftment into bone marrow, circulation, and cornea was monitored. Immunohistochemistry, using proliferating cell nuclear antigen (PCNA), P63, vimentin, and alpha-smooth muscle actin (alpha-SMA) was carried out to assess the cell proliferative and differentiative ability. RESULTS: At the time of 1-month follow-up, Group II rabbits showed the best clinical results with a clearer healed cornea compared with other groups. Well-formed neovascularization appeared on day 14 after alkali burn in Group II, that coincided with the maximum engraftment of MSCs. PCNA, P63, vimentin were more strongly expressed in Group II at multiple time points. DiI-labelled MSCs were differentiated into myofibroblast by the expression of alpha-SMA. Delayed and insufficient cell engraftment, with malformed neovascularization and retarded corneal wound healing was found in Groups III and IV. CONCLUSIONS: Systemically transplanted MSCs can engraft to injured cornea to promote wound healing, by differentiation, proliferation, and synergizing with haemotopoietic stem cells.
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Yeo S, Jeong S, Kim J, Han J S, Han Y M and Kang Y K. Characterization of DNA methylation change in stem cell marker genes during differentiation of human embryonic stem cells. *Biochem Biophys Res Commun* 2007;3:536-42

Pluripotent human embryonic stem cells (hESCs) have the distinguishing feature of innate capacity to allow indefinite self-renewal. This attribute continues until specific constraints or restrictions, such as DNA methylation, are imposed on the genome, usually accompanied by differentiation. With the aim of utilizing DNA methylation as a sign of early differentiation, we probed the genomic regions of hESCs, particularly focusing on stem cell marker (SCM) genes to identify regulatory sequences that display differentiation-sensitive alterations in DNA methylation. We show that the promoter regions of OCT4 and NANOG, but not SOX2, REX1 and FOXD3, undergo significant methylation during hESCs differentiation in which SCM genes are substantially repressed. Thus, following exposure to differentiation stimuli,

OCT4 and NANOG gene loci are modified relatively rapidly by DNA methylation. Accordingly, we propose that the DNA methylation states of OCT4 and NANOG sequences may be utilized as barometers to determine the extent of hESC differentiation.
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Yokoo S, Yamagami S, Shimada T, Usui T, Sato T A, Amano S, Araie M and Hamuro J. A novel isolation technique of progenitor cells in human corneal epithelium using non-tissue culture dishes. *Stem Cells* 2008;7:1743-8

The existence of adult stem cells or progenitor cells in the human corneal epithelium (i.e., self-renewing squamous cells) has long been suggested, but these cells have not yet been isolated. Here we describe a novel isolation technique using non-tissue culture dishes to enrich progenitor cells, which are able to reconstitute a three-dimensional human corneal epithelial equivalent from single cells in serum-, feeder-, and bovine pituitary extract-free medium. These cells showed original tissue-committed differentiation, a high proliferative capacity, and limited self-renewal. Laminin-5 was measured by mass spectrometric analysis. Pretreatment of cells with anti-laminin-5 antibody demonstrated that laminin-5 was important in allowing corneal epithelial progenitor cells to adhere to non-tissue culture dishes. Hydrophilic tubes (used for cell collection throughout this study) are essential for efficient isolation of adherent corneal epithelial progenitor cells expressing laminin-5. These findings indicate that our new technique using non-tissue culture dishes allows the isolation of progenitor cells from human corneal limbal epithelium and that laminin-5 has a critical role in the adhesion of these cells.
Department of Corneal Tissue Regeneration, Tokyo University Graduate School of Medicine, Tokyo, Japan.

Yokoo S, Yamagami S, Yanagi Y, Uchida S, Mimura T, Usui T and Amano S. Human corneal endothelial cell precursors isolated by sphere-forming assay. *Invest Ophthalmol Vis Sci* 2005;5:1626-31

PURPOSE: To isolate precursors of human corneal endothelial cells (HCECs) in vitro. METHODS: HCECs were subjected to a sphere-forming assay in which spheres floated in serum-free medium containing growth factors. To promote differentiation, the isolated sphere colonies were plated in dishes coated with poly-L-lysine (PLL)/laminin or fetal bovine endothelium extracellular matrix. Marker expression of neural and mesenchymal cells was examined in the sphere colonies and their progenies by immunocytochemistry and/or reverse transcription-polymerase chain reaction (RT-PCR). Adherent differentiated cells from the sphere colonies were evaluated morphologically and functionally. RESULTS: HCECs formed primary and secondary spherical colonies, as shown by sphere-forming assay in vitro. The colonies expressed nestin, beta3-tublin, glial fibrillary acidic protein, and alpha-smooth muscle actin on immunocytochemistry. The progeny, proliferating on extracellular matrix derived from bovine corneal endothelium, but not on PLL/laminin-coated and noncoated dishes, expressed nestin and beta3-tublin. These markers were confirmed by RT-PCR. Adherent differentiated cells from the sphere colonies had an HCEC-like hexagonal shape and satisfactory transport activity that is essential in HCECs. CONCLUSIONS: These findings indicate that the HCEC contains precursor cells with a propensity to differentiate into HCECs and that these cells can also produce neuronal and mesenchymal cell proteins.
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Zhang X, Sun H, Li X, Yuan X, Zhang L and Zhao S. Utilization of human limbal mesenchymal cells as feeder layers for human limbal stem cells cultured on amniotic membrane. *J Tissue Eng Regen Med* 2010;1:38-44

Various cell culture techniques for limbal epithelial cells are currently being used for the transplantation of cultured limbal stem cells. In this study, we explored the possibility of using human limbal mesenchymal cells (HLMCs) as feeder layer for the human limbal epithelial cells (HLECs). Single cell suspension of HLECs was seeded onto denuded amniotic membranes with inactivated 3T3 fibroblasts or HLMCs as feeder layer. Expressions of Cytokeratin 3, Np63 and connexin 43 (Cx43) of the cultured epithelial cells were determined at 28 days and the ultrastructure of the epithelium was examined by transmission electron microscope after 14 days and 28 days of cultivation. In both groups, cells were differentiated into multilayer epithelium at 28 days. Basal cells of the cultured epithelium showed a strong nuclear labeling of Np63, but lacked CK3 and Cx43 expression. Transmission electron microscopy examination showed that there were abundant desmosomal contacts between the cells. The key feature the cultured epithelium was occurrence of a typical basement membrane. These results suggested that HLMCs can be used as an alternative feeder layer for HLECs, which makes the bioengineering product biologically safer for the clinical applications.
Tianjin Medical University Eye Centre, People's Republic of China.

Zhou S, Schuetz J D, Bunting K D, Colapietro A M, Sampath J, Morris J J, Lagutina I, Grosveld G C, Osawa M, Nakauchi H and Sorrentino B P. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. *Nat Med* 2001;9:1028-34

Stem cells from bone marrow, skeletal muscle and possibly other tissues can be identified by the 'side-population' (SP) phenotype. Although it has been assumed that expression of ABC transporters is responsible for this phenotype, the specific molecules involved have not been defined. Here we show that expression of the Bcrp1 (also known as Abcg2 murine/ABCG2 human) gene is a conserved feature of stem cells from a wide variety of sources. Bcrp1 mRNA was expressed at high levels in primitive murine hematopoietic stem cells, and was sharply downregulated with differentiation. Enforced expression of the ABCG2 cDNA directly conferred the SP phenotype to bone-marrow cells and caused a reduction in maturing progeny both in vitro and in transplantation-based assays. These results show that expression of the Bcrp1/ABCG2 gene is an important determinant of the SP phenotype, and that it might serve as a marker for stem cells from various sources.
Division of Experimental Hematology, Department of Hematology/Oncology, St. Jude Children's Research Hospital, Memphis, Tennessee, USA.

Zhu C and Joyce N C. Proliferative response of corneal endothelial cells from young and older donors. *Invest Ophthalmol Vis Sci* 2004;6:1743-51

PURPOSE: To compare the effect of epidermal growth factor (EGF), nerve growth factor (NGF), platelet-derived growth factor-BB (PDGF-BB), bovine pituitary extract, and fetal bovine serum (FBS), alone or in combination, on proliferation of human corneal endothelial cells (HCEC) cultured from young (<30 years old) and older donors (>50 years old). METHODS: Corneas from donors 2 to 79 years old were obtained from the National Disease Research Interchange. Descemet's membrane with intact endothelium was dissected. Cells were isolated by EDTA treatment and cultured to confluence. The HCEC marker, antibody 9.3.E, tested for pure endothelial populations. Antibody Ki67 and ZO-1 tested either before or after cultured cells reached confluence to indicate cell proliferation and cell-cell contact formation. Cell morphology was documented by inverted phase-contrast microscopy. Passages I through VII were used to test the effect of various factors on cell proliferation. For each study, equal numbers of cells were seeded, maintained overnight in 4% FBS to permit cell attachment, washed, and incubated for up to 3 weeks in one of the following: modified Eagle's Minimum Essential Medium (Opti-MEM-I) alone; Opti-MEM-I plus EGF, NGF, PDGF-BB, bovine pituitary extract, or FBS; or a combination of factors. At various times after seeding, cell numbers were determined by electronic cell counter. For each condition, three separate wells were tested and each sample was counted three times. Studies were repeated at least twice using cells from different donors and age groups. Within each study, a one-way ANOVA test was performed to analyze statistical significance. RESULTS: Cells stained positively with antibody 9.3.E, indicating isolation of HCEC and lack of contamination with epithelial cells or keratocytes. Positive staining of Ki67, indicating cycling cells, was found in subconfluent cultures. Plasma membrane-associated ZO-1 staining and lack of Ki67 staining indicated that cultured cells formed a contact-inhibited monolayer. Cultured cells decreased in density, increased in size, and became more heterogeneous depending on donor age and on the number of passages. Incubation in OptiMEM-I promoted attachment and induced a moderate proliferative response above that of MEM ($P < 0.001$). In general, proliferative responses to growth stimuli were relatively slow, with cell counts generally plateauing 10 to 14 days after exposure to growth-promoting agents. EGF yielded a broad, dose-dependent effect and, at 5-50 ng/mL, peak cell counts were significantly higher ($P < 0.001$) than basal levels. EGF consistently stimulated proliferation in cells from younger donors, but was less effective in stimulating growth of cells from older donors. NGF did not show a consistent significant stimulatory effect at any concentration tested. PDGF-BB (25 ng/mL) tended to stimulate growth to a greater extent than EGF ($P < 0.05$) in cultures from the same donor. Pituitary extract significantly increased counts at 1.0 ($P < 0.05$) to 100 ug/mL ($P < 0.001$).

PDGF-BB plus pituitary extract demonstrated greater stimulation than pituitary extract (P < 0.01) or PDGF-BB alone (P < 0.01). FBS (1%-8%) increased cell numbers in a dose-dependent manner, and, at 4%-8%, yielded counts significantly higher (P < 0.001) than that of any single growth-promoting agent tested. CONCLUSIONS: HCEC from both young and older donors can proliferate in vitro in response to growth-promoting agents. Proliferation in the presence of multiple mitogens ceased when confluence was reached, indicating the formation of a contact-inhibited monolayer. In general, cells cultured from young donors were more responsive to the agents tested, but the relative response of HCEC to these agents was similar, regardless of donor age. The relative difference in the extent of the response of the same cell population to different mitogens suggests that these mitogens induce different downstream signals. The relatively robust proliferative response of HCEC to FBS may involve stimulation of multiple downstream signaling pathways may involve stimulation of multiple downstream signaling pathways and/or induce more sustained downstream signaling than the other growth-promoting agents tested.
Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, Massachusetts 02114, USA.

Zhu C, Rawe I and Joyce N C. Differential protein expression in human corneal endothelial cells cultured from young and older donors. *Mol Vis* 2008;1805-14

PURPOSE: To establish a baseline protein fingerprint of cultured human corneal endothelial cells (HCEC), to determine whether the protein profiles exhibit age-related differences, and to identify proteins differentially expressed in HCEC cultured from young and older donors. METHODS: Corneas were obtained from five young (<30 years old) and five older donors (>50 years old). HCEC were cultured, and protein was extracted from confluent passage 3 cells. Extracts from each age group were pooled to form two samples. Proteins were separated on two-dimensional (2-D) gels and stained with SyproRuby. Resultant images were compared to identify protein spots that were either similarly expressed or differentially expressed by at least twofold. Protein spots were then identified by matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry. RESULTS: Protein spots were well resolved, and patterns were reproducible on 2-D gels using either pH 3-10 or pH 4-7 IPG strips. Two-dimensional gels prepared with pH 4-7 IPG strips were used for differential display analysis, which was reproduced on three separate pairs of gels. MALDI-TOF identified 58 proteins with similar expression; 30 proteins were expressed twofold higher in HCEC from young donors; five proteins were expressed twofold higher in cells from older donors; and 10 proteins were identified in gels from young donors that did not match in gels from older donors. Several proteins expressed at higher levels in younger donors support metabolic activity, protect against oxidative damage, or mediate protein folding or degradation. CONCLUSIONS: This is the first proteomic comparison of proteins expressed in HCEC cultured from young and older donors. Although restricted to proteins with isoelectric points between pH 4.0 and pH 7.0, the data obtained represent an initial step in the investigation of molecular mechanisms that underlie physiologically important age-related differences in cultured HCEC, including differences that may affect proliferative capacity. Results indicate that HCEC from older donors exhibit reduced expression of proteins that support important cellular functions such as metabolism, antioxidant protection, protein folding, and protein degradation. These differences may affect the ability to consistently obtain a sufficient number of healthy cultured HCEC for use in preparing bioengineered endothelium as an alternative method for the treatment of endothelial dysfunction.
Schepens Eye Research Institute and Department of Ophthalmology, Harvard Medical School, Boston, MA 02114, USA.

Zou J, Yu X F, Bao Z J and Dong J. Proteome of human colon cancer stem cells: a comparative analysis. *World J Gastroenterol* 2011;10:1276-85

AIM: To isolate and identify the biological characteristics of human colon cancer stem cells (SW1116 cells) and further study their proteome. METHODS: SW1116 cells were isolated and cultured with a serum-free medium (SFM). Sphere formation was assayed to observe the formation of colon cancer stem cell spheres. SW1116 cells were inoculated into a serum-containing medium for observing their differentiation characteristics. Proliferation curve and cross-resistance of SW1116 cells to different drugs were detected by MTT. Percentage of SP cells in SW1116 cells was detected with Hoechst33342 staining. Telomerase activity in SW1116cells was checked by polymerase chain reaction (PCR)-enzyme linked immunosorbent assay. Expressions of stem cell relevant genes and proteins were detected by reverse transcription-PCR and Western blot, respectively. Total protein was isolated from SW1116 cells by two-dimensional gel electrophoresis (2-DE) and differentially expressed proteins were identified by tandem mass spectrometry (MALDI-TOF/TOF). RESULTS: The isolated SW1116 cells presented as spheroid and suspension growths in SFM with a strong self-renewal, proliferation, differentiation and drug-resistance ability. The percentage of SP cells in SW1116 cells was 38.9%. The SW1116 cells co-expressed the CD133 and CD29 proteins. The telomerase activity in SW1116 cells was increased. The expressions of different stem cell relevant genes and proteins were detected. The proteomic analysis showed that the 26 protein spots were differently expressed in SW1116 cells and 10 protein spots were identified as ubiquitin fusion-degradation 1-like protein, nuclear chloride channel protein, tubulin beta, Raichu404X, stratifin, F-actin capping protein alpha-1 subunit, eukaryotic translation elongation factor 1 delta isoform 2, hypothetical protein, glyceraldehyde-3-phosphate dehydrogenase and guanine nucleotide binding protein beta polypeptide 2-like 1, respectively. CONCLUSION: SW1116 cells are biologically characterized by self-renewal, proliferation and differentiation, and the differently expressed proteins in SW1116 cells may be essential for isolating cancer stem cells.
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PARTIE EXPERIMENTALE

Publication 1 (en review Optics Letter, IF 3,31) Fabrication of optical mosaics to optimize corneal endothelial cell quality determination in eye banks

Publication 2 (soumis à Molecular Vision IF 2,51) Optimization of immunolocalization of cell cycle proteins in human corneal endothelial cells

Publication 3 (en preparation pour Journal of Histochemistry and Cytochemistry IF 2,38) Effects of fixatives temperature on immunolocalization in flat mounted human corneal endothelium.

Publication 4 (publié dans Ophthalmic Research IF 1,29) Ex vivo gene electrotransfer to the endothelium of organ cultured human corneas

Publication 5 (soumis à Stem Cells, IF 7,87) Revisited microanatomy of the corneal endothelial periphery: new evidences for a continuous centripetal migration of endothelial cells in human

Publication 1 (en review Optics Letter, IF 3,31) Fabrication of optical mosaics to optimize corneal endothelial cell quality determination in eye banks

Fabrication of optical mosaics to optimize corneal endothelial cell quality determination in eye banks

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The determination of endothelial cell density (ECD) is a crucial activity in eye banks for the assessment of corneal tissue quality. These cells are responsible of corneal transparency and ECD correlates with graft survival. ECD is mainly evaluated with a manual "naked-eye" procedure under a transmitted light microscope in Europe and using a specular microscope in the USA. Inter and intra bank variability has been previously demonstrated. In order to facilitate training and continuous education of technicians and reliability assessment of eye banks ECD determination, we fabricate with technologies employed in micro-optics test mosaics exactly reproducing the image of human corneal endothelium. The description of the fabrication process is detailed and comparisons are made between amplitude and phase mosaics. © 2010 Optical Society of America

OCIS Codes: 170.0180, 170.4470, 220.4000

Endothelial cell density (ECD expressed in cells/mm^2) is the decisive factor for quality evaluation of corneal tissue in eye banks prior to graft. Corneal Endothelial Cells (EC) are differentiated cells, incapable of self-replication in human, that form a monolayer of hexagons at the posterior surface of the cornea. They play a major role in corneal transparency by regulating stromal hydration. In recipients of corneal grafts, ECD determines the survival of the graft (transparency during time). It is consequently crucial to have an accurate and reproducible method to determine ECD. An ECD of 2000 to 2400 cells/mm^2 is considered by most eye banks worldwide to be the cut-off for corneal delivery. This large range is explained by the empirical method used to define the value that corresponds to the ratio between acceptable results for patients and medico-economic consideration of eye banks. A higher threshold would mean higher graft quality but less graft available, and a lower threshold would mean lower graft quality with shorter survival. A second explanation for this rather large range is that it also reflects the relative imprecision of the methods used in eye banks to determine ECD.

ECD is estimated on a small area comprising 50 to 300 cells (depending on the eye bank) among the 3 to 400 000 that a cornea bears. In Europe, where the graft storage method called organ culture consists in preserving tissue viability during several weeks in cell culture derived medium at physiologic temperature, the endothelium is evaluated directly under a Transmitted Light Microscope (TLM) (either bright field or phase contrast) after osmotic preparation. This consists of a short incubation of the endothelial side with sodium chloride or sucrose to temporarily dilate the intercellular spaces and enables the visualization of EC borders spontaneously not visible. In the rest of the world, a short-term cold storage technique is used allowing a maximum of 8-10 days storage. The endothelium is evaluated thanks to a Specular Microscope (SM) that does not require a specific cell preparation [1]. Beside ECD, EC morphometry (regularity in cell area and shape) constitute supplementary data to qualify corneas which ECD are close to the threshold values.

Previous studies analyzing ECD measurements in the 22 eye banks in France have underlined the need for standardization of counting methods [2, 3]. Lack of microscope calibration and differences in manual counting techniques and strategies had been identified as main factors responsible for large variations in ECD between eye banks. Until now, the only available method of calibration was a certified graduated micrometer, a usual method in biological imaging. Essentially, this measures only the magnification ratio between the observed tissue and its projected image in a calibrated reticule or by image analysis software. Contrary to what is routinely performed to assess (and sometimes certify) reliability of biological tests in laboratories, no standards can be easily sent to eye banks. Each human cornea is unique in term of ECD and ECD decreases with time, both characteristics that hinders from doing a mass analysis of several eye banks at a given time. We had therefore developed the concept of sending specific original test slides (called keratotests) to eye banks. Each eye bank technician had to determine ECD on the keratotest with the method he routinely used. In a first generation keratotest, we used isolated real human corneal endothelium with EC borders distinctly stained with alizarin red and fixed on a certified calibrated glass graticule (leica) made of 1 mm^2 squares. Only 1 square was left visible and ECs were counted with two calibrated methods to determine the reference ECD. Each mosaic was unique but keratotests were inalterable. Four slides

with various ECD were chosen and successively sent to eye banks [3]. To remedy the impossibility to exactly reproduce the same mosaic we made the proof of concept of a 2nd generation keratotest using laser beam direct writing photolithography of hexagonal patterns with chosen sizes on a chromium (Cr) coated glass wafer [4]. Nevertheless the perfect regular hexagons mathematically generated did not reflect the reality of ECs.

We therefore developed a 3rd generation keratotest combining the advantages of both previous generations: imitation of true human corneal endothelial mosaics and possibility of mass production with the aim of developing a tool devoted to the periodic reliability assessment of eye banks as well as the training and continuing education of technicians. The prototype contained a two dimension virtual endothelial structure coming from a simple TLM image. Human corneas retrieved at our laboratory of Anatomy (body donation to Science, Faculty of Medicine) were used. ECs were stained for 1 min with 0.5% Alizarin red (Sigma, St Louis, MO) dissolved in 0.9% sodium chloride with a pH adjusted at 5.2. After a rinse in phosphate buffer saline, corneas were flat mounted on a glass slide and endothelial images taken on a light microscope (IX81, Olympus, Tokyo, Japan) equipped with a digital camera, using a x4 objective. Tagged format file images (2576 x1932 pixels) were then treated using Adobe Photoshop CS 8.0 to extract a Region Of Interest (ROI), touch up all imperfect cell boundaries and remove staining artefacts (see Fig 1). ROI with variable contours were selected in order to reduce the possibility of 'cheating' by counting all ECs in a known area to deduce ECD (easy if ROI were squares of 1mm^2, as in our first generation keratotest).

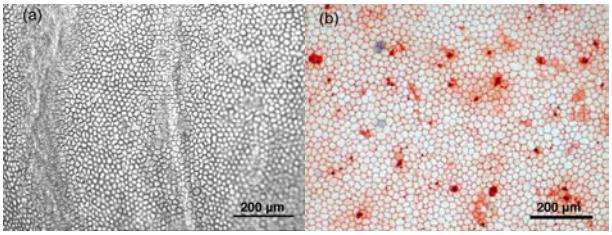

Fig. 1. (Color online) Example of endothelial mosaic of a human cornea. (a). Usual transmitted light microscopy (bright field) after osmotic dilation of intercellular spaces in eye banks. Note that corneal curvature and endothelial folds create a 3D surface that hinders the image from being entirely focused (b). Light microscopy after alzarin red staining that reveals cell borders. In this experimental staining, the cornea can be flat mounted to avoid defocusing.

We then used a set of micro-fabrication techniques borrowed from traditional Integrated Circuit foundries [5]. Once the target mosaic has been finalized, the file was binarized and saved as a non-compressed raster binary black and white image (.raw file) to make sure there are no grey scale pixels included in the file. This binary file was then read by a vector fracture program which read only the white pixels (black pixels were not patterned) and produced a continuous contour over neighboring white pixels (see Fig. 2). This contour had a maximum of 200 vertices, which is a constrain in traditional Graphic Database System GDS2 mask layout file formats. This fracture process is attractive since it reduces the price of the mask patterning without sacrificing any resolution of the pattern. If the original file were to remain raster (every pixel being fractured in an individual 4 edge polygon), the patterning costs would increase by a non negligible factor, since most of the process performed by the E-Beam Process Generator (EBPG) system would be to read the individual shapes on the hard disk (see Fig. 2).

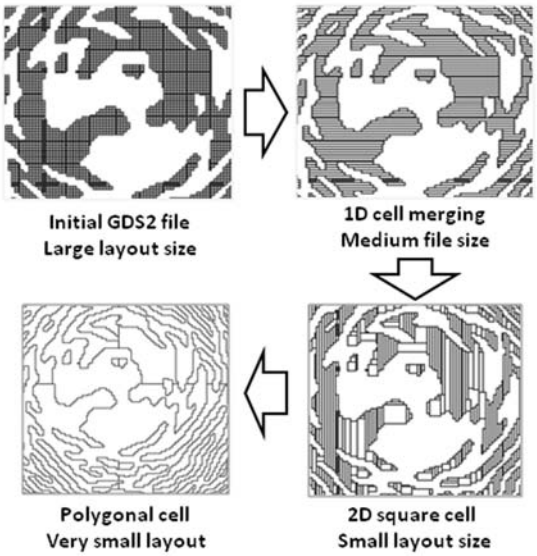

Fig. 2. Vector fracture of binary black and white file into optimized GDS2 file.

The patterning costs is a direct function of the overall patterning time, including « on the fly » read time performed by the EBPG system. The critical dimension for our prototype mask was 1.25µm, which was the dimension of one edge of a pixel. The GDS2 mask layout file was sent to a mask patterning shop (in other words a mask shop). The mask blank consisted of a 5 inches square quartz substrate, 2.28mm thick, covered with a 250nm thick high reflectivity Cr layer. An 800nm thick e-beam resist was spun over the quartz blank.

An EBPG was then used to pattern the GDS2 file on the resist. It used the smallest spot size of 800nm, with an addressing grid of 100nm. After development of the e-beam resist and hard bake, the exposed Cr layer was etched away, and the remaining resist stripped off through an oxygen plasma etch (plasma ashing). The lithography step involves the use of a Canon FPA 501 mask aligner which was used either in hard contact or in proximity mode.

For our elements, we employ a small batch of 4 inches quartz wafers (fused silica), on which we have sputtered a 200nm layer of Cr. The batch of wafers was then inserted in an adhesion promoter vapor phase coater for 45 min, which coated the wafers (front and back side) with a very thin hexamethyldisilazane adhesion promoter layer. Following this process, we span a 1µm thin resist layer (Fuji Film OCG835 G-line type positive resist). After a 2 min soft bake at 60°C, the wafers were ready to be exposed. The exposure process was critical since we are trying to produce a smooth pattern definition on the Cr,

from a binary raster image (mask layout in GDS2 format). There were 2 ways to achieve this step: one solution was to overexpose the wafer and the other was to use the aligner in the proximity mode. We chose the proximity mode since the overexposure would in addition enlarge a bit the resulting pattern. We employed a 10µm proximity mode, which provided a smooth profiling of the binary raster shapes. After exposure of the wafer batch, we developed them by using an OCG835 developer in a 4/5 dilution with distilled water, for 2 min with medium shaking. Following the development process, the wafers were cleaned in a Spin Rinse Dry (SRD) system using hot nitrogen gas and sent through a hard bake process at 145°C for 45 min, to prepare them for the wet etch. The wafer batch was then inserted in a plasma O_2 asher for 4 min with 450W Radio Frequency power in order to remove any remaining resist (scum) on the surface of the pattern (resist strains). The Cr wet etch process was then used to transfer the resist patterns into the underlying Cr layer, by using an acetic acid/ceric amonium nitrate bath for 90 sec.

Following this process, the wafers were washed in distilled water and again in the SRD. The wafers intended to be used as amplitude targets went then through a hot sulfuric acid bath heated up to 128°C activated with Hydrogen Peroxide (H_2O_2) for 5 min. This process got rid of all the remaining resist and leaved a clean Cr on quartz wafer (see Fig. 3). These wafers were diced out through diamond dicing on sticky tape using a Disco Dicing DAD5h/Li dicer. After dicing, the dies were cleaned again in hot sulfuric acid to get rid of any sticky tape residues and rinsed in distilled water with hot nitrogen jets.

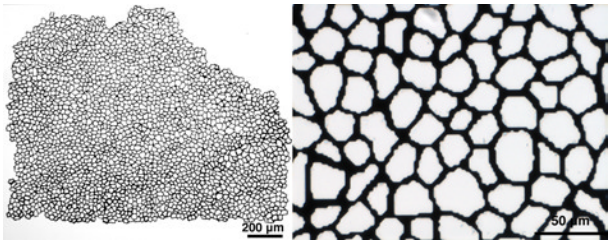

Fig. 3. Amplitude component developed from a real human corneal endothelial image It can be visualized by both transmitted light and specular microscopes.

For other wafers to be used as phase modulation targets, the resist layer on top of the Cr layer was not removed. They were inserted in an oven for a dry etch hard bake process at 115°C for 30 min. After this process (which got rid of all the moisture in the resist), they were ready for dry Reactive Ionic Etch (RIE). The Cr layer acted as an etch stop and the resist acted as a catalyser of the etching process. The RIE etcher used was a 8110 series Applied Materials etcher under 55mtorr with 8ccm of oxygen, 65ccm of CHF3 and 10ccm of CF4. Figure 4 presents an example of phase component. This process allowed an etch rate of around 39nm per min in the fused silica. The etch depth has been set to 550nm (producing an optimal phase shift in the central visible spectrum – one wave phase shift in quartz - yielding a optimal visual contrast under phase contrast microscopy). The etch depth was measured by a KLA Tencor Surfscan 200 (stylus profilometer with a 12.5µm end tip). The remaining resist was removed by a hot sulfuric bath at 128°C activated by H_2O_2, and rinsed in distilled water. The remaining underlying Cr was also removed by a 10 min Cr wet etch bath.

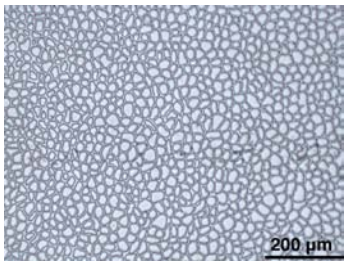

Fig. 4. (Color online) Phase component observed under phase contrast microscopy, useful for several eye banks that choose this method of observation.

After these processes, the wafer was rinsed in distilled water and underwent a final high speed SRD spin rinse dry process with hot nitrogen before being sent to the diamond wafer dicing machine. Both sets of targets (amplitude and phase targets) were stored in Gel packs ready to be used in the laboratory.

In conclusion, we demonstrate the possibility to use microtechnology for micro-optics to create virtual EC mosaics from images of real human ECs. The chosen technology allowed producing a large number of mosaics with the same process in a reduced time, compared to other possibilities (laser writing, ablation etching, etc…). Virtual mosaics are realistic and can be visualized by both TLM (bright field and phase contrast) and SM to cover all eye bank types worldwide. Several mosaics with different ECD and/or EC morphology can be chosen to cover all the range of ECD susceptible to be analyzed in eye banks. We will soon start a European research project to assess the reliability (reproducibility and sharpness) of ECD count among approximately 150 European Eye Banks.

The next step will consist in developing 3D virtual mosaics mimicking corneal curvature and endothelial folds to completely fit with the reality.

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Publication 2 (soumis à Molecular Vision IF 2,51) Optimization of immunolocalization of cell cycle proteins in human corneal endothelial cells

Optimization of immunolocalization of cell cycle proteins in human corneal endothelial cells

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Short title:

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ABSTRACT

Purpose: *En face* observation of corneal endothelial cells (ECs) using flat-mounted whole corneas is theoretically much more informative than observation of cross-sections that show only a few cells. Nevertheless, it is not widespread for immunolocalization (IL) of proteins, probably because the endothelium, a superficial monolayer, behaves neither like a tissue in immunohistochemistry (IHC) nor like a cell culture in immunocytochemistry (ICC). In our study we optimized IL for EC of flat-mounted human corneas to study the expression of cell cycle-related proteins.

Methods: We systematically screened 15 fixation and five antigen retrieval (AR) methods on 108 human fresh or stored corneas (organ culture), followed by conventional immunofluorescence labeling. Firstly, in an attempt to define a universal protocol, we selected combinations able to correctly localize four proteins that are perfectly defined in ECs (ZO-1 and actin) or ubiquitous (hnRNP L and histone H3). Secondly, we screened protocols adapted to the revelation of 10 cell cycle proteins: Ki67, PCNA, MCM2, cyclin D1, cyclin E, cyclin A, $p16^{Ink4a}$, $p21^{Cip1}$ and $p27^{Kip1}$, and ZONAB/DppA. Primary antibody controls (positive controls) were performed on both epithelial cells of the same, simultaneously-stained whole corneas, and by ICC on human ECs in *in vitro* non-confluent cultures. Both controls are known to contain proliferative cells. IL efficiency was evaluated by two observers in a masked fashion. Correct localization at optical microscopy level in ECs was define as clear labeling with no background, homogeneous staining, agreement with previous works on ECs and/or protein functions, as well as a meaningful IL in proliferating cells of both controls.

Results: The conventional fixation with 4% formaldehyde (gold standard for IHC) failed to reveal 13 of the 14 proteins. In contrast, they were all revealed using either 0.5% formaldehyde at room temperature (RT) during 30 minutes alone or followed by AR with sodiumdodecyl sulfate or trypsin, or pure methanol for 30 minutes at RT. Individual optimization was nevertheless often required to optimize the labeling. Ki67 was absent in both fresh and stored corneas, whereas PCNA was found in the nucleus, and MCM2 in the cytoplasm, of all ECs. Cyclin D1 was found in the cytoplasm in a paranuclear pattern much more visible after corneal storage. Cyclins E and A were respectively nuclear and cytoplasmic, unmodified by storage. P21 was not found in ECs with three different antibodies. P16 and p27 were exclusively nuclear, unmodified by storage. ZONAD/Dppa was nuclear and did not colocalize with ZO-1.

Conclusions: IL in ECs of flat-mounted whole human corneas requires a specific sample preparation, especially to avoid overfixation with aldehydes that probably easily masks epitopes. *En face* observation allows easy analysis of labeling pattern within the endothelial layer and clear subcellular localization, neither of which had previously been described for PCNA, MCM2, cyclin D1 or ZONAB/DppA.

INTRODUCTION

The corneal endothelium functions as a permeability barrier that restricts the movement of water and solutes into the hydrophilic stroma in order to control corneal transparency [1]. It is composed of a monolayer of flat (approximately 5µm) tessellated joint cells forming a mosaic of mainly hexagonal elements (Figure 1). In the adult human, the proliferative capacities of endothelial cells (ECs) are practically nil and cannot offset cell losses in physiological or pathological cases. Indeed, no increase in EC number has been clinically documented. Although in physiological conditions the number of ECs decreases very slowly, by about 0.6% per year during adulthood [2, 3], this pace accelerates dramatically in several corneal diseases (mainly Fuchs' dystrophy, the most frequent endothelial primary dystrophy) and after accidental or surgical traumatisms: endothelial cell density (ECD) falls below a threshold of 300 to 500 cells/mm^2 (depending on the kinetics of loss) and an irreversible corneal oedema occurs, causing permanent visual loss. During corneal storage by eye banks, be it in short-term cold storage at 4°C in Optisol or in long-term storage at 31°C to 34°C in organ culture (OC) media, EC loss also accelerates strongly, causing a decrease in graft quality. In the past two decades Joyce and colleagues have highlighted, mainly based on proteomic studies, that adult ECs *in vivo* are arrested in the G1 phase of the cell cycle (reviewed in [4]). However in parallel, they also demonstrated, with other, that ECs retain proliferative capacity [5] even in the elderly, but with an age-related decrease [6, 7]. Primary EC cultures from adult donors of all ages can also be routinely obtained *in vitro* [8].

Stimulation and control of EC proliferation would have high therapeutic impact, making it possible to increase ECD directly *in vivo* in patients with low ECD and to improve the quantity and quality of donor corneas by increasing ECD *ex vivo* during storage in eye banks, or by facilitating large-scale development of bioengineered endothelial grafts. Identification of the proteins involved in cell cycle arrest is key to these future endothelial cell therapies.

Immunolocalization (IL) of proteins has been previously used, as a basic tool, on corneal endothelium. Generally, EC can be observed either on corneal cross-sections or on flat-mounts, as the EC monolayer of 300,000 to 400,000 tessellated cells on the innermost face of the cornea is easily visible by *en face* observation. Although standard immunohistochemistry (IHC) on corneal cross-sections is the prevalent method in the literature, it allows visualization of only a few cells, not a complete view of the unmodified endothelium (Figure 1). Moreover, it does not always give a clear subcellular localization of proteins of interest, a condition required for a proper interpretation [9]. On the contrary, IL on flat-mounted corneas theoretically appears far more informative. It gives access to the whole intact tissue with a possibility to detect regional differences in expression patterns (for example a centripetal gradient) as well as to detect rare isolated cells among an apparently homogeneous cell population. Nevertheless, to date it has been used in very few studies and for few proteins. Moreover, in the

only paper reporting the use of both cross-sections and flat-mounts with the same antibodies, immunostaining was not identical, with generally fewer positive results on flat-mounts [10].

In the present study, we systematically screened immunostaining protocols to select those able to correctly reveal proteins in ECs of intact flat-mounted corneas. Fixation and antigen retrieval (AR) are two fundamental steps in all immunostaining processes. They strongly influence the ability of specific antibodies to recognize their targets (retention of antigenicity) while offering good preservation of ultrastructure. Cross-linking fixatives (aldehydes) act by creating covalent chemical bonds between proteins, mainly between lysine residues [11]. They also anchor soluble proteins to the cytoskeleton. Formaldehyde is the most common fixative in histology. Four percent formaldehyde (equivalent to the neutral-buffered formalin (10%) used for clinical pathology) appears as a gold standard in most studies, including those on corneal endothelium. Precipitating, also called denaturing, fixatives (ethanol, methanol, and acetone) reduces the solubility of proteins and disrupts the hydrophobic interactions involved in the tertiary structure of proteins. They are commonly used for immunocytochemistry (ICC), alone or in combination. Metallic fixatives, which denature the proteins and create weak bonds between them, are rarely employed alone in ICC or IHC. Nevertheless, $HgCl_2$ or $ZnCl_2$ containing formaldehyde solutions improve nuclear morphology with various effects on antigenicity [12]. AR, either by heating in specific buffers, enzymatic digestion or chemical reagents, helps unmask certain hidden epitopes. It can be helpful or even crucial during IHC of formalin-fixed paraffin-embedded tissues [13]. Heat-induced antigen retrieval (HIAR), known to hydrolyse protein cross-linking that occurred during aldehyde fixation (but not only [14]), is the most common AR technique [15], but enzymatic digestion by trypsin that cleaves peptide chains mainly at the carboxyl side of lysine, and protein denaturants like sodium dodecyl sulfate (SDS) and urea, have also been used.

In this study, we present optimization of fixation and antigen retrieval (AR) methods for ECs of flat-mounted whole human corneas, and results obtained on a very large series of corneas for IL of proteins involved in cell cycle control.

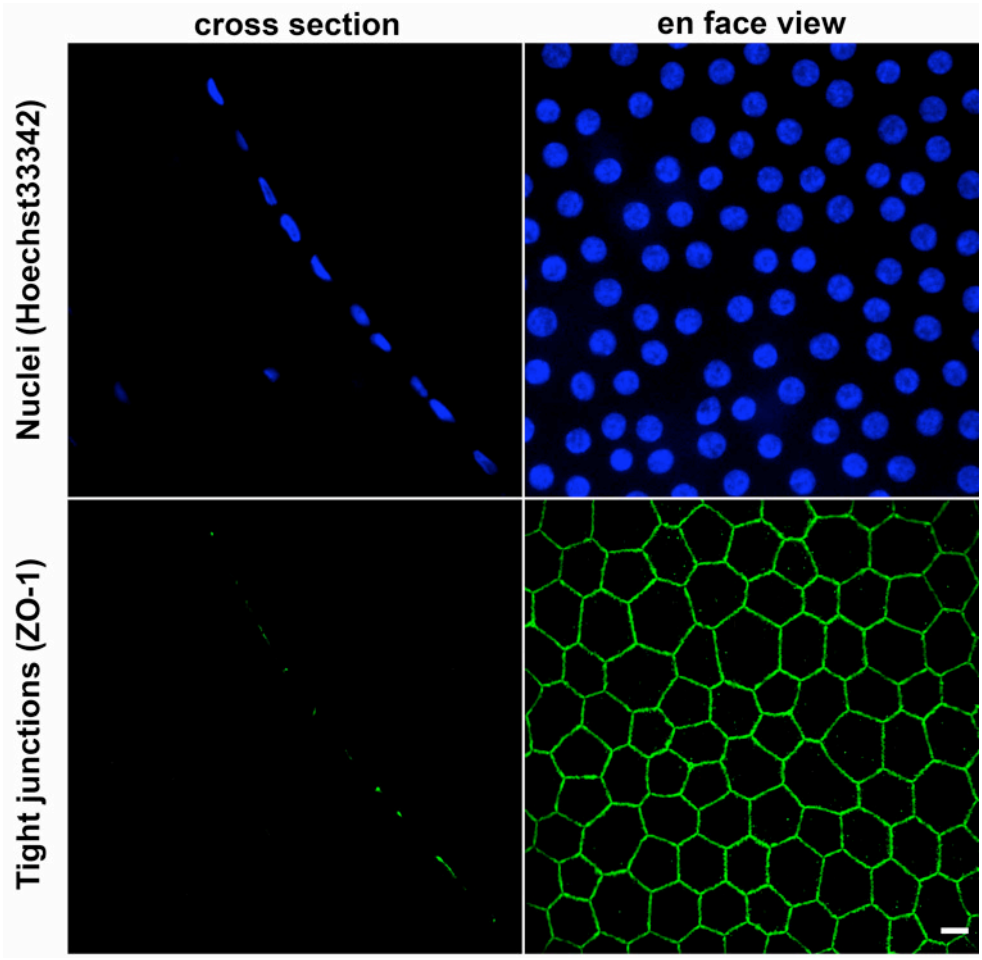

Figure 1. Hoechst 33342 nuclei staining and tight-junction immunostaining with ZO-1 antibody illustrate the differences between conventional immunohistochemistry on a corneal cross-section where only a few endothelial cells are partially visible, and en face view showing a wide field of intact cells. Original magnification x40. Bar 10 µm.

METHODS

Human corneas

Corneas were retrieved by in situ excision, the routine method for clinical use. Fifty corneas were obtained from the Department of Anatomy of the Faculty of Medicine of Saint-Etienne (body donation to science). Bodies were refrigerated at 4°C in conditions comparable with those used for donation for clinical use. Donor age (mean±standard deviation, range, median) was 81±11 years, (46 to 99, 82), and time from death to procurement was 27±14 hours (7 to 48, 23). They were fixed immediately after procurement and further processed (group 1 of fresh corneas). Seventy-six other corneas were obtained from the Eye Bank of Saint-Etienne. They were not suitable for corneal graft because of inconclusive donor serologies, but had a normal endothelium. They were stored in OC at 31°C for 25±9 days (7 to 35, 27) in CorneaMax medium (Eurobio, Les Ulis, France) before fixation and processing for immunostainings (group 2 of stored corneas). Donor age was 73±12 years (42 to 94, 76), and death-to-procurement time always less than 24 hours (13±6 hours (4 to 24, 12). Handling of the donor tissues adhered to the tenets of the Declaration of Helsinki of 1975 and its 1983 revision in protecting donor confidentiality.

Primary endothelial cell cultures

Using eight OC corneas from the group described above, ECs were isolated and cultured according to protocols published elsewhere [8]. After primary cultures reached confluence, cells were transferred to a non-precoated T25 flask and subcultured until confluence. Then, 2000 cells per well were seeded in non-precoated 96-well tissue culture plates and cultured until subconfluence before being used for ICC. These cultures were used as controls (see below).

Study design

The work comprised two steps. Firstly, we studied whether a universal corneal preparation existed for IL in ECs of flat-mounted corneas. We therefore chose four proteins known to be expressed by ECs independently of their proliferative status and localized in four different cell compartments. They were either perfectly characterized in human ECs or ubiquitous proteins: (1) ZO-1 is a peripheral membrane protein associated with tight junctions [16]; (2) actin is one of the most conserved cytoplasmic proteins in eukaryotes and is distributed in linear circumferential strands that form a hexagonal belt beneath plasmic membrane in ECs [17]; (3) heterogeneous ribonucleoprotein L (hnRNP L) is present in the nucleoplasm as part of the hnRNP complexes that play a major role in the formation, packaging, processing, and function of mRNA; (4) bound with DNA, histone H3 is one of the basic nuclear proteins responsible for the nucleosome structure of chromosomal fibres in eukaryotes.

Considering the large number of fixatives and AR methods to assess, a systematic experiment matrix was built to minimize the number of human corneas necessary. Fixatives and AR were chosen from the general histology and cytology literature [18, 19] as well as from current laboratory practices. They were classified as shown in Table 1. In order to increase the number of experiments while minimizing the number of human corneas necessary, each cornea was cut into eight pie-shaped wedges before or after fixation. Fixatives were then successively assessed and preselected once correct localization seemed to be achieved. Also in order to reduce the number of corneas necessary, secondary controls and labeling controls (see below) were done only once a protocol had been preselected. When labeling of ECs and positives controls seemed specific in terms of localization but faint and/or non-homogeneous (not all cells), AR methods were assessed successively with the preselected fixative. In case of complete negativity for all fixation protocols, AR methods were assessed only with 0.5% formaldehyde fixation (which, during the previous experiments in the study, had proved to be the most efficient fixative overall).

Secondly, we applied the previously optimized protocols to 10 proteins involved in cell cycle control: three proteins known as proliferation markers (Ki67, proliferating cell nuclear antigen (PCNA), and mini chromosome maintenance protein 2 (MCM2)); five proteins involved in controlling cell cycle phase G1 (cyclin D1, cyclin E, $p16^{INK4a}$ (p16), $p21^{WAF1/Cip1}$ (p21), and $p27^{Kip1}$ (p27)); cyclin A for the S phase (all had previously been studied in human corneas, but only Ki67 and MCM2 on flat-mounted whole corneas); and ZONAB/DbpA, a transcription factor implicated in G1-phase arrest when sequestrated by ZO-1 in several cell types [20], but never before studied in ECs. Protocols selected after step one were applied to each of the 10 target proteins. If staining proved unsatisfactory, the systematic selection process was applied from the beginning as in step one, excluding the fixatives and AR methods that consistenly had negative results during step one. For ZONAB/DbpA, after selection of the optimized protocol, double staining with ZO-1 was performed. Incubations with both primary antibodies were done successively, with three rinses in-between.

Table 1. Fixation protocols and antigen retrieval methods assessed on whole corneas.

No.	Fixation protocols
1	4% formaldehyde*, in PBS, pH 7.45 30 min at 4°C
2	0.5% formaldehyde*, in PBS, pH 7.45 30 min at 4°C
3	4% formaldehyde*, in PBS, pH 7.45 30 min at RT
4	0.5% formaldehyde*, in PBS, pH 7.45 30 min at RT
5	100% methanol 30 min at -20°C
6	100% methanol 30 min at RT
7	100% acetone 30 min at -20°C
8	100% acetone/100% methanol (1:1 v/v) 30 min at -20°C
9	100% acetone/100% methanol (1:1 v/v) 30 min at RT
10	Carnoy I** 30 min at -20°C
11	Carnoy I** 30 min at RT
12	Bouin modified*** 45 min at RT
13	0.5% $Zn(OAc)_2$ + 0.5% $ZnCl_2$ + 0.05% $Ca(OAc)_2$ in 0.1M Tris base buffer 45 min at RT (Tris-Zinc)
14	$HgCl_2$ 6g in formalin (37%-40%) 10mL + distilled water 90ml 45 min at RT
15	$ZnCl_2$ 0.16g + PFA 4g + NaCl 5g in distilled water 100mL 45 min at RT

No	Antigen retrieval methods
1	0.1M citrate buffer pH6, 4 hours at 55°C[§]
2	0.01M EDTA buffer pH10, 4 hours at 55°C[§]
3	0.1% trypsin in PBS, 5 min at 37°C
4	4M urea in water, 4 hours at RT
5	0.5% sodium dodecyl sulfate in water, 5 min at RT

PBS phosphate buffer saline
RT room temperature
***** always made fresh from paraformaldehyde
****** 100% ethanol/100% acetic acid (3:1 v/v)
******* 75 mL picric acid saturated in water+25 mL 4% PFA+5mL acetic acid
[§] the highest temperature that did not induce shrinking of fixed corneas

Immunolocalization on flat-mounted whole corneas

Corneas were rinsed in PBS before fixation. All tissues were fixed immediately after retrieval or after being removed from OC medium, as a delay between retrieval and fixation is detrimental for immunostaining results [11]. To increase the number of experiments, each cornea was cut into eight pie-shaped wedges before or after fixation, but we occasionally verified that the same results were observed for entire corneas (data not shown) to eliminate artefacts caused by the cut and border effects. Cell membranes were permeabilized by 1% Triton x-100 in PBS for 5 min at room temperature (RT) after fixation. This step was omitted when methanol, acetone or SDS was used, as they alter lipids by themselves. AR methods were then applied each time necessary (for details, see below). The non-specific binding sites were then saturated by incubation with goat serum for 30 minutes at 37°C. Primary and secondary antibodies were diluted in PBS containing 2% bovine serum albumin (BSA) and 2% goat serum. Primary antibodies were diluted in the same blocking buffer and incubated for 1 hour at 37°C. Corneal pieces were totally immersed in 100 µl of this solution in eppendorf microtubes in order to expose both endothelial and epithelial surfaces. Incubation with secondary antibodies diluted 1:500 in PBS containing 2% BSA and 2% goat serum was done for 45 minutes at 37°C. Nuclei were finally counterstained with Hoechst 33342 (Sigma) 10µg/mL in PBS at RT for 2 minutes. Three rinses in PBS were performed between all steps, except between saturation of non-specific protein binding sites and incubation with primary antibody. The corneal piece was finally placed on a glass slide, covered with PBS and gently flattened using a large glass coverslip retained by adhesive tape. Experiments were done at RT unless otherwise stated. Images were captured by a fluorescence inverted microscope IX81 (Olympus, Tokyo, Japan) equipped with the Cell^P imaging software (Soft Imaging System GmbH, Munster, Germany). This non-confocal microscope was chosen to obtain large-field images able to tolerate the residual 3D aspect of cell layers after flat-mounting.

The primary antibodies and their dilutions are listed in Table 2. Whenever possible, they were chosen from among antibodies validated for ICC by their manufacturers, which seemed closest to our technique. Non-specific rabbit and/or mouse IgG (Zymed, Carlsbad, CA, respectively ref 02-6102 and 02-6502) were used as primary antibodies for negative controls (i.e. secondary antibody control [21]). Complementarily, labeling control

was performed by omitting primary and secondary antibodies, to identify potential autofluorescence. Secondary antibodies were Alexa Fluor 488 goat anti-mouse IgG or/and Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen, Eugene, OR).

Table 2. List of primary antibodies.

Target proteins	Animal source	Clonality	Laboratory	Reference	Dilution
ZO-1	mouse	monoclonal	Zymed, Carlsbad, CA	33-9100	1/200
actin	rabbit	polyclonal	Sigma, Saint Louis, MO	A2066	1/200
hnRNP L	mouse	monoclonal	Abcam, Cambridge, UK	ab6160	1/200
histone H3	rabbit	polyclonal	Abcam	ab1791	1/200
Ki67 clone MIB-1	mouse	monoclonal	Dako, Glostrup, Denmark	M7240	1/100
PCNA	rabbit	polyclonal	Abcam	ab18197	1/200
MCM-2	rabbit	polyclonal	Abcam	ab31159	1/200
*cyclin D1	rabbit	monoclonal	Abcam	ab16663	1/200
*cyclin D1	rabbit	polyclonal	Abcam	ab31450	1/200
cyclin E	rabbit	polyclonal	Abcam	ab7959	1/100
cyclin A	mouse	monoclonal	Abcam	Ab38	1/200
$p16^{INK4a}$	rabbit	polyclonal	Interchim, Fremont, CA	#RB-025-PABX	1/200
*$p21^{Cip/WAF1}$	mouse	monoclonal	Cell Signaling, Danvers, MA	#2946	1/200
*$p21^{Cip/WAF1}$	rabbit	polyclonal	Santa Cruz, Santa Cruz, CA	sc-397	1/200
*$p21^{Cip/WAF1}$	mouse	monoclonal	Zymed, San Francisco, CA	33-7000	1/200
$p27^{kip1}$	mouse	monoclonal	Sigma	P2092	1/200
ZONAB	rabbit	polyclonal	Zymed	40-2800	1/200

*For cyclin D1 and p21, different antibodies were tested because of initial negative results.

Controls for primary antibody specificity

In order to perform the equivalent of a primary antibody control [21] as an indicator for antibody specificity, immunostaining results in ECs of flat-mounted corneas were compared with those of two types of cells: epithelial cells of the same flat-mounted corneal piece, and primary cultures of ECs studied with ICC. Epithelial cells, known to form a highly proliferative layer during OC [22], served as positive controls for the whole process with predictable patterns of expression for proliferation markers and proteins involved in cell cycle control. Primary EC cultures served as positive controls of the same cell type, using conventional ICC techniques.

Immunocytochemistry on endothelial cell cultures

Nonconfluent cell cultures were rinsed in PBS and fixed with 4% formaldehyde in PBS at RT for 15 min without any subsequent AR. Permeabilization, saturation of the non-specific binding sites, immunoreactions, and counterstaining were performed as described above for flat-mounts.

Efficiency criterion

Protocols were deemed optimized once labeling exhibited correct localization at light microscopic level, with homogeneous staining, without zone effect (the first 100 µm near the cutting edges were excluded), and

absence of staining in secondary antibody and labeling controls. Defining correct localization was easy for ZO-1, Actin, hnRNP L and H3. For the 10 cell cycle-related proteins, for which no absolute certainty existed, localization was considered correct when it agreed with their fundamental characteristics and by comparison with the staining patterns in epithelial cells and EC cultures (both primary antibody controls). Homogeneous staining was the last factor required. In case of equivalence between protocols in terms of specificity, the brightest staining was selected. Whatever the result (positive or negative), each combination was assessed on three independent corneas. The experiment was done twice more before a protocol was rated “optimized”. Immunostainings were evaluated independently by two observers experienced in immunohistochemical assessment (ZH, GT) on digital pictures blind to the preparation used. Subcellular localization, extent, homogeneity and intensity (signal/noise ratio) of immunostainings were considered. Intensity of staining was judged weak, moderate, or strong. In case of disagreement, a third judge (PG) reviewed the pictures.

RESULTS

Optimization of immulocalization

ZO-1, actin, hnRNPL, and histone H3

Standard IHC fixation with 4% formaldehyde (at 4°C or RT) gave unacceptable results for the four proteins: absence of staining for actin, very weak staining or heterogeneous pattern for hnRNPL and ZO-1, or aberrant pattern (cytoplasmic instead of nuclear) for H3. These results clearly demonstrated the need for optimization. Using systematic testing, we found several protocols that provided the expected localization for the four proteins in ECs and epithelial cells, at least for the most superficial layers. (Figure 3 and Table 3). Results were similar for both fresh and stored corneas (data not shown). Moreover, results of each protocol, whether correct or not, were always coherent between endothelium and epithelium: no discrepancy (correct localization on one side with aberrant staining on the other) was ever observed, confirming that epithelium can serve as a primary antibody control. Negative controls (with irrelevant antibodies and without antibodies) remained constantly unstained. Fixation in pure methanol at RT for 30 min appeared the best compromise to localize the four proteins present in four different cell compartments, even if not optimal for both nuclear proteins. AR greatly improved H3 labeling.

Table 3. Optimization of immunolocalization of ZO-1, actin, hnRNP L, and histone H3 in endothelial cells on flat-mounted whole human corneas.

Target protein	Classification	Selected protocols
ZO-1	best	methanol at -20°C
	second choice	methanol at RT; 0.5% formaldehyde at RT; Carnoy I at -20°C
Actin	best	methanol at RT
	second choice	Carnoy I at -20°C
hnRNP L	best	0.5% formaldehyde at RT
	second choice	methanol at RT
histone H3	best	0.5% formaldehyde + AR with 0.5% SDS in water 5 min at RT *
	second choice	methanol at RT

* SDS: Sodium dodecyl sulfate. In these cases fixation was prolonged during 1 hour to avoid endothelial cell detachment induced by SDS with short time fixation. For all other cases, fixation during 30 minutes.

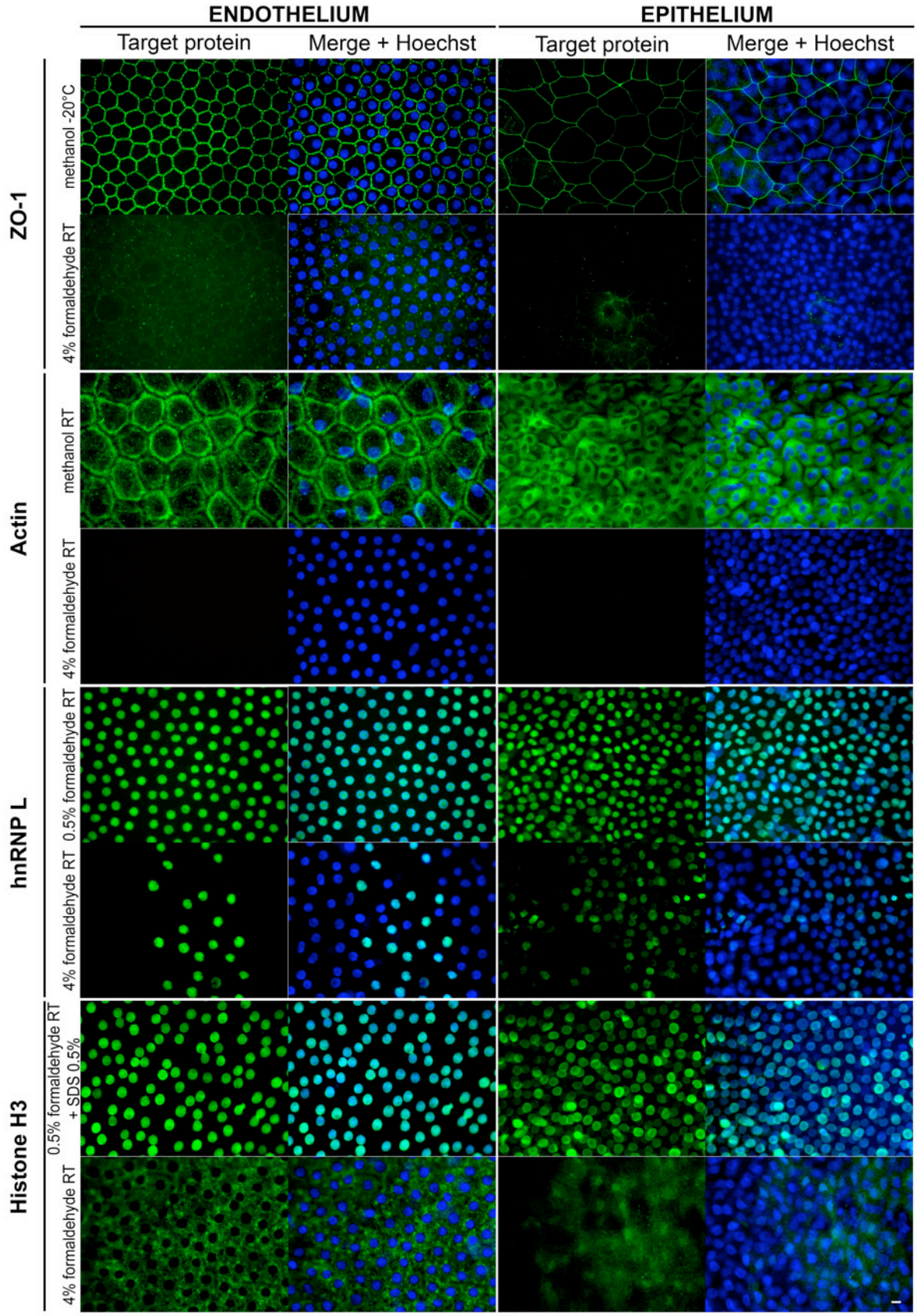

Figure 2. Optimization of immunolocalization of ZO-1, actin, hnRNP L and histone H3 in endothelial cells on flat-mounted whole human corneas. Only the best combination is shown in the first line, compared with the 4% formaldehyde usually considered as the gold standard in immunohisto- and cytochemistry. Original magnification x40. Bar 10 µm.

Cell cycle-related proteins

Methanol for 30 min at RT, found to be the best compromise in the previous step, was not able to provide correct IL for the 10 proteins of interest, indicating that antibody/antigen specific preparations were probably necessary. Selected protocols are summarized in Table 4. There was no ubiquitous protocol but 0.5% formaldehyde alone was nevertheless usable for nine out of 10 cases, even if it was not optimal for four antibodies. Notably, 0.5% formaldehyde alone was the only solution for p21, p27 and ZONAB antibodies. AR strongly improved immunostaininq quality (brightness and contrast) for PCNA, cyclin E, and p16. It was even the only efficient solution for cyclin A. Each time AR was necessary, it increased the risk of EC detachment from Descemet membrane, so fixation time was increased to 1 hour to reduce this risk. As for ZO-1, Actin, hnRNPL, and H3, results of all assessed combinations were coherent between endothelial and epithelial cells of the same cornea. As we could not obtain cyclin D1 or p21 staining in ECs of flat-mounts with any of the protocols with the antibodies initially chosen, we assessed other antibodies using the same procedure. Staining of positive controls was always comparable between antibodies of the same target, using the same protocols. EC results are presented bellow.

Table 4. Optimization of immunolocalization for cell cycle-related proteins in endothelial cells on flat-mounted whole human corneas.

Target protein	Classification	Selected protocols
Ki67	best	0.5% formaldehyde
	alternatives	4% formaldehyde; methanol
PCNA	best	0.5% formaldehyde 60 min at RT + AR with 0.5% SDS 5 min at RT
	alternatives	0.5% formaldehyde; methanol
MCM-2	best	0.5% formaldehyde
	alternatives	/
cyclin D1 (abcam 31450)	best	Carnoy I at -20°C
	alternatives	0.5% formaldehyde
cyclin E	best	0.5% formaldehyde 60min at RT + AR with 0.1% trypsin 5 min at 37°C
	alternatives	0.5% formaldehyde
cyclin A	best	0.5% formaldehyde 60min at RT + AR with 0.5% SDS 5 min at RT
	alternatives	/
P16	best	0.5% formaldehyde for 60min at RT + AR with 0.5% SDS 5 min at RT
	alternatives	0.5% formaldehyde
P21 (cell signaling #2946)	best	0.5% formaldehyde
	alternatives	/
P27	best	0.5% formaldehyde
	alternatives	/
Zonab	best	0.5% formaldehyde
	alternatives	/

Fixation was at RT during 30 minutes, unless otherwise stated.
/ = absence of second choice protocol

For ICC on primary EC cultures, 4% formaldehyde gave reproducible correct IL for all proteins tested except for cyclin A, which required additional AR with 0.5% SDS in water for 5 minutes at RT.

Summary of optimized protocols obtained on flat-mounted whole corneas

Fixation with 0.5% formaldehyde at RT with or without AR was efficient for 13 antibodies/proteins out of 14, and considered as the best protocol for 11 out of them. Fixation with 0.5% formaldehyde alone for 30 minutes was efficient for 11 antibodies and considered as the best protocol for six antibodies. Two AR methods combined with 0.5% formaldehyde improved detection for four antibodies: 0.5% SDS in water at RT for 5 min and 0.1% trypsin in PBS at 37°C for 5 min. Fixation with methanol alone was efficient for six antibodies, and considered as the best protocol for two antibodies.

Cell cycle protein expression

Proliferation markers

We were first interested in analyzing the presence and localization of proliferation markers widely used in clinical histological studies, and selected Ki67, PCNA and MCM2 (Figure 3 and table 5). Ki67 positive cells were not detected in ECs of whole corneas. A few positive cells were observed in the epithelium of fresh corneas with death-to-procurement time under 24 hours. The number of Ki67 positive cells always increased dramatically in the epithelium of stored corneas. In *in vitro* ECs, Ki67 positive cells were numerous. Staining pattern characteristics of Ki67 were clearly visible in nuclei. Occasionally epithelial mitosis were observed, confirming completion of the cell cycle.

PCNA was essentially detected with an invariable signal in the nucleus of all ECs on both fresh and stored corneas and of *in vitro* ECs. Both nuclear and weak cytoplasmic expression was found in a few epithelial cells of fresh corneas. After storage, PCNA was detected in the nucleus of all epithelial cells.

The staining pattern of MCM2 differed totally in ECs and epithelial cells. It was mainly cytoplasmic in the vast majority of ECs of both fresh and stored corneas. It filled the lateral expansions of cytoplasm located at the basal Descemetic pole of ECs, giving a characteristic hairy aspect. MCM2 was preferentially detected in the nucleus of *in vitro* ECs, but cytoplasmic staining was also observed. In the epithelium, MCM2 was exclusively found in the nucleus of only a few cells on fresh corneas but in those of almost all cells on stored corneas.

G1 and S phase markers

We next analysed the presence and localization of cyclins involved in the G1/S transition and S-phase, cyclin D1, cyclin E and cyclin A (Figure 4 and Table 5). The expression pattern of both cyclin D1 and cyclin E also differed totally in ECs and epithelial cells. Surprinsingly, with the monoclonal rabbit antibody (abcam, ab16663) no staining was observed in ECs whatever the sample preparation. With the polyclonal rabbit antibody

(abcam, ab31450) cyclin D1 was absent or had dim paranuclear staining in fresh corneas' ECs, which increased, after storage. Nuclear and weak cytoplasmic staining was found in in vitro ECs. In epithelium, with both antibodies, cytoplasmic localization was observed on fresh corneas. After storage, cyclin D1 had a predominant nuclear localization in almost all epithelial cells.

Cyclin E was homogeneously detected in the nucleus of all ECs on both fresh and OC corneas but exclusively in the cytoplasm of *in vitro* ECs. In contrast, in the epithelium it was heterogeneously detected either in the cytoplasm, the nucleus or both and OC seemed to increase nuclear staining.

Cyclin A was found in almost all ECs on both fresh and stored corneas with a cytoplasmic localization. Hexagonal cytoplamsic patterns were observed in ECs on fresh corneas. Nuclear staining was predominant in *in vitro* ECs. In the epithelium, cyclin A was mostly cytoplasmic on fresh corneas, but accumulated in the nucleus in numerous cells after storage.

Cell cycle inhibitors

We next analysed the presence and localization of cyclin-dependent kinase inhibitors, p16, p21 and p27 (Figure 5 and Table 5).

p16 , a Cdk4 and Cdk6 inhibitor that play a role at the G1/S transistion was detected homogeneously in the nucleus of all ECs of both fresh and stored corneas. Weak cytoplasmic or both nuclear and cytoplasmic staining was observed in *in vitro* ECs as well as in epithelial cells on fresh corneas. After storage, the nuclear accumulation of p16 disappeared in all epithelial cells.

We found no p21, a Cdk1 and Cdk2 inhibitor, in ECs or in fresh or stored corneas, with any of the three antibodies assessed under the various sample preparations. Nevertheless, p21 was found in the nucleus of *in vitro* ECs in an identical manner with all three antibodies, but the best results were with the mouse monoclonal antibody (Cell Signaling #2946). In epithelial cells, p21 was predominantly found in the cytoplasm of fresh corneas, with a particular granular pattern in the most superficial cells (especially with death-to-procurement time below 24 hours). After storage, p21 was detected exclusively in the nucleus of epithelial cells.

p27, also a Cdk1 and Cdk2 inhibitor, was homogeneously detected in the nucleus of all ECs of both fresh and stored corneas. It was detected in both the nucleus and cytoplasm of in vitro ECs. In epithelial cells on fresh corneas, p27 was heterogeneously detected in cytoplasm, nuclei or both. Similarly to p21, cytoplasmic localization had a particular granular pattern in epithelial cells on fresh corneas with a short death-to-procurement time. After storage, p27 staining dramatically decreased and concerned only a few nuclei.

ZONAB was expressed predominantly in nucleus of all ECs on both fresh and stored corneas. It did not co-localize with ZO-1 (Figure 7).

Table 5. Subcellular localization of the proteins involved in the cell cycle in endothelial cells and in both controls (in vitro endothelial cells and epithelial cells of whole flat-mounted corneas).

	EC			Epithelial cells	
	Fresh	**Stored**	**In vitro cultures**	**Fresh**	**Stored**
Ki67	A	A	N	N	N
PCNA	N	N	N, N+C	N, C, N+C	N
MCM2	C	C	N, N+C	N	N
cyclin D1	C	C	N, N+C	C, N+C	N
cyclin E	N	N	C	N, C, N+C	N, C, N+C
cyclin A	C	C	N, N+C	C	N, C, N+C
p16	N	N	N, C, N+C	N, C, N/C	C
p21	A	A	N	N, C	N
p27	N	N	N, C, N+C	N, C, N+C	N, C, N+C
ZONAB	N	N	nd	nd	nd

A: absence of detection; N: nuclear; C: cytoplasmic, N+C: both nuclear and cytoplasmic; nd: not done

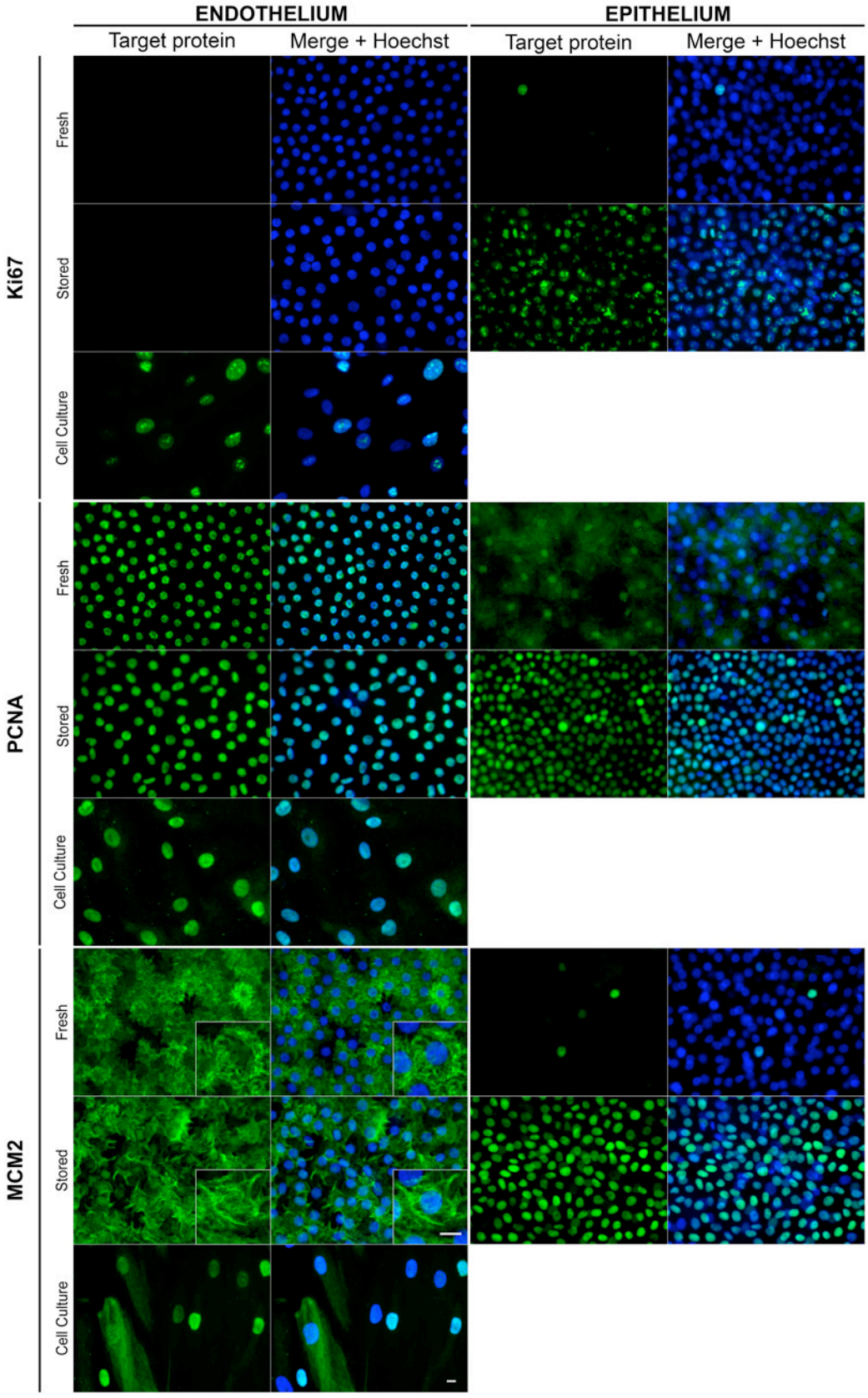

Figure 3. Optimized immunolocalization of proliferation markers in endothelial cells on flat-mounted whole human corneas. Only the best combination of fixative and epitope retrieval is shown for each antibody (see Table 4). Original magnification x40 except insets (x96). Bar 10 µm.

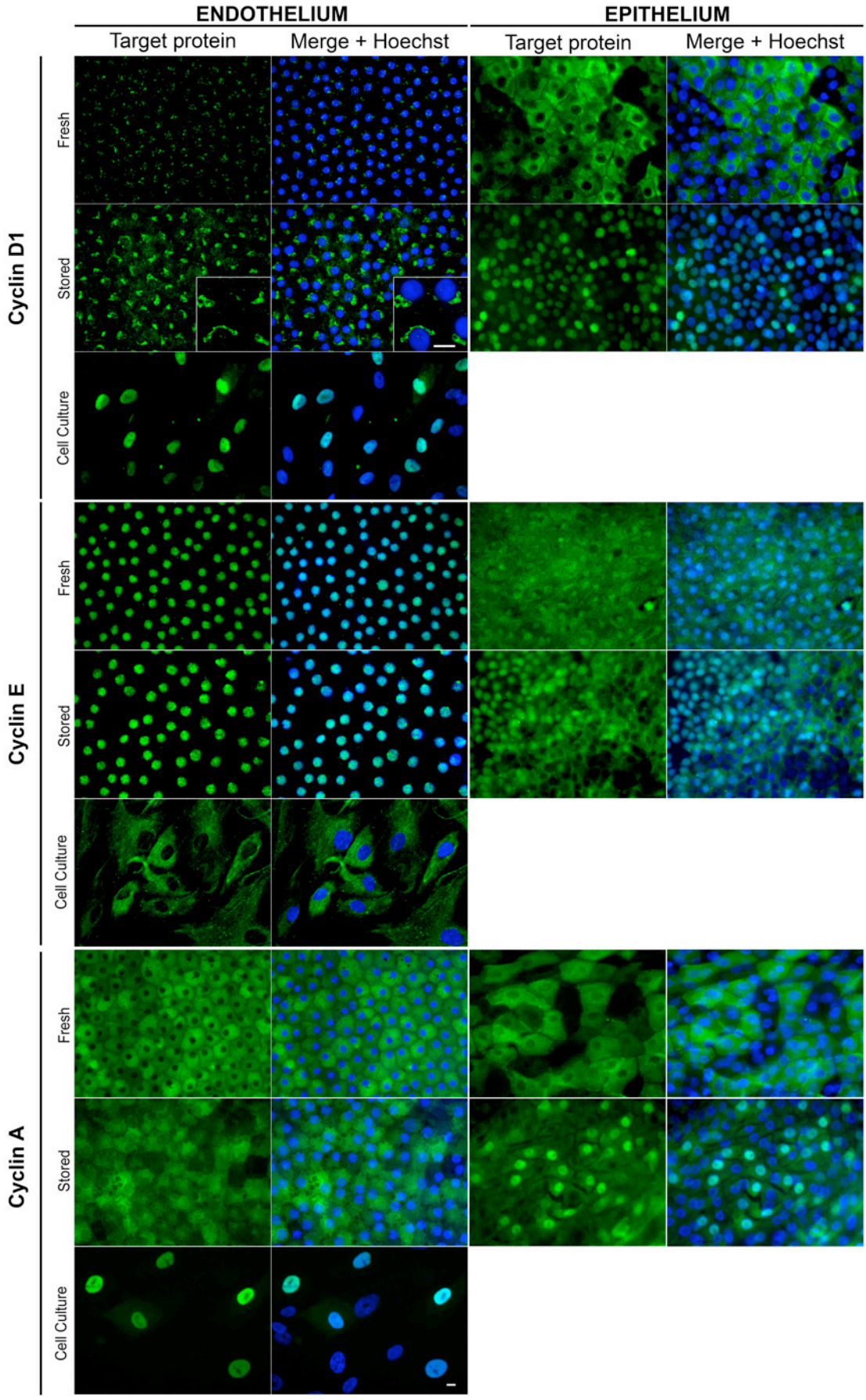

Figure 4. Optimized immunolocalization of cyclins D1, E and A in endothelial cells on flat-mounted whole human corneas. Only the best combination of fixative and epitope retrieval is shown for each antibody (see Table 4). Original magnification x40 except insets (x96). Bar 10 µm.

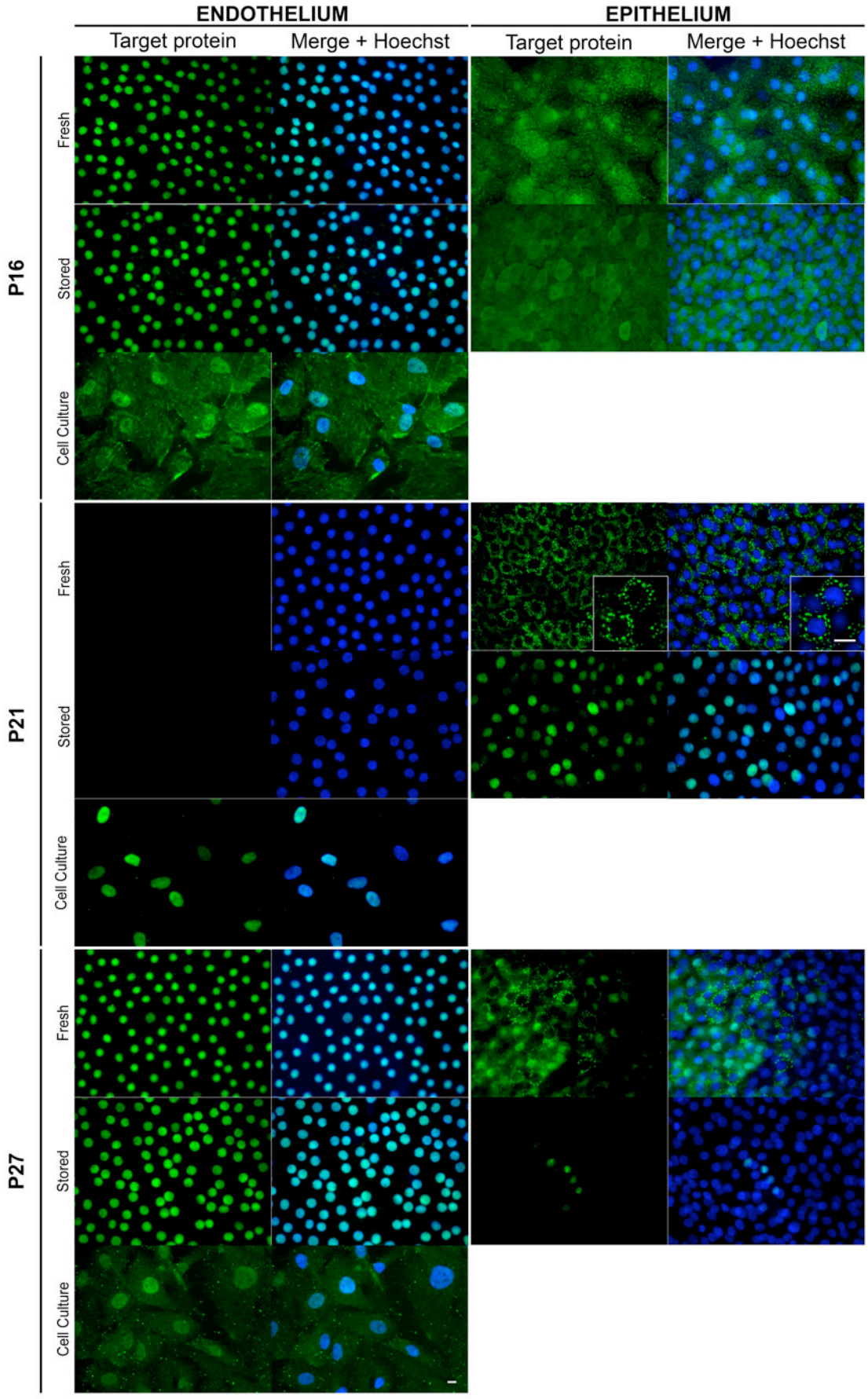

Figure 5. Optimized immunolocalization of three cyclin dependent kinase inhibitors p16, p21, and p27 in endothelial cells on flat-mounted whole human corneas. Only the best combination of fixative and epitope retrieval is shown for each antibody (see Table 4). Original magnification x40 except insets (x96). Bar 10 µm.

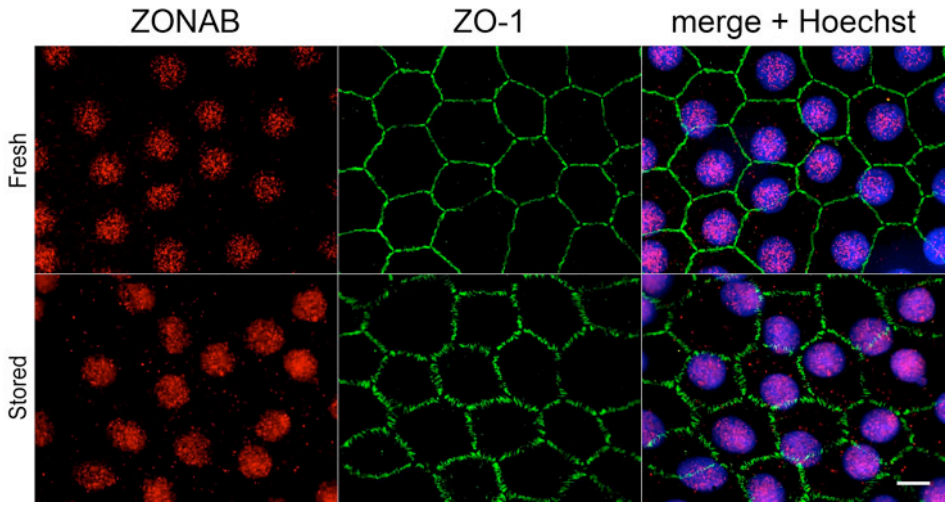

Figure 6. Optimized immunolocalization of ZONAB/BdpA in endothelial cells on flat-mounted whole human corneas, combined with ZO-1. Only the best combination of fixative and epitope retrieval is shown (see Table 4). Original magnification x40. Bar 10 µm.

DISCUSSION

Through systematic testing, on a very large series of human corneas, of most of the fixatives and AR methods that we felt were applicable to the cornea, we determined the optimal preparations for several cell cycle control proteins.

Optimization of immunolocalization on flat-mounted whole corneas

Endothelial immunostaining on flat-mounted corneas offers many advantages over that done on conventional histological cross-sections, but requires adaptation of the usual protocols due to the specificities of the endothelium. IL on flat-mounted whole corneas (intact cell monolayer on intact tissue) possesses the characteristics of both IHC (tissue) and ICC (intact cells). Contrary to IHC, where the content of cross-sectioned cells is easily visible, membrane permeabilization is required for intact ECs of whole flat-mounted corneas to diffuse bulky antibodies and secondary labels. This may be achieved by certain fixatives alone (altering lipids) or require an additional step of membrane permeabilization. Moreover, while dangers of over- [11] and underfixation [23] have been highlighted for IHC on formalin-fixed tissue, they have not been discussed for a superficial cell layer such as ECs. These form a monolayer of cells that are strongly joined together but adhere relatively poorly to their Descemet membrane. They withstand certain reagents better than cells cultured in vitro on slides, but less well than cells nested in tissue mass. To preserve the integrity of this fragile monolayer, we found a compromise between tissue fixation, permeabilisation of cell membranes to give access to antibodies, and restricted use of AR techniques, which, though useful, carry a high risk of cell detachment or tissue retraction, especially those involving high temperature. In particular, we showed that the most common fixation method, 4% formaldehyde, did not achieve correct localisation of most of the proteins studied, irrespective of their cell compartment, probably because there is high and rapid cross-linking of antigenic targets in the directly acccesible superficial cells [24].

We did not find a universal protocol able to satisfactorily reveal all antigens in ECs. Nevertheless, we selected several efficient solutions that could help standardize immunostaining of ECs and facilitate inter-laboratory comparisons. Notably, 0.5% formaldehyde with or without AR or methanol at RT for 30 minutes are the three simplest and most efficient protocols, as all antigens tested seemed correctly localized by one or another of the protocols. The efficacy of a low concentration of formaldehyde had been described for immunostaining on cytologic preparations [18]. It seems therefore that overfixing was the main obstacle during IL on ECs of flat-mounted corneas.

Interestingly, AR with SDS greatly improved immunolocalization of the three nuclear proteins H3, PCNA and P16. SDS is a detergent, used in the SDS-PAGE technique and DNA extraction, because it can disrupt non-covalent bonds between proteins, and between proteins and DNA, a property also useful for AR. Histone H3 is

one of the proteic constituents of chromatin, while PCNA can be presented in chromatin-associated form [25], and P16 can directly interact with PCNA in nuclei [26]. Our data suggest that SDS enhanced staining quality after formaldehyde fixation by unmasking the epitopic fragments of histone H3 or PCNA, which are probably hidden by their physical interaction with DNA.

We used two complementary positive controls for primary antibodies [21]: epithelial cells of the same cornea, observed at the same time on the opposite side of the flat-mount, and human corneal EC in primary cultures. Both were useful to distinguish negative staining of EC due to absence of expression or due to an unadapted protocol. Observation of the epithelial layer was particularly useful because it was submitted to exactly the same preparation as EC, as corneal pieces were entirely immersed in the different solutions. The difficulty relating to the multilayered organization of the corneal epithelium was taken into account during interpretation. Use of a non-confocal microscope was in this respect an advantage, as we could observe two or three layers of cells at the same time. In vitro ECs did not behave in the same way: stronger fixation (4% formalhedyde) was necessary to avoid cell detachment during the rest of the process. Why 4% formaldehyde allows perfect localization in *in vitro* ECs and not *in situ*, where a lesser concentration (0.5%) is far more appropriate, remains unclear. The surface coating layer present *in vivo* on top of the EC plasma membrane, comprising, among other molecules, membrane proteins, proteoglycans and glycoproteins[27] may form after 4% formaldehyde fixation, a layer relatively impermeable to most of the antibodies.

Expression of the ten cell cycle proteins

To the best of our knowledge, investigation of a large range of proteins involved in the cell cycle had never been performed in fresh human corneas fixed immediately after procurement in donors, without being stored before study. Only Ki67 (clone SP6, Labvision, Fremount, CA) and PCNA (clone PC 10, Dako, Glostrup, Denmark) had been studied on 4% PFA-fixed cross-sections of fresh corneas, but with extended postmortem time (29 to 163 hours) [28] and only PCNA on cross-sections of fresh corneas (postmortem time not available) [29]. Modifications in protein expression profile induced by any storage medium (4°C or OC), even when storage is short, cannot be ruled out. By avoiding this storage time, one is sure to stay as close as possible to physiologic conditions, except for the postmortem metabolic changes that inevitably also occur. Only the study of corneas retrieved from enucleated globes for posterior tumor would ensure true physiological conditions, but these cases are fortunately scarce and do not provide a sufficient source of human tissue.

We compared fresh corneas with similar corneas after several weeks of storage in OC and highlighted several differences never described before. Conventional OC media are derived from Dulbecco's modified Eagles minimum essential medium supplemented with 2% FCS necessary to ensure EC survival up to 5 weeks. They also stimulate wound healing by cell migration and enlargement [30] but are not known to stimulate EC

proliferation. Only in a very specific OC medium containing 8% FCS has EC proliferation been described *in situ* [28, 29]. On the contrary, non-confluent *in vitro* cultures of human adult ECs contain numerous proliferating cells, explaining a profile totally different from EC *in situ*. Overall, the profile of cultured ECs is very similar to that of epithelial cells after OC, both being used as primary antibody controls [21]. During OC, the epithelial layer is highly activated and may completely regenerate even after long death-to-retrieval time [22]. Nevertheless, although cells proliferate, the number of cell layers gradually decreased during OC [31], explaining why we observed mainly basal cells where higher proliferative activity occurs. In this article, we have described both a method for systematically assessing antibodies on whole mount en face cornea, and staining patterns for a set of antibodies directed against cell-cycle proteins. We are well aware that the use of a single technique does not allow for definitive interpretation of data, and that additional primary antibody controls should be added. Several methods could be applicable to ECs, such as immunostaining after knocking down the specific protein expression with siRNA, a combination of immunostaining and fluorescent in situ hybridization, or immunoblots, and will be the subjects for future studies, as they require substantial technical developments. The following interpretation of our data should therefore be seen as open avenues for further investigations.

The Ki67 antibody was originally identified as a reliable marker for a nuclear antigen present in proliferating cells, from late G1 to M phase, and is therefore approved as a ubiquitous proliferation marker [32, 33]. As expected, no Ki67 staining was observed in ECs of fresh and stored corneas, whereas it was abundant in epithelial cells and in vitro ECs, two situations where cells proliferate. Ki67 positive ECs and even mitosis have previously been described on wholemounts of human corneas, with clone MIB-1 by Zymed (San Francisco, CA), in experiments of wound healing in presence of 10% FBS and growth factors [34]. In this study, corneal pieces were fixed for 10 minutes in ice-cold methanol and incubated in 1% Triton X-100 to permeabilize the cells. We also found methanol to be a possible fixative for Ki67, but 0.5% formaldehyde without subsequent cell permeabilization seemed better. From the Ki67 pattern, it appears that whereas the epithelium is highly proliferative in OC, the endothelium is devoid of proliferating cells either in fresh or in stored corneas.

PCNA is another proliferation marker in a wide variety of cell types whose accumulation in the nucleus begins early in the G1 phase. It is a cofactor of DNA polymerase delta [35] but also has numerous other functions in response to DNA damage, cell cycle control, chromatin assembly and cohesion, and in other functions [25]. In ECs, PCNA had only been studied on cross-sections [28, 29]: PCNA positive ECs were found in long-term OC corneas but not in fresh corneas [29]. On the contrary, we observed homogeneous nuclear PCNA staining in all ECs of fresh and stored corneas, indicating that PCNA may not be considered as a proliferation marker in this population, whereas staining was more typical in epithelial cells with an increase of nuclear staining after OC. The role of PCNA in ECs remains however to be determined but could be related to nuclear oxidative DNA damage that accumulates in ECs throughout their life [36]. Furthermore, there could be a

relationship with the ubiquitous nuclear localization of p16 that we found in ECs of fresh and stored corneas, as p16 can interact directly with PCNA and inhibit DNA polymerase delta activity [26].

MCM2–7 are highly conserved proteins, which form a hexameric complex with replicative helicase activity [37] involved in the regulation of DNA duplication [38]. MCM2 is undetectable in G0, and accumulates in the nucleus during G1, peaking on S-phase entry [39], which explains its role as a proliferation marker. MCM2 is then eliminated from the nucleus during the S phase. In ECs *in situ*, we found no nuclear MCM2 but dense fibrillary cytoplasmic staining, unmodified after storage. The irregular shape of ECs was due simply to the filling by fluorescent markers of the irregular basolateral cytoplasmic expansions previously described [40, 41]. In contrast, MCM2 was always detected exclusively in nuclei of the proliferative epithelial cells and preferentially in nuclei of in vitro ECs. Incidentally, a similar pattern in cultured ECs had already been described [42]. Interestingly, the sequestration of MCM proteins in cytoplasm and therefore the inability of MCM to initiate DNA synthesis is one of the mechanisms by which progesterone inhibits estrogen-induced uterin epithelial cell proliferation [43]. We propose that MCM2 sequestration in ECs could play a role in G1-phase arrest. Nuclear localization of MCM2 had previously been described in nuclei of ECs using corneal pieces fixed for 10 minutes in ice-cold methanol and incubated in 1% Triton X-100 to permeabilize the cells (MCM2 antibody: BD Pharmingen, San Diego ,CA) [7]. In the latter experiments, designed to analyse proliferation in response to a calibrated wound, corneas initially stored at 4°C for several days were subsequently incubated at 37°C in culture medium supplemented with 8% FCS and various growth factors. MCM2 positive nuclei were observed between 36 to 72 hours after wounding and only in ECs close to the wound. These results do not contradict our own, since our OC conditions differ greatly from these proliferation-promoting conditions. In our hands, only 0.5% formaldehyde was able to make the Abcam MCM2 antibody react. Methanol did not help reveal cytoplasmic MCM2.

Cyclins and their catalytic partner cyclin-dependent kinases (CDKs), regulate cell cycle progression in eukaryotes. The three D cyclins (D1, D2, D3) accumulate during G1 phase in response to mitogenic stimulation, and assemble with CDK4 and 6. Cyclin D1 is an important actor in G1 to S progression by inactivating the cell cycle-inhibiting functions of the retinoblastoma protein through phosphorylation. In addition, cyclin D/CDK complexes sequester CDK inhibitors such as p21 and p27. Nevertheless, once assembled with CDK within the cytoplasm, complexes need to be imported into the nucleus to be active. For example, it has been demonstrated that the prevention of their nuclear import plays a critical role in the maintenance of cardiomyocytes in their non-proliferative state acquired soon after birth [44]. It has also been shown that postmitotic normal neurons lose their ability to import cyclin D/CDK complexes into the nucleus and that this ectopic cytoplasmic localization plays a role in neuron survival [45]. Aside from its role in cell cycle control, cyclin D1 also possesses other CDK-independent functions [46] such as chromatin remodelling, cell metabolism, and cell migration [47]. The

exclusive cytoplasmic localization of cyclin D1 that we found in all ECs, without nuclear translocation after storage whereas it localized in nuclei of proliferating in vitro ECs and in basal epithelial cells after storage, agrees with its non-proliferative status but suggests that cyclin D1 has CDK-independent functions in ECs. It also suggests that ECs might have passed the restriction point (after which, in a normal cell cycle, cyclin D1 is exported from the nucleus).

Cyclin E/CDK2 complexes act normally after cyclin D/CDK4-6 complexes during G1 phase. Their activity rises in late G1 phase, peaks at G1/S transition, and declines quickly during the S phase [48, 49]. Nevertheless, cyclin E accumulation in nuclei with inactive cyclinE/cdk2 complexes is compatible with arrest in the G1 phase related to senescence [50] and when overexpressed in nontransformed HC11 mouse mammary epithelial cell line, cyclin E localizes exclusively in nuclei, induces an increase in p27 expression, and inhibits cell proliferation [51]. Our results, showing constant localization of cyclin E in nuclei of all ECs in fresh and stored corneas, are therefore compatible with non-proliferative status, and suggest again that ECs *in situ* might be arrested after the restriction point and before the entry in S-phase. In epithelial cells, staining was heterogeneous and nucleocytoplasmic in most cells in fresh corneas with a predominant nuclear translocation after storage, suggesting progression through the cell cycle. In *in vitro* ECs cyclin E was mostly localized in the cytoplasm, suggesting that they crossed the restriction point of the cell cycle.

Cyclin A is nomally expressed from late G1 to late G2 phases. Cyclin A/CDK complexes have substrates in both the nucleus and the cytoplasm and shuttle between the two compartments [52]. It initially assembles with CDK2 to form a complex imported in the nucleus, where it phosphorylates nuclear substrates implicated in DNA replication, thus promoting progression through the S phase [53]. In proliferating epithelial cells and in vitro ECs, cyclin A is mainly nuclear which is a hallmark of proliferating cells. We found it exclusively expressed in the cytoplasm of ECs of fresh and stored corneas, which again hints at cells having passed the restriction point. It is possible that the cytoplasmic retention of cyclin A hinders progression through S-phase. It would be interesting to assess cyclin A/CDK complexes activity in ECs in situ, as cytoplasmic substrates of the complexes might play a role in ECs physiology.

Our results for cyclins D1, E and A in whole corneas were consistent with the previous studies by Joyce et al. on cross-sections of corneas from seven donors aged 6 weeks to 67 years and stored at 4°C during 24 to 36 hours (Antibodies from Upstate Biotechnology (Lake Placid, NY)) [54, 55]. Flat mounts now allow clearer subcellular localization as well as observation of a larger population of ECs.

We detected p16 in the nuclei of ECs of both fresh and stored corneas. This is consistent with previous results of IHC done on cross-sections of human corneas, although subcellular localization was in either cytoplasm [55] or nuclei [56, 57] of ECs. P16 inhibits cyclin D/CDK4-6. It is thus considered to have a pivotal role

in G1-phase arrest of ECs and stress-induced premature senescence [58]. Furthermore, p16 could interact directly with nuclear PCNA found in ECs and inhibit DNA polymerase delta activity [26]. In epithelial cells, cytoplasmic translocation occurred after OC, corresponding to prominent proliferative status. In *in vitro* ECs, p16 was found in both nuclei and cytoplasm, indicating cytoplasmic translocation compatible with cell cycle progression.

We did not detect p21 in ECs of flat-mounted fresh or OC-stored corneas despite systematic assessment of a wide variety of fixatives and AR and the use of three antibodies. P21 positivity in the nuclei of epithelial cells of the same corneal piece and in *in vitro* ECs eliminates a false negative result in ECs. This apparently contradicts two previous studies that used IHC on cross-sections of 4°C stored human corneas. In one case, a p21 antibody C-19 from Santa Cruz Biotechnology was used on six corneas stored at 4°C and confirmed by western blots of proteins extracted from in vitro EC cultures [56]. In the second case, a p21 mouse monoclonal antibody from Lab Vision Corp, (Fremont, CA) was used on four corneoscleral rims left off after corneal graft and confirmed with quantitative RT-PCR on RNA extracted from other corneo-scleral rims [57]. Both found nuclear staining for p21. It remains unclear why we did not reveal p21 in ECs in flat-mounted cornea methods. It is possible that fresh and OC corneas with preserved metabolic activities may behave differently from corneas stored for several days at 4°C. As we found that in vitro EC cultures expressed p21 in nuclei, it is not surprising that WB was found positive by Enomoto et al. [56]. Moreover, the presence of p21 transcripts in ECs from whole corneas is not incompatible with post-transcriptional alterations resulting in absence of protein expression. At present, no other study has revealed p21 expression using immunolocalization in ECs. A direct comparison of paired corneas, one stored at 4°C and the other either studied fresh or after OC, seems necessary to answer the question. We detected p21 in the nucleus of proliferative cells: epithelial cells of OC corneas and in vitro EC cultures. P21 is one of the main CKIs implicated in late G1-phase arrest by inhibiting cyclin E/CDK2 complexes [59], but p21 can also contribute to initial G1 progression by promoting the nuclear import of cyclin D/CDK4-6 complexes [60] and by preventing the nuclear export of cyclin D [61].

P27, another CIP/KIP family member, also strongly inhibits cyclin E/CDK2 complexes and arrests the cell cycle in G1 [62]. It is implicated in cell cycle arrest triggered by contact inhibition and TGFβ [63]. Its role has been demonstrated in rats [64] and the protein has been detected in nuclei of ECs on cross-sections of two corneas from donors aged 18 and 74 years [56] and in one cornea from a 54-year-old donor [57]. The three corneas had been stored shortly at 4°C beforehand. In our study, in epithelial cells, the near-complete disappearance of p27 after OC strongly suggests cell cycle progression as, at the end of the G1 phase, cyclin E/CDK2 complexes (which we found in epithelial cell nuclei and cytoplasm) phosphorylate p27 that is subsequently ubiquitinated by Skp1/Skp2 and degraded by the proteasome [65]. In cultured ECs, p27 in both nuclei and cytoplasm also suggests possible progression beyond the restriction point.

ZONAB/DbpA is a transcription factor susceptible to play an inhibitory role during G1 progression in cells having tight junctions. Canine ZONAB is a homologue of human DbpA, an E2F target gene. ZONAB/DbpA specifically binds to the SH3 domain of ZO-1, which inhibits its function. ZO-1 can consequently sequestrate ZONAB/DbpA or ZONAB/DbpA-CDK4 complex at tight junctions, thus inhibiting G1/S transition [66]. Here we detected ZO-1 at the tight junctions and ZONAB strictly in the nucleus, suggesting that this mechanism is not involved in ECs's cell-cycle arrest. Furthermore, as it has been shown that accumulation of ectopic nuclear ZONAB/DbpA facilitates the G1/S phase transition by upregulating expression of cyclin D1 and PCNA in the human nontransformed mammary epithelial cell line MCF-10A [67], and by increasing nuclear CDK4 level in the MDCK (Madin-Darby canine kidney) cell line [20], it is possible that ZONAB in ECs in situ could facilitate passage of restriction point.

In conclusion, IL on flat-mounted whole corneas seems possible for a large number of proteins, provided that substantial adaptations in corneal fixation and further processing are adopted. Continued efforts to refine methods of antigen preservation or retrieval in human corneas will ultimately allow standardization of IL and elimination of inter-laboratory discrepancies in results, thus allowing accurate research. 0.5% formaldehyde or methanol without AR should be recommended as first-line fixatives during experimental developments, given that in situ ECs seem particularly sensitive to overfixation by aldehydes and that AR methods often harm the integrity of the endothelial monolayer. Conventional OC medium containing 2% FCS does not modify the non-proliferative status of ECs, which is compatible with G1-phase arrest. Nevertheless, our data suggest that ECs are arrested after the restriction point in the G1 phase. The role of MCM2 in cytoplasm, the subcellular localization of cycle D1, and the absence of p21 are the main characteristics highlighted by our optimized protocols. Further studies are necessary to explain these particularities of fresh and OC-stored corneas.

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Publication 3 (en preparation pour Journal of Histochemistry and Cytochemistry IF 2,38) Effects of fixatives temperature on immunolocalization in flat mounted human corneal endothelium.

Brief report

Effects of fixatives temperature on immunolocalization in flat-mounted human corneal endothelial cells.

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Summary

Corneal endothelial cells (ECs) control corneal hydration and are essential for the maintenance of corneal transparency. They are devoid of proliferative capacities, and severe dysfunctions leading to corneal blindness can only be treated by a corneal graft. Most of fundamental research works done on ECs require study of proteins expression. Imunolocalization in ECs of flat-mounted whole corneas present multiple advantages over conventional cross-sections. It allows observation of the entire intact cell mosaic and precise subcellular protein localization. It nevertheless requires several technical adaptations compared with conventional immunohisto or cytochemistry because it concerns a fragile cell monolayer at the surface a tissue. Optimal fixation and antigen retrieval are necessary to obtain reliable immunolabeling. In this experiment pure methanol and 0.5% formaldehyde were used at four increasing temperature. Four proteins with well-characterized expression patterns in ECs (Zo-1, hnRNPL, actin and histone H3) were chosen in order to easily identify staining differences. Results show that temperature strongly influenced immunolabeling quality In particular, conventional temperature (-20°C for methanol and 4°C for formaldehyde) were mostly inefficient. Room temperature appears at first-line temperature for fixation during development of immunolocalization protocols.

Introduction

The monolayer of tightly packed hexagonal flat cells forming the endothelium at the posterior surface of the cornea is crucial for the maintenance of its transparency. By controlling the stromal hydration it ensure stability of the perfect collagen fibers organization that explain tissue transparency (Maurice 1957). Despite their fundamental role in vision, endothelial cells (ECs) loose their proliferating capacities at birth (Murphy et al. 1984), which explains the limited possibilities of wound healing in case of pathological processes. In the healthy cornea, there is an important EC reserve, which explains why, despite a physiological EC loss of 0.6% per year (Bourne et al. 1997), there is no age-related corneal endothelial disease. On the contrary, in pathological processes, beyond a certain threshold of EC loss, residual cells cannot achieve their normal functions and cornea become irreversibly edematous causing permanent visual loss, only accessible to a corneal graft. Endothelial dysfunctions constitute therefore one of the first indication for corneal graft (Peh et al. 2011). The molecular mechanisms leading to the arrest of ECs in the G1 phase of the cell cycle are being stepwise understood (Joyce et al. 2002; Joyce et al. 2011) but require continuous efforts in order to develop strategies of cell therapy able to trigger and control ECs re-proliferation. The possibility to expand ECs pool would have considerable therapeutic impact. Expansion could be useful during ex vivo corneal storage by eye banks in order to increase both the number and the quality of cornea available, in vitro with a view of developing bioengineered endothelial allogenic graft, or even in vivo to cure endothelial dysfunctions.

Because of the limited quantity of ECs (300,000 to 500,000 cells per cornea) protein extractions directly from whole human corneal endothelium in view of performing immunoblots remains problematic and has only exceptionally been reported after pooling of ECs from several donors (Joyce et al. 2011). Consequently, up to now, immunolocalization techniques were the only methods used to detect proteins directly in situ in ECs. Because of its simplicity, immunohistochemistry (IHC) on cross-sections is currently the main technique used in the literature, despite it allows visualization of only a few ECs without a clear subcellular localization due EC morphology. Moreover, because of the superficial situation of the endothelium, immunostainings of ECs on cross-sections may be subject to staining artifacts frequently encountered at the tissue borders. In a recent study we have systematically developed specific immunolocalization protocols performed directly on the intact endothelium of whole cornea. The *en face* observation that we use, presents two advantages: a perfect visualization of the whole intact endothelium allowing detection of regional or local difference in staining patterns (going up to the easy detection of isolated rare cells with a specific expression profile), and a precise subcellular localization. The technique is hybrid between IHC and immunocytochemistry (ICC), because it concerns a cell monolayer on top of an intact tissue. We demonstrated that ECs were easily over-fixed when treated with conventional 4% formaldehyde, resulting in poor quality immunostaining. Instead, we showed that 0.5% formaldehyde of pure methanol, both for 30 minutes at room temperature were first choice fixatives to perform immunolocalization on intact ECs of flat-mounted whole corneas. Additional adaptation such as antigen retrieval (AR) with 0,5% sodium dodecyl sulfate (SDS) was nevertheless necessary after formaldehyde fixation for a few antigens to achieve a better result.

During this previous study, we noticed that temperature during fixation might influence staining quality. For diverse immunolocalization techniques, like IHC and ICC, the conventional fixation temperature is -20°C for methanol and 4°C for formaldehyde, although the influence of the temperature during the fixation on staining quality had never been clearly reported. In order to improve immunolocalization in ECs of flat-mounted corneas, we systematically assessed a wide range of temperature during methanol and formaldehyde fixation, using four antibodies that had previously proven efficient to reveal perfectly characterized proteins located in the different cell compartments.

Materials and Methods

Human corneas

Ten corneas were obtained from the Eye Bank of Saint-Etienne. They were initially stored in 100 mL organ culture medium (CorneaMax, Eurobio, Les Ulis, France) at 31°C without medium renewal for 22±10 days (7 to 35, median 21). Endothelilal cell density (ECD) was 1976±618 cells/mm^2 (1229 to 2898, median 2004). They were obtained from 7 donors whose age was 75±8 years (54 to 81, median 77), with time from death to retrieval always less than 24 hours. Handling of the donor tissues adhered to the tenets of the Declaration of Helsinki of 1975 and its 1983 revision in protecting donor confidentiality.

Antibodies

We chose four different proteins known to be consistently expressed in ECs with a characteristics pattern. They were either perfectly characterized in human ECs or ubiquitous proteins: (1) ZO-1 is a peripheral membrane protein associated with tight junctions (Barry et al. 1995); (2) actin is one of the most conserved cytoplasmic proteins in eukaryotes and is distributed in linear circumferential strands that form a hexagonal belt beneath plasmic membrane in ECs (Kim et al. 1992); (3) heterogeneous ribonucleoprotein L (hnRNP L) is present in the nucleoplasm as part of the hnRNP complexes that play a major role in the formation, packaging, processing, and function of mRNA; (4) bound with DNA, histone H3 is one of the basic nuclear proteins responsible for the nucleosome structure of chromosomal fibres in eukaryotes. Specific primary antibodies and their dilutions used for this study are presented Table 1. Whenever possible, they were chosen from among antibodies validated for ICC by their manufacturers, which seemed closest to our technique. Non-specific rabbit or/and mouse IgG (Zymed, Carlsbad, CA. Respectively ref 02-6102 and 02-6502) were used as primary antibodies for negative controls (i.e. secondary antibody control (Burry 2011)). Secondary antibodies were Alexa Fluor 488 goat anti-mouse IgG or/and Alexa Fluor 555 goat anti-rabbit IgG (invitrogen, Eugen, OR).

Table 1 List of primary antibodies

Target proteins	Animal source	Clonality	Laboratory	Reference	Dilution
ZO-1	mouse	monoclonal	Zymed, Carlsbad,USA	33-9100	1/200
actin	rabbit	polyclonal	Sigma, Saint Louis, MO	A2066	1/200
hnRNP L	mouse	monoclonal	abcam, Cambridge, UK	ab6160	1/200
histone H3	rabbit	polyclonal	abcam, Cambridge, UK	ab1791	1/200

Immunolocalization on flat-mounted whole corneas

The general processing of corneas followed the protocols previously described (He et al. 2011). Briefly, corneas were rinsed in PBS, cut into eight pie-shaped wedges to increase the number of experiments while saving rare tissues and fixed immediately after being removed from OC medium. Double stainings were chosen, again in order to save human corneas. Fixation was performed for 30 minutes either in formaldehyde 0.5% in PBS pH 7,45 at 4°C, 23°C (room temperature, RT), 37°C or 50°C, or in methanol at -20°C, RT, 37°C or 50°C. Fifty degrees was the highest temperature usable on whole corneas without trigerring tissue shrinking (data not shown). Cell membranes were permeabilized by 1% Triton x-100 in PBS for 5 min at room temperature (RT) after fixation. This step was omitted when SDS was used as AR method or for methanol fixation, as they alter lipids by themselves (We had previously shown that AR with 0.5% SDS in

water for 5 min at RT was necessary after formaldehyde fixation, only to reveal histone H3). The non-specific binding sites were then saturated by incubation with heat-inactivated goat serum for 30 minutes at 37°C. Primary and secondary antibodies were diluted in PBS containing 2% bovine serum albumin (BSA) and 2% heat-inactivated goat serum. Corneal pieces were totally immersed in 100 µl of this solution in eppendorf microtubes to avoid dehydration of specimens, and incubated at 37°C during 1 hour. Incubation with secondary antibodies diluted 1:500 in PBS containing 2% BSA and 2% goat serum was done for 45 minutes at 37°C. Nuclei were finally counterstained with Hoechst 33342 (Sigma) 10µg/mL in PBS at RT for 2 minutes. Three rinses in PBS were performed between all steps, except between saturation of non-specific protein binding sites and incubation with primary antibody. The corneal piece was finally placed on a glass slide, covered with PBS and gently flattened using a large glass coverslip retained by adhesive tape. Experiments were done at RT unless otherwise stated. Images were captured by a fluorescence inverted microscope IX81 (Olympus, Tokyo, Japan) equipped with the Cell^P imaging software (Soft Imaging System GmbH, Munster, Germany). This non-confocal microscope was chosen to obtain large-field images able to tolerate the residual 3D aspect of cell layers after flat-mounting. Each assay was repeated at least three times on three different corneas.

Evaluation of immunostaining

Protocols were deemed optimized once labeling exhibited correct localization at light microscopic level, with homogeneous staining in all ECs, without zone effect (the first 100 µm near the cutting edges were excluded), and absence of staining in controls. In case of equivalence between protocols in terms of specificity, the brightest staining was selected. Evaluation of immunostaining was done independently by two observers experienced in immunofluorescence (IF) assessment, on digital pictures blind to the preparation used. The intensity of staining was rated as null, weak, medium, good and excellent. In case of disagreement, a third judge reviewed the pictures.

Results

By optimizing the temperature for each of the four proteins, we obtained perfect typical staining patterns in ECs, in term of signal/noise ratio and precise subcellular localization (Figure 1 and Table 1). There was no unique temperature that gave best results for all antigens and with both fixatives. Fixation at RT gave nevertheless acceptable staining for all but one situation.

Best stainings were globally obtained with methanol. During methanol fixation, the conventional temperature of -20°C was optimal only for ZO-1 and totally inefficient for histone H3. RT was optimal for hnRNPL, 37°C for actin and 50°C for histone H3. Other combinations were sub-optimal (Figure 2 and Table 1). SDS always triggered partial or complete detachment of ECs.

During 0.5% formaldehyde fixation, the conventional temperature of 4°C was optimal only for ZO-1 and totally inefficient for actin and histone H3. RT and 37°C were optimal for hnRNPL and histone H3 but inefficient for actin, and 50°C was never optimal. Formaldehyde did not allow revealing actin at any temperature (Figure 3). AR with SDS induced almost complete detachment of the ECs from their basement membrane after 4°C fixation. Increase of the temperature at 50°C allow to keep ECs attached during subsequent treatment with SDS but the staining improvement was weak in this condition.

Discussion

In this study, we demonstrated the strong influence of temperature during fixation for immunolocalization in ECs of flat-mounted human corneas. Prolonged fixation at low temperature ensures a slow regular diffusion within tissue, intended to better preserve tissue and cell morphology. On the contrary, an elevated temperature during fixation increases the rapidity of fixation. In case of a superficial cell layer as ECs, directly accessible to fixatives, the fixation process is supposed to be extremely rapid in all cases.

Results after methanol fixation were particularly dependent on the temperature. The well-accepted cold fixation at -20°C only reveals the most superficial antigen (ZO-1) whereas increasing temperatures were required to detect the deeper antigens located in the cytoplasm (actin), nucleoplasm (hnRNPL) or bound with DNA (histone H3). The highest temperature tolerable by the cornea was indeed optimal for the deepest antigens (histoneH3). Methanol is commonly used during ICC on in vitro-cultured cells and during IHC on tissue frozen cross-sections. Classified as precipitating (or agglutinating) fixatives, it reduces proteins solubility by creating non-covalent bonds between them. Furthermore, its hydrophilic group (-OH) disrupts the hydrogen bonds between amide groups in the secondary protein structure and its hydrophobic group (R-CH2-) disrupts the intramolecular hydrophobic interactions of protein core and thus denature the proteins by changing their secondary and tertiary structure (Zellner et al. 2005). The temperature of -20°C, commonly used for tissue fixation during immunolocalization, exploits the precipitating properties of methanol while avoiding protein denaturation. This temperature was efficient only to detect ZO-1. By increasing the temperature we may increase the denaturation of proteins, especially by modifying proteins' tertiary structures, giving access to hidden epitopes. Warm methanol could therefore ensure both fixation and AR. Further study with alcohol fixatives at high temperature should be necessary to confirm this effect on other cells and tissues.

Formaldehyde cross-link proteins amino groups with other nearby nitrogen atoms in proteins or DNA through a -CH2- covalent linkage (Kiernan 2000). Four percent formaldehyde is almost a universal concentration for ICC and IHC, but we recently suggested that it results in ECs overfixation leading to poor immunostainings, and therefore recommend using 0.5% formaldehyde instead (our ref). In this study, we went one step ahead, by showing that RT and 37°C improve staining results during 0.5% formaldehyde fixation compared with 4°C which is however the usual temperature for immunolocalization techniques. The absence of improvement with 50°C may be due to accelerated protein cross-linking

resulting in overfixation. Lower performance of formaldehyde to reveal actin compared to that of methanol fixation has previously been reported without clear explanation (Allan 2000).

SDS disrupts the non-covalent bonds in proteins, between proteins and between proteins and other molecules such as DNA, allowing changes in structure conformation of proteins and antigen unmasking (Brown et al. 1996; Rajamannan et al. 2002). It is however incompatible with methanol fixation that does not create covalent bonds between ECs and their underlying basement membrane. Consequently, all ECs are rapidly lost when subsequently exposed to SDS. On the contrary, formaldehyde can allow ECs to resist AR with SDS provided a sufficient protein cross-linking occurs. The almost complete ECs detachment after fixation at 4°C proves a limited protein cross-linking. Consequently, when AR is required for a specific antigen (histone H3 in this study), an increase of temperature up to 50°C during fixation allows a better adherence of ECs that stay intact. The different possible combinations that we assessed, now allows to find the best compromise between preservation of morphology of the fragile endothelium and maintenance of a good antigenicity using chemical AR whenever necessary.

If the choice for the type and concentration of fixatives and AR methods are the most crucial steps during the assessment of a new antibody on ECs of flat-mounted whole cornea, the temperature during fixation may also be determinant to improve staining quality. RT, but not -20°C for methanol fixation, nor 4°C for formaldehyde 0.5% fixation, may be recommended as first-line fixatives temperature during development of experiments. Secondarily, if necessary, increase in temperature up to 50°C and use of AR with SDS may be useful to optimized staining quality. Such recommendations should be applicable to other cell types localized at the surface of tissue that require an en face observation.

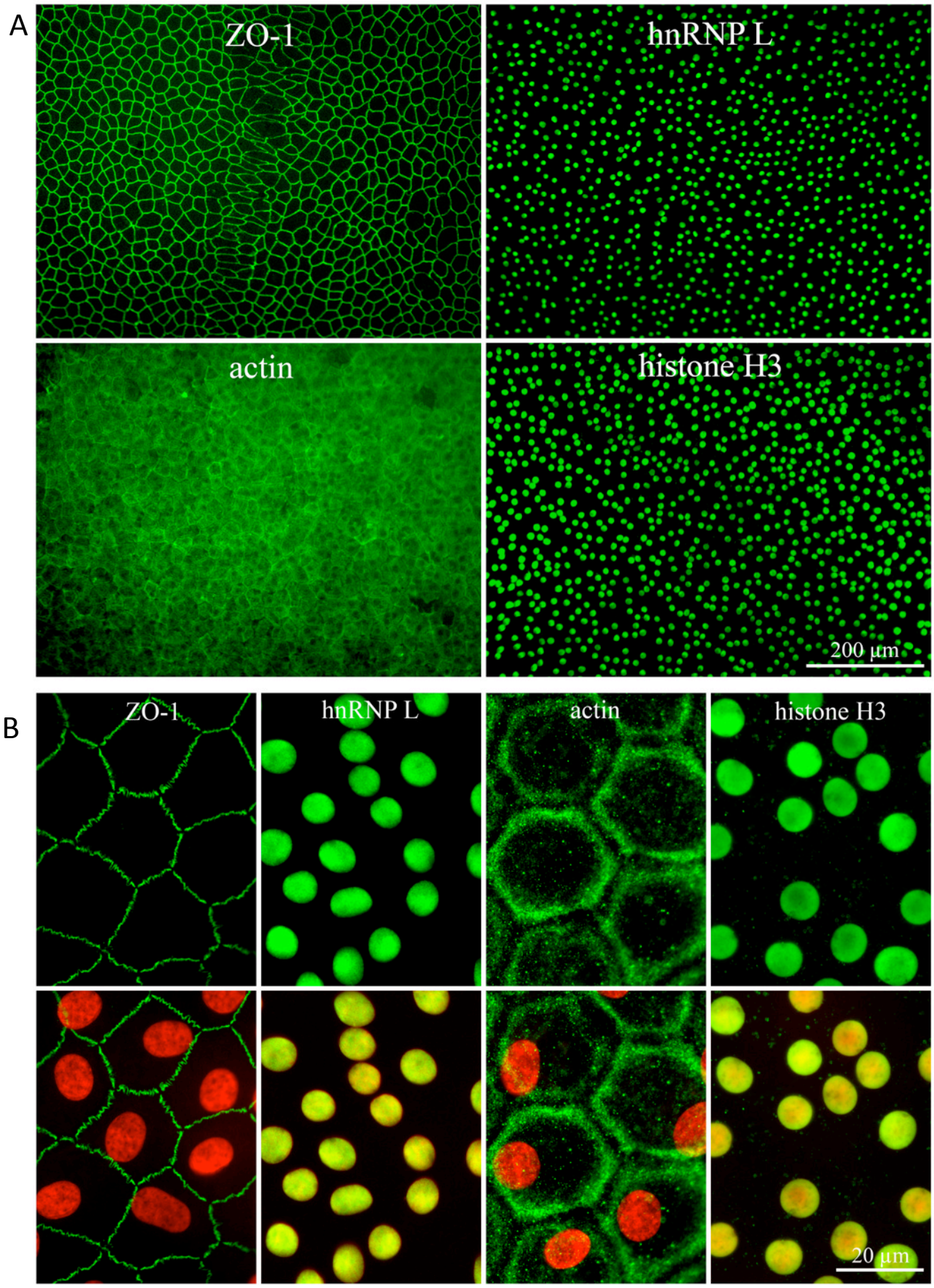

Figure 1 Best results obtained after optimization of fixative temperature for ZO-1(methanol at -20°C), hnRNPL(formaldehyde at RT), actin (methanol at 37°C) and histone H3(formaldehyde at RT) detection in endothelial cells of flat-mounted whole human corneas. A. low magnification (x10 objective) showing homogenous staining in all ECs. B; high magnification (x96 objective) showing the typical expected staining patterns for each target protein. Upper line: immunostaining alone; lower line: merge with Hoechst 33342.

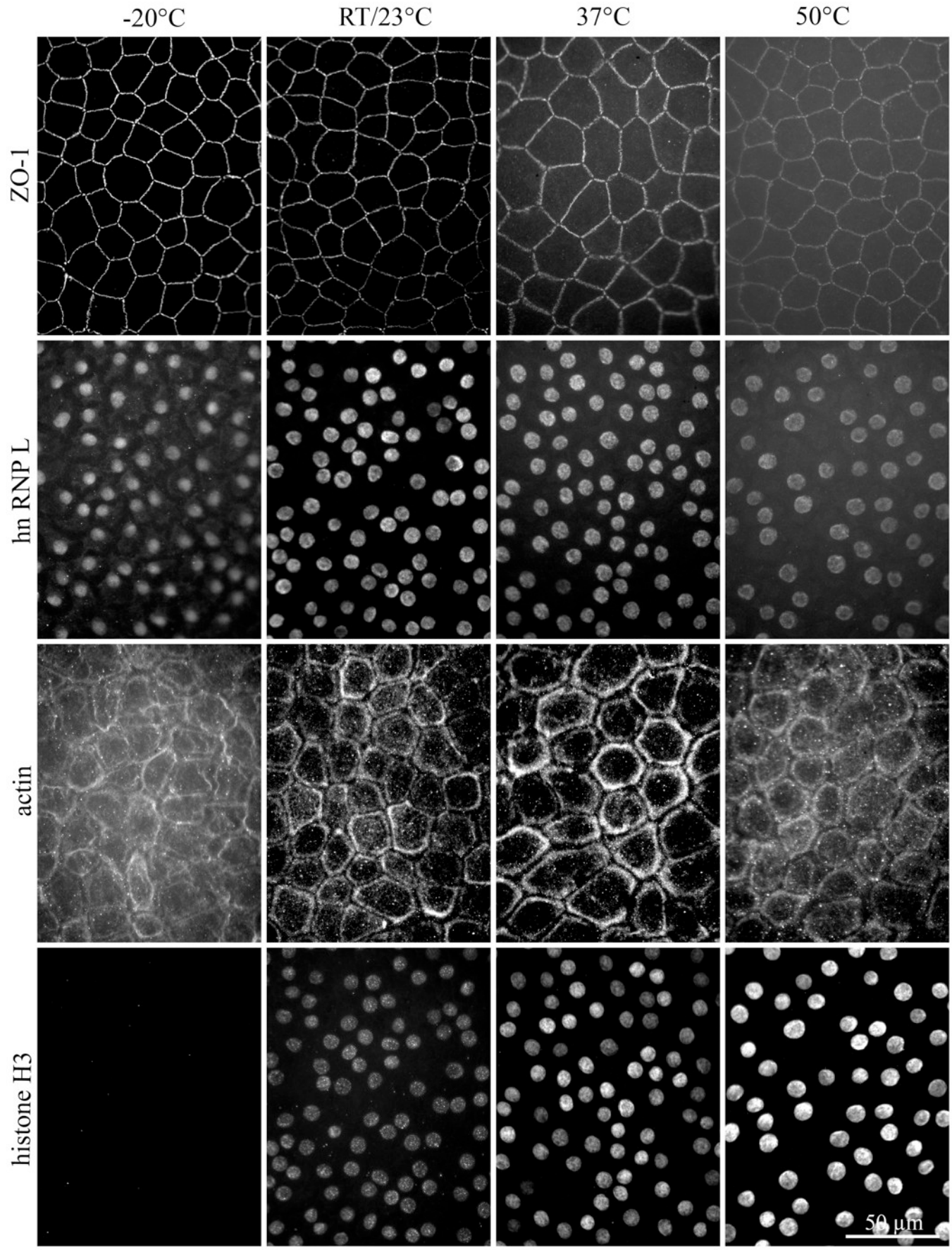

Figure 2 Influence of the temperature during methanol fixation. All corneal pieces were fixed for 30 minutes. No nuclear counterstaining. Original magnification x40

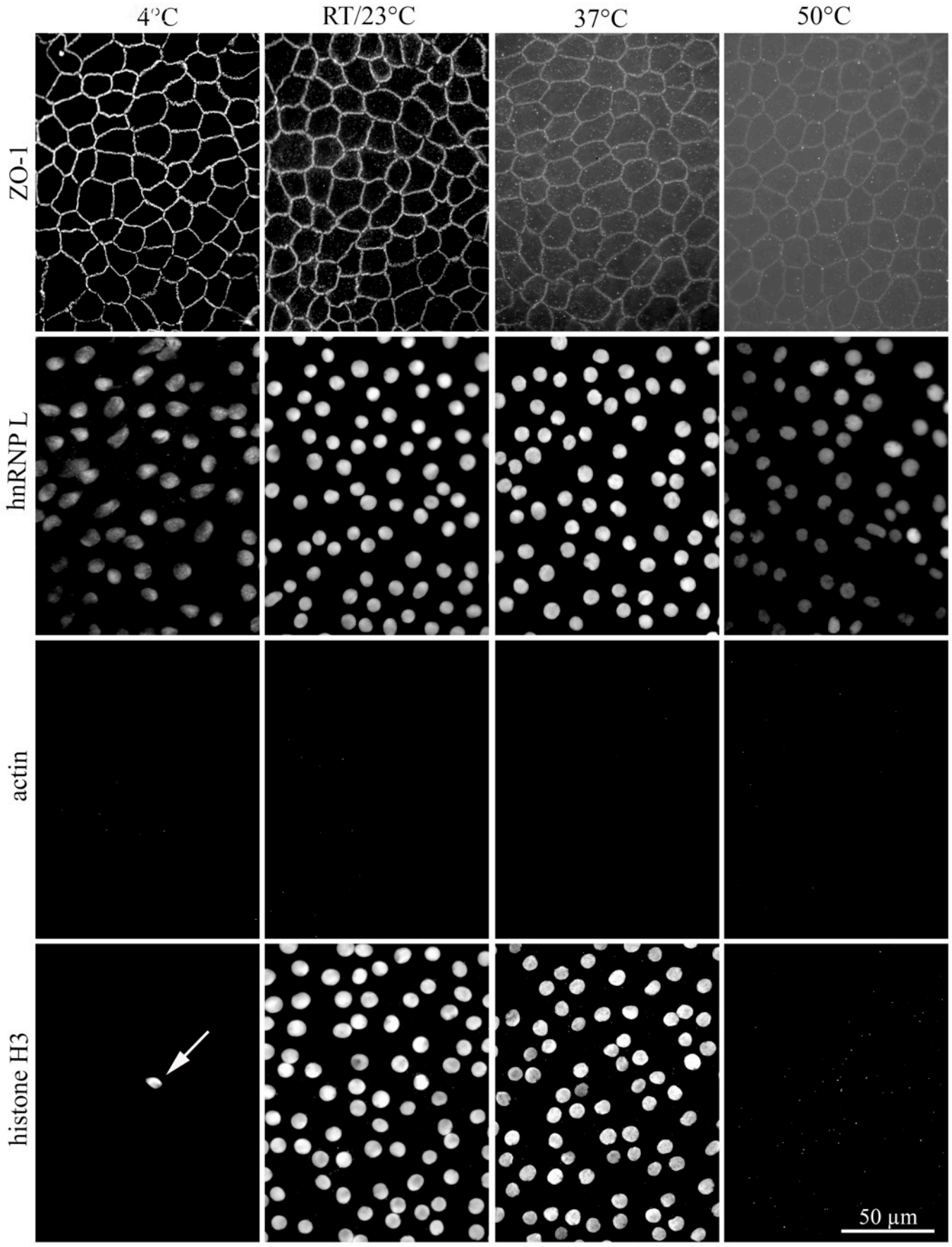

Figure 2 Influence of the temperature during 0.5% formaldehyde fixation. All corneal pieces were fixed for 30 minutes. Fixation alone without antigen retrieval (AR) was used excepted for histone H3 that required AR with SDS; SDS detached almost all endothelial cells from their basement membrane after fixation at 4°C as indicated by the arrow that indicates the single cell still attached. All the ECs were present for actin at the four temperatures and for histone H3 at 50°C; the counterstaining with Hoechst is not shown. Original magnification x40

Table 1 Evaluation of immunostaining at different temperatures during methanol and formaldehyde fixation

	Methanol				**0.5 % formaldehyde**			
	-20°C	RT	37°C	50°C	4°C	RT	37°C	50°C
ZO-1	++++	+++	++	+	++++	+++	++	+
hn RNP L	++	+++	++	+	++	++++	++++	+
actin	+	+++	++++	++	-	-	-	-
Histone H3	-	++	+++	++++	#	++++	++++	-

- : null ; + : weak, ++ : medium ; +++ : good ; ++++ : excellent ; #: detachment of EC by SDS

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Ex vivo Gene Electrotransfer to the Endothelium of Organ Cultured Human Corneas

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Key Words

Corneal endothelium · Corneal transplantation · Storage · Organ culture · Gene therapy · Gene transfer technique · Electroporation · Human

Abstract

Aims: To describe an innovative device that allows gene electrotransfer to human corneal endothelial cells (EC) during storage in organ culture. ***Methods:*** Customized electrodes without endothelial contact were developed. Two plasmids containing the cytomegalovirus promoter and reporter genes [enhanced green fluorescent protein (eGFP) or beta-galactosidase (β-gal)] were electroporated in 2 series of human corneas with eight 1-Hz 100-ms pulses of 125 mA square current. Controls were exposed to naked DNA without electric pulses. eGFP-transduced corneas were used to determine the transgene expression kinetics, whereas β-gal measured transfection efficiency using image analysis tools. Overall, endothelial toxicity was determined by: (1) cytotoxicity tests using triple staining with Hoechst 33342, ethidium homodimer III, and calcein AM, 3 h and 3 and 14 days after electroporation on the series of 15 eGFP-transfected paired corneas; (2) anti-ZO-1 staining to assess tight junctions' integrity. ***Results:*** All electroporated corneas carried transfected ECs, whereas the controls carried none. eGFP expression was observed 3 h after electrotransfer, and was then present from days 1 to 28. Transfection efficiency determined on 63 corneas transfected with β-gal ranged from 0.1 to 54% of the transfected ECs (mean ± SD: 7 ± 11%, median: 2.9%) with significant reproducibility for paired corneas from the same donor. Electroporation produced low early EC death. Anti ZO-1 staining revealed no dramatic change in EC mosaic continuity, neither 1 and 3 nor 28 days after electroporation. ***Conclusions:*** Gene electrotransfer to the endothelium of organ-cultured human corneas with custom-designed electrodes allows rapid and easy EC transfection. However, further optimization is required to ensure reproducible results.

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Introduction

Corneal preservation in organ culture (OC) for several weeks in a nutritive medium at 31–37°C facilitates the programming of transplantation as well as quality control and microbiological screening of graft tissue [1].

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However, this technique, like any other storage method, is responsible for progressive endothelial cell (EC) death higher than in physiological conditions [2]. Likewise, despite the high immune tolerance of the cornea, accelerated EC death is observed in recipients [3–5]. Use of grafts with a very high EC density (ECD) or enhancing the resistance of EC to cell death mechanisms in the postoperative period are 2 strategies that may improve graft survival in the recipient. By virtue of its prolonged duration lasting several weeks, corneal OC provides enough time to modify the ECs while allowing pre-graft quality assessment.

Gene transfer is a useful biological tool for improving our understanding of the pathophysiology of the human corneal EC. Overexpression of certain genes of interest and the inactivation of others may allow determination of the role and interactions of certain proteins in the corneal endothelium. This is all the more important as insight into the genes expressed most specifically in the ECs of healthy subjects and in patients with Fuchs' dystrophy was provided several years ago [6–10]. In the long term, the prospect of local gene therapy for management of early stages of Fuchs' dystrophy and for modulation of accelerated EC loss during corneal preservation and post-graft rejection is equally attractive. In vivo, human corneal ECs are non-proliferative in adults, are adherent, and do not migrate: 3 properties that theoretically eliminate the risk of systemic complications from this therapy and facilitate its acceptance on ethical grounds [11]. Gene transfer to the corneal endothelium is also facilitated by the arrangement of the EC mosaic in a single layer easily accessible ex vivo, particularly during corneal storage.

Gene transfer to the endothelium of whole ex vivo human corneas has already been described using viral [12–19] and non-viral vectors [20–22], but never using gene electrotransfer. In numerous tissues or cell models, electroporation has proved simpler and less immunogenic than viral methods, but also more efficient than most non-viral methods. Its principle is based on both permeabilization of cell membranes and DNA electrophoresis inside the cells [23–26]. It has been shown for rodent muscle tissue that the application of electric pulses strongly increases the transfection rate compared to injection of naked DNA [27–30]. Electroporation has proved efficient in vivo in rat corneal endothelium [31, 32] and in vitro on human corneal ECs in primary culture [33]. Here, we present the very first application of gene electrotransfer to the endothelium of human corneas preserved in OC, using a custom-designed electrode.

Methods

Electrodes

Stainless steel electrodes were custom-designed for electroporation of human corneas (fig. 1). The anode had a convex surface (13.0 mm diameter, 8.40 mm radius of curvature) that covered the entire endothelial surface, and the facing cathode had a complementary concave surface. The 2 electrodes were parallel and 2.50 mm apart, to ensure a homogeneous electric field. A screw thread on the anode shaft allowed adjustment of this distance. The electrode insulating base was derived from an autoclavable plastic Barron trephine (Katena, Denville, N.J., USA). The cornea was placed epithelium down on the cathode, and the anode was placed close to the endothelium without contact. Electric continuity was assured by the buffer placed in the corneal concavity. The whole device was autoclavable to anti-prion standards. The scleral rim systematically overlapped the electrodes and was always interposed between them, preventing direct anode-cathode contact.

Characteristics of the Human Corneas

All procedures conformed to the tenets of the Declaration of Helsinki for biomedical research involving human cadaver tissue. Scientific corneas were procured from bodies donated to the Laboratory of Anatomy in the Faculty of Medicine and from the Eye Bank of Saint-Etienne, including 78 paired corneas (same donor). Mean donor age was 73 ± 15 years (SD; range: 40–99, median: 75). The male/female ratio was 0.37. Mean time from death to procurement was 24 ± 10 h (range: 3–60, median: 21). The corneoscleral rims (16–18 mm diameter) excised in situ were immediately placed in 100 ml of OC medium (Inosol, Opsia Bausch and Lomb, Labège, France or CorneaMax, Eurobio, Les Ulis, France) at 31 °C in a dry incubator. Mean storage time before electroporation was 13 ± 9 days (range: 1–33, median: 10). ECD was without exception determined 24 h before treatment using the SambaCornea endothelial analyser [34–37] (TribVn, Châtillon, France) after osmotic preparation in NaCl 0.9% to render the ECs visible by optical microscope. The cell count was conducted on at least 300 ECs.

Gene Electrotransfer Protocol

Two plasmids with the same cytomegalovirus (CMV) immediate early promoter [*E. coli lacZ* gene of β-galactosidase (pCMV/β-gal) and enhanced green fluorescent protein (pCMV/eGFP)] were multiplied in thermocompetent *E. coli* JM109 and then purified after alkaline bacterial lysis with an endotoxin-free plasmidic purification kit (Qiagen, Dorking, UK). One hundred twenty-five microlitres of 0.9% NaCl (Sigma, St Quentin Fallavier, France) containing 1.25 μg of plasmidic DNA were added into the corneal concavity. The GET42 generator (Electronique et Informatique du Pilat, Jonzieux, France) delivered 8 square pulses lasting 100 ms, at 1 Hz frequency and 125 mA intensity. The entire operation was performed under a laminar flow hood at room temperature. The corneas were then returned to their OC preservation medium. Control corneas with similar donor characteristics were subjected to the same manipulations without electric pulses.

Transfection with the eGFP Gene: Transgene Expression Kinetics and Duration

Ten corneas were transfected with the eGFP plasmid. Seven were observed hourly for the first 6 h after electroporation to de-

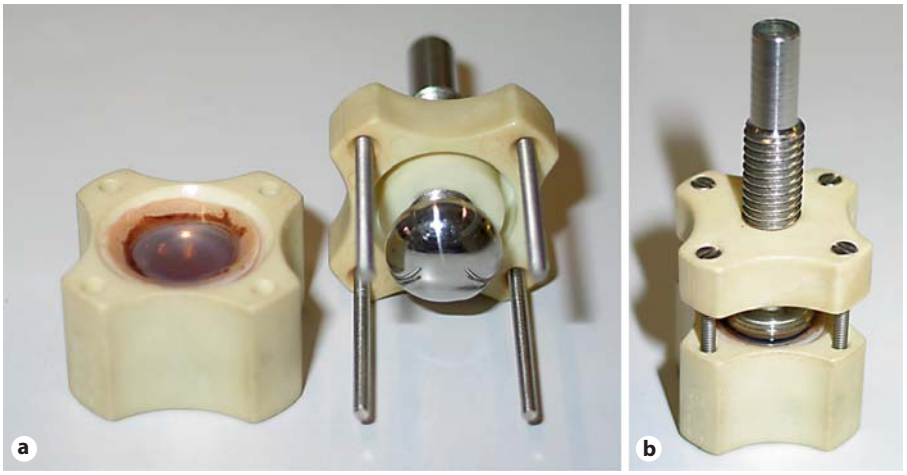

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Fig. 1. Custom designed electrodes for ex vivo electroporation of human corneas. **a** The concave cathode touched the cornea (epithelial side), while the convex anode did not (endothelial side). **b** Device with a human cornea in place. The screw thread allowed adjustment of the inter-electrode gap to obtain 2 parallel surfaces 2.5 mm apart. The electrodes were sterilizable, thereby allowing organ culture preservation to be continued after electroporation.

termine the minimum time required to synthesize the neoprotein. After each observation, in a sterile Petri dish under a fluorescent microscope (Olympus IX81, Tokyo, Japan), the corneas were returned to their OC preservation medium. Three other corneas were observed at hours 24, 48 and 72 then days 7, 14 and 28 to determine transfection stability and the maximum duration of expression. For some corneas (chosen at random among the 10), at the end of the observation period the endothelium was covered for 15 minutes with a solution of bis-benzimidazole Hoechst 33342 (Sigma) of 1 μg/ml concentration, to counterstain all nuclei. To determine whether the underlying stromal keratocytes were also transfected, a small part of the Descemet membrane was gently peeled off using micro-forceps under an operating microscope. The portion of denuded stroma, as well as the Descemet membrane fragment, were again observed under the fluorescent microscope.

Transfection with β-Galactosidase: Measurement of Transfection Efficiency

Sixty-three corneas transfected with the β-galactosidase gene served to determine mean transfection efficiency. Seven negative controls were performed. Efficiency was measured after 1 (n = 6), 3 (n = 31), 5 (n = 17) and 14 (n = 9) days. Corneas were fixed for 30 min in phosphate-buffered saline (PBS) containing 1% glutaraldehyde, then rinsed thrice in cold PBS containing 2 mM of $MgCl_2$. Corneas were then incubated for 30 min at 37°C in a solution containing 0.2% X-Gal (5-bromo-4-chloro-3-inodyl-β-galactosidase, Sigma), 10 mM sodium phosphate buffer (pH = 7.0), 150 mM NaCl, 1 mM $MgCl_2$, 3.3 mM $K_4Fe(CN)_63H_2O$ and 3.3 mM $K_3Fe(CN)_6$, which gave a blue coloration to the transfected ECs. After rinsing in distilled water, corneas were placed on a backlit table and photographed at low magnification. The digital images were analysed to determine the percentage of transfected ECs using the Scion Image analysis program for Windows (freeware available at www.scioncorp.com). The colour images were transformed into 256 shades of grey in an 8-bit greyscale bitmap and thresholded at a constant predefined level for all corneas. The percentage of transfected ECs corresponded to the ratio of X-Gal blue-stained surface area to the total endothelial surface area. EC nuclei were counterstained with Meyer's hematoxylin, and whole corneas were flat mounted using a technique previously described [38] and observed under the microscope (×10). ECD was determined by the Sambacornea corneal endothelial analyser by manually pointing to cells' nuclei.

Epithelial and Endothelial Toxicity

Overall cell toxicity was assessed at several levels. Epithelial changes were assessed on corneal cross-sections. Two pairs were observed 3 days after transfection, and 2 pairs after 28 days. Corneas were fixed in 10% formaldehyde, embedded in paraffin and 7 μm cross sections were stained with hematein-eosin-safran. The epithelial layer was observed with a ×40 objective. Endothelial toxicity was assessed using a live/dead assay on a series of 15 pairs of corneas. ECD was assessed using the Sambacornea analyser 24 h before electroporation to verify comparability between electroporated and control corneas. One cornea of each pair was electroporated with eGFP using the same protocol, whereas the mate cornea (negative control) remained untreated. Five pairs were assessed 3 h after electroporation, 5 pairs after 3 days and 5 pairs after 14 days to evaluate acute, delayed and late toxicity, respectively. All corneas were then briefly checked under the fluorescent microscope for verification of eGFP expression. Corneas were rinsed in balanced salt solution (BSS) to eliminate the serum-containing OC medium, and then immediately incubated with Hoechst 33342 (H) (Sigma) (10 μM), ethidium homodimer III (E) (4 μM) and calcein AM (C) (2 μM) (both from Biotium, Hayward, Calif., USA) for 45 min at 31°C in the dark. After a brief rinse in BSS, they were flat mounted using 4 radial cuts and fluorescent mounting medium (DakoCytomation Glostrup, Denmark). Images of the 3 channels were analysed using the Cell P image analysis software (Olympus Soft Imaging System, Berlin, Germany) after appropriate calibration. Briefly, instant EC death was defined as the ratio of E+ nuclei (dead cells with permeable damaged membranes, unable to exclude the intercalating agent) to the total of (H+ and E+) nuclei. It was measured in a region of interest (ROI) located in the central field of all corneas, taking account of at least 1,000 nuclei. ECD was calculated as the ratio of the total number of nuclei (H+ and E+) to the ROI area. The area of live cells exhibiting metabolic activity (esterase activity able to esterify the non-fluorescent calcein AM into a bright fluorescent

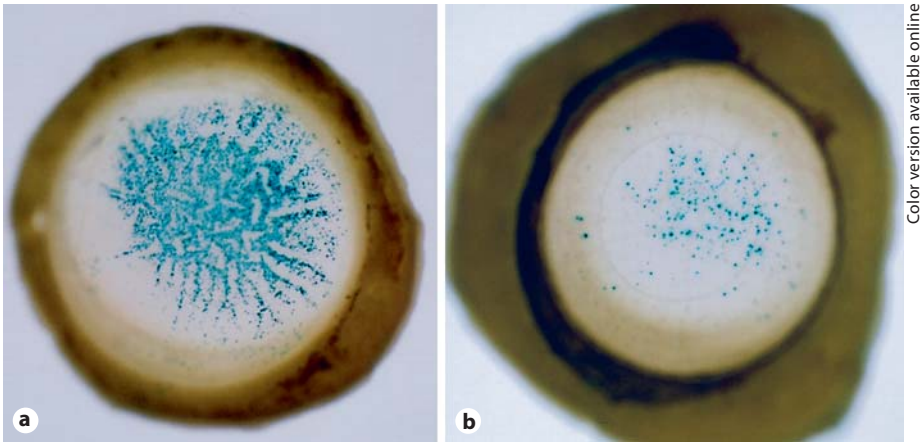

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Fig. 2. Representative example of 2 corneas transduced with β-galactosidase gene showing high (**a**; 28.6% of cells, ECD = 2,180 cells/mm^2) and low levels of transfection (**b**; 0.8% of cells, ECD = 1,831 cells/mm^2). Such images were analysed to determine transfection efficiency.

substrate) was determined in the same central zone, in an ROI of approximately 5 mm^2 similar for all the corneas. We used a simple algorithm to delineate and measure the area of intense fluorescence after thresholding. Viability was expressed as the ratio of the fluorescent area to the whole of the ROI. Paired data were compared using the Wilcoxon non-parametric test, with $p < 0.05$ considered as significant. Complementary to the previous tests, apical tight junction integrity of ECs was assessed on 6 pairs of corneas (2 after 1 day, 2 after 3 days, and 2 after 28 days) using immunostaining of the occludin protein (anti-ZO-1 antibody) using a protocol described elsewhere [39]. For the 30 corneas assessed with the live/dead assay, the presence of possible corneal opacities was checked by observation of corneas placed on a backlit chart, before exposure to the live/dead assay reagents, using a method previously described [40, 41].

Results

All the corneas subjected to electroporation expressed the protein of interest, whether eGFP or β-gal. The transfected ECs were randomly and more or less homogeneously spread over the entire surface of the endothelium, in the centre as well as in the periphery. In several corneas, distribution tended to follow the top of the Descemet folds (fig. 2a). The transduced cells were isolated or were arranged in small groups. No expression was observed in the endothelium of the negative control corneas exposed to naked DNA only (see below).

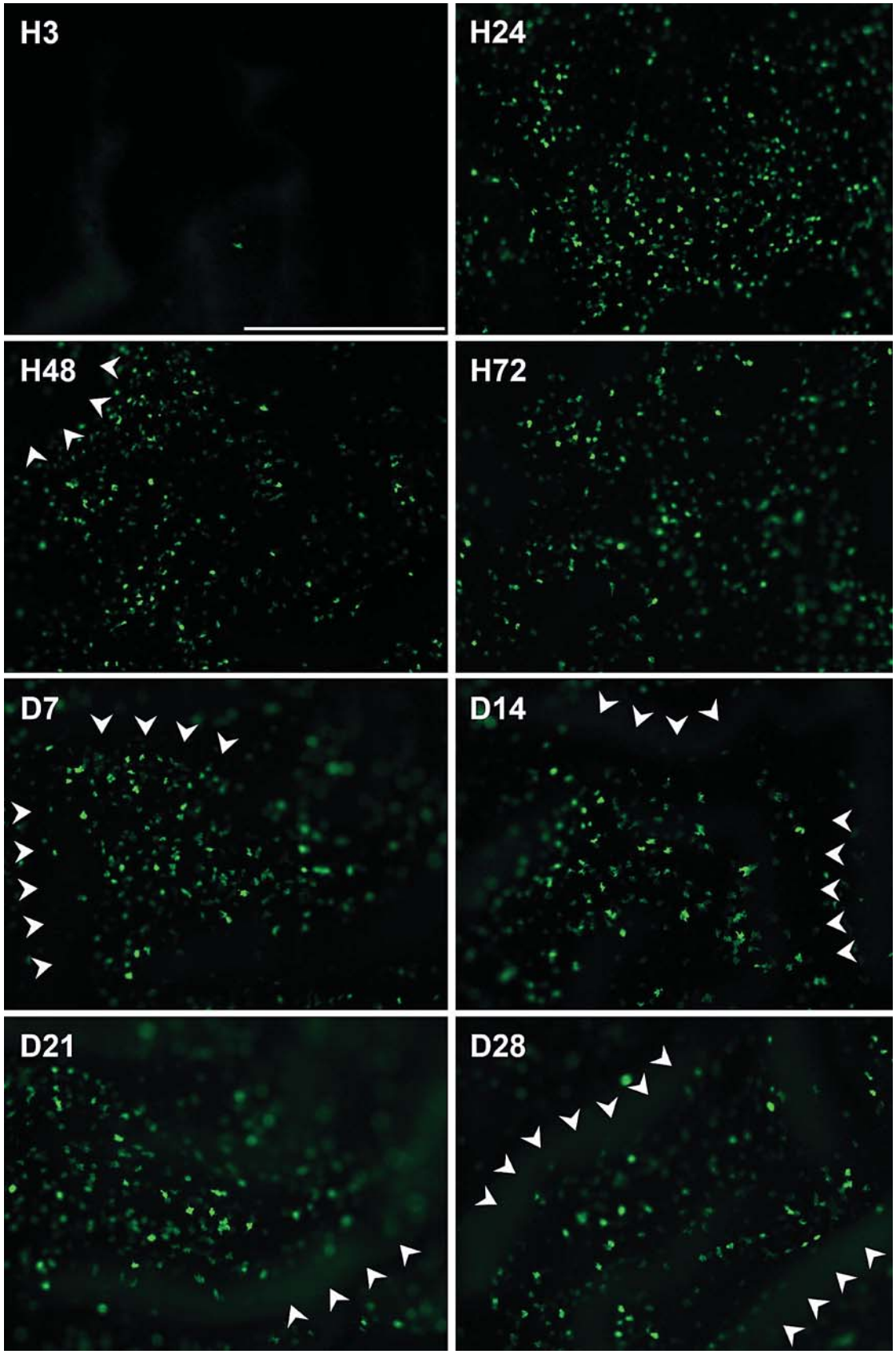
Enhanced Green Fluorescent Protein

Expression of eGFP was detected from the third hour after electroporation. After 24 h, the number of positive cells dramatically increased and remained stable until day 28 (fig. 3). As was observed with β-gal, distribution tended to follow the top of the Descemet folds, and was mostly absent from the bottom. The location of the neoprotein was most often in both the nucleus and cytoplasm. Homogeneous staining of the cytoplasm made it possible to distinguish the morphology of the transfected cells with their irregular lateral cytoplasmic expansions (fig. 4). Transfection was strictly limited to the endothelium with no fluorescence detectable in the stromal keratocytes after ablation of the endothelium or in epithelial cells (fig. 5).

β-gal

Expression peaked at 3 days after electrotransfer, and was still detectable in some ECs 14 days after transfection. Efficiency ranged from 0.1 to 54% of ECs, with a median of 2.9 and a mean of 7.1 (SD: 11.0; fig. 2). Details of efficiency according to time are presented in table 1. For the 7 control corneas, the thresholding method of image analysis showed, as expected, 0% efficiency. Intercorneal efficiency varied greatly, but there was significant reproducibility between the corneas from the same donor observed after the same post-electroporation period (Pearson's correlation coefficient, $r = 0.72$, $p < 0.001$). Median overall efficiencies (expressed as median, mean ±

Fig. 3. Representative example of the centre of the same cornea observed at hours (H) 3, 24, 48 and 72 then at days (D) 7, 14, 21 and 28 after electroporation with the eGFP transgene. First positive cells appeared after 3 h. The maximal expression (intensity and number of positive cells) was observed between H24 and H72, then expression was continuously observed until at least D28. Fold bottoms are indicated by arrowheads. ×4. Scale bar = 1 mm.



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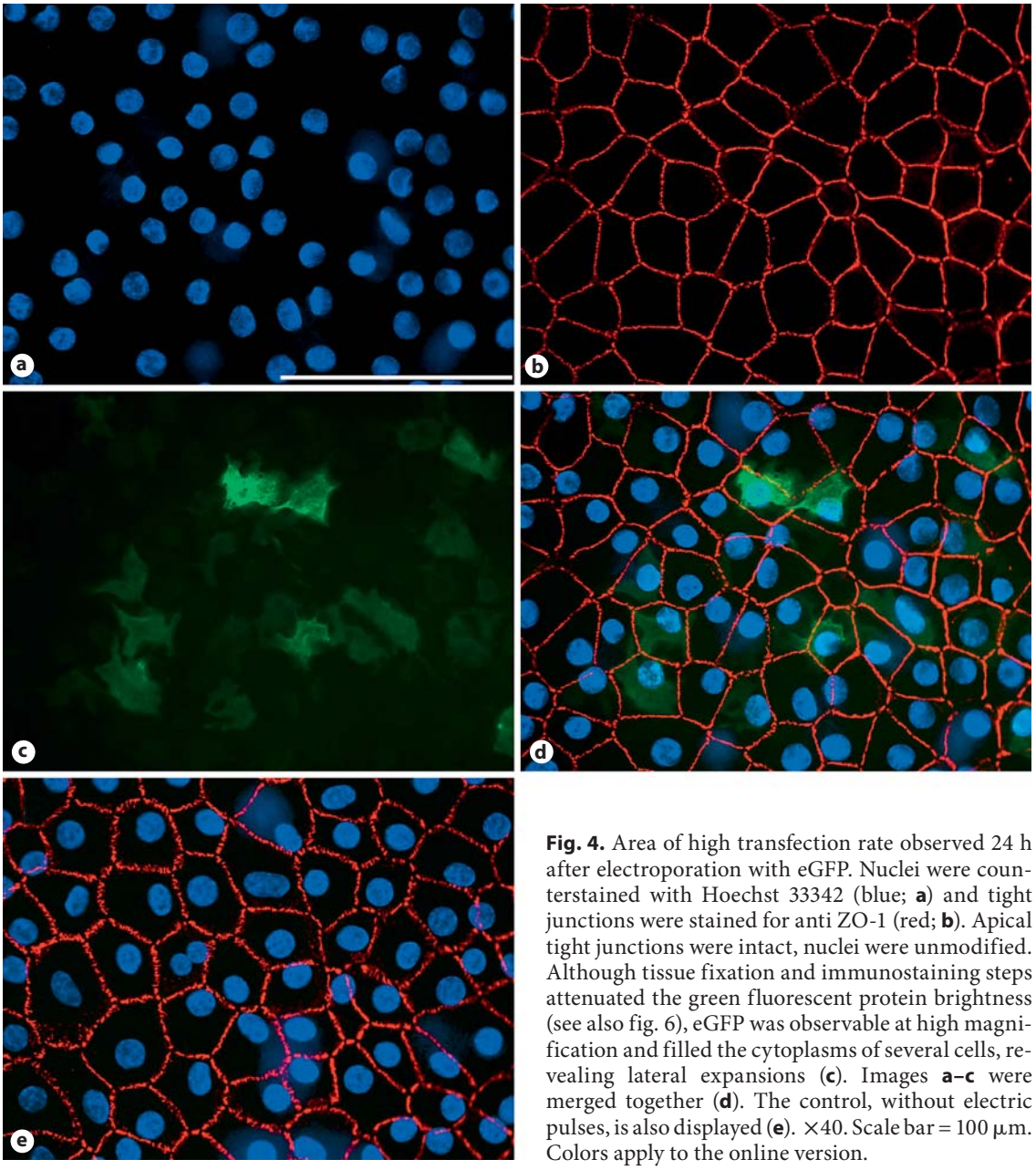

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Fig. 4. Area of high transfection rate observed 24 h after electroporation with eGFP. Nuclei were counterstained with Hoechst 33342 (blue; **a**) and tight junctions were stained for anti ZO-1 (red; **b**). Apical tight junctions were intact, nuclei were unmodified. Although tissue fixation and immunostaining steps attenuated the green fluorescent protein brightness (see also fig. 6), eGFP was observable at high magnification and filled the cytoplasms of several cells, revealing lateral expansions (**c**). Images **a–c** were merged together (**d**). The control, without electric pulses, is also displayed (**e**). ×40. Scale bar = 100 μm. Colors apply to the online version.

SD, range) for the 24 paired corneas available were 2.6 (mean ± SD: 5.4 ± 8.4, range: 0.1–31.3%) for the right corneas and 1.5 (mean ± SD: 6.7 ± 9.8, range: 0.1–33.6) for the left corneas (p = 0.530). At transfection, the mean ECD of the 63 corneas was 2,043 ± 571cells/mm^2 (range: 870–3,002, median: 1,968). Transfection efficiency seemed to be uncorrelated with ECD (p = 0.056), donor age (p = 0.435) or cornea preservation time prior to electroporation (p = 0.095).

Toxicity

Compared with the paired control corneas, the epithelium of several electroporated corneas presented fewer cell layers and several shrunk basal cells, be it after 3 or 28 days. Epithelial modifications seemed unrelated to endothelial transfection efficiency. Despite these features, no complete epithelial desquamation was observed at any time point (fig. 6).

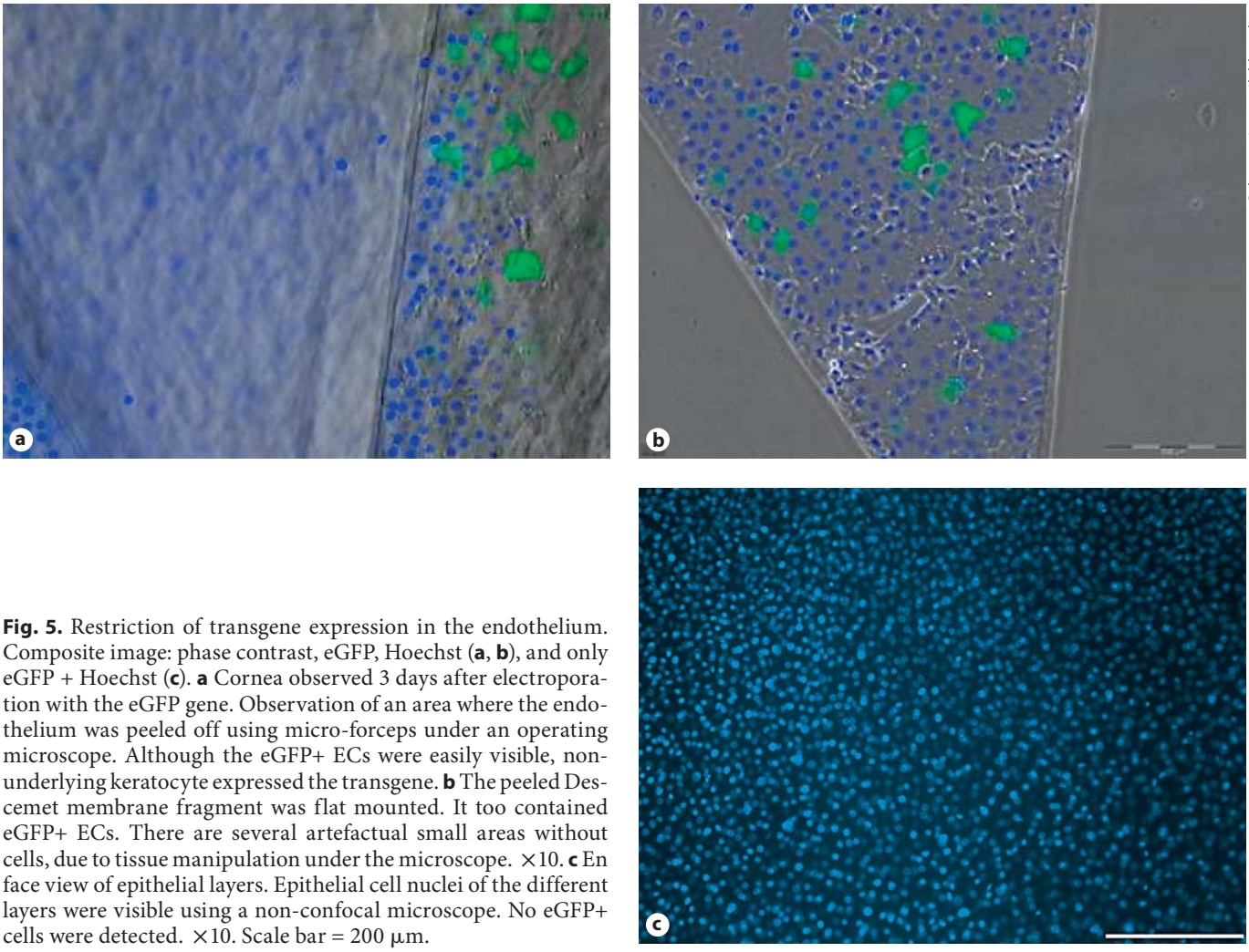

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Fig. 5. Restriction of transgene expression in the endothelium. Composite image: phase contrast, eGFP, Hoechst (**a**, **b**), and only eGFP + Hoechst (**c**). **a** Cornea observed 3 days after electroporation with the eGFP gene. Observation of an area where the endothelium was peeled off using micro-forceps under an operating microscope. Although the eGFP+ ECs were easily visible, non-underlying keratocyte expressed the transgene. **b** The peeled Descemet membrane fragment was flat mounted. It too contained eGFP+ ECs. There are several artefactual small areas without cells, due to tissue manipulation under the microscope. ×10. **c** En face view of epithelial layers. Epithelial cell nuclei of the different layers were visible using a non-confocal microscope. No eGFP+ cells were detected. ×10. Scale bar = 200 μm.

Occasionally, in the series of corneas electroporated with β-gal, a few corneas presented localized major endothelial damage due to accidental contact with the electrode (in both the control and the electroporated groups). EC toxicity and viability are expressed in table 2. All electroporated corneas carried eGFP positive ECs. Although comparisons did not reach the level of significance, acute cytotoxicity involving a limited number of cells seemed to occur after 3 h. This phenomenon was observed to persist at day 3. After 2 weeks, only the consequences of the initial cell damage were observed. At the 3 time points, the area covered by metabolically active cells tended to be slightly lower in electroporated corneas than in paired control corneas. ZO-1 immunostaining did not reveal dramatic changes within the apical tight junction system at any time point (fig. 7). Polymorphism and polymege-

Table 1. Efficiency of transfection according to time after gene electrotransfer

Days after electroporation (corneas)	Mean ± SD	Min.	Max.	Median
1 (n = 6)	7.7 ± 10.8	0.4	28.6	4.2
3 (n = 31)	11.1 ± 13.8	0.1	54.4	4.2
5 (n = 17)	2.8 ± 4.0	0.1	13.4	0.8
14 (n = 9)	0.6 ± 1.0	0.1	2.8	0.1

Efficiency was determined using image analysis of 63 corneas transfected with the β-gal gene, and expressed as a percent of blue-stained surface area to total endothelial surface area.

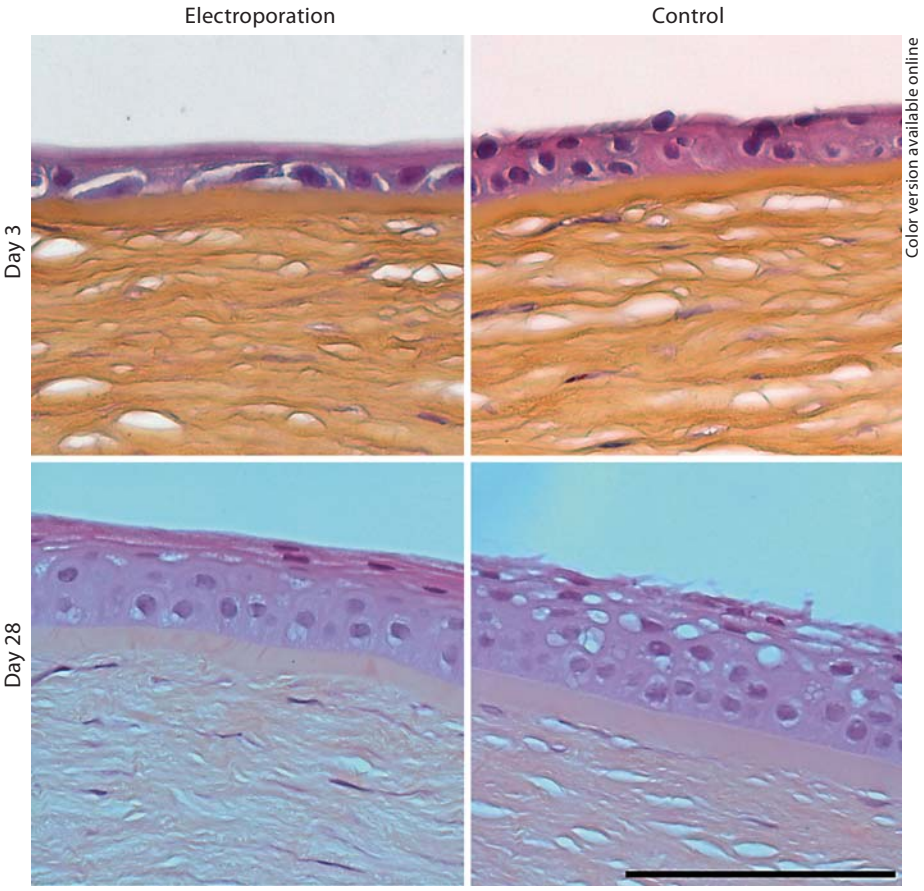

Fig. 6. Cross sections of corneal epithelium and anterior stroma 3 and 28 days after electroporation. Representative example of 2 pairs of corneas. Minor modifications of epithelial layers were observed after electroporation. Hematein-eosin-safran. ×40. Scale bar = 100 μm.

thism sometimes appeared greater, but overall endothelial integrity was preserved. No electroporated cornea displayed any stromal opacity (data not shown).

Discussion

Our system enables efficient gene electrotransfer to the endothelium of human corneas in OC storage. Electroporation offers a gene transfection rate to corneal endothelium ex vivo that is lower than for viral vectors, but higher than for several chemical vectors. This ultra-fast technique, unlike viral and other physicochemical methods, does not require prolonged incubation out of the graft tissue preservation medium. The medium of incubation is similar to that used for routine OC containing 2% fetal calf serum (FCS). Considering the possibility of future clinical application, we chose to respect the standard conditions of OC, and not to increase the percentage of FCS in particular, in contrast to other techniques which, for human corneas, necessitate incubation in medium supplemented with 10% FCS or a cocktail of growth factors [12, 15, 18, 21, 22]. These characteristics also constitute advantages over other methods for laboratory research work where the effects of FCS growth factors are undesirable.

Corneal storage in OC makes it possible to maintain the metabolic activities of ECs [1]. This explains the speed of translation of the neoproteins, from the third hour after electrotransfer, unlike low-temperature preservation at +4°C [42]. These transgene expression kinetics are comparable to those obtained in animals after electroporation of corneal epithelial cells [43] or of stromal keratocytes [43, 44]. These kinetics are also characteristic of the CMV immediate early promoter used for our plasmids [43, 45]. This powerful promoter may also be partially responsible for the transient expression of some proteins of interest [46]. This phenomenon has been ascribed to silencing of the viral-derived promoter by methylation [47]. Blair-Parks et al. [43] have also shown in mouse corneas that this promoter and enhancer is well suited to short-term expression studies, but is not the

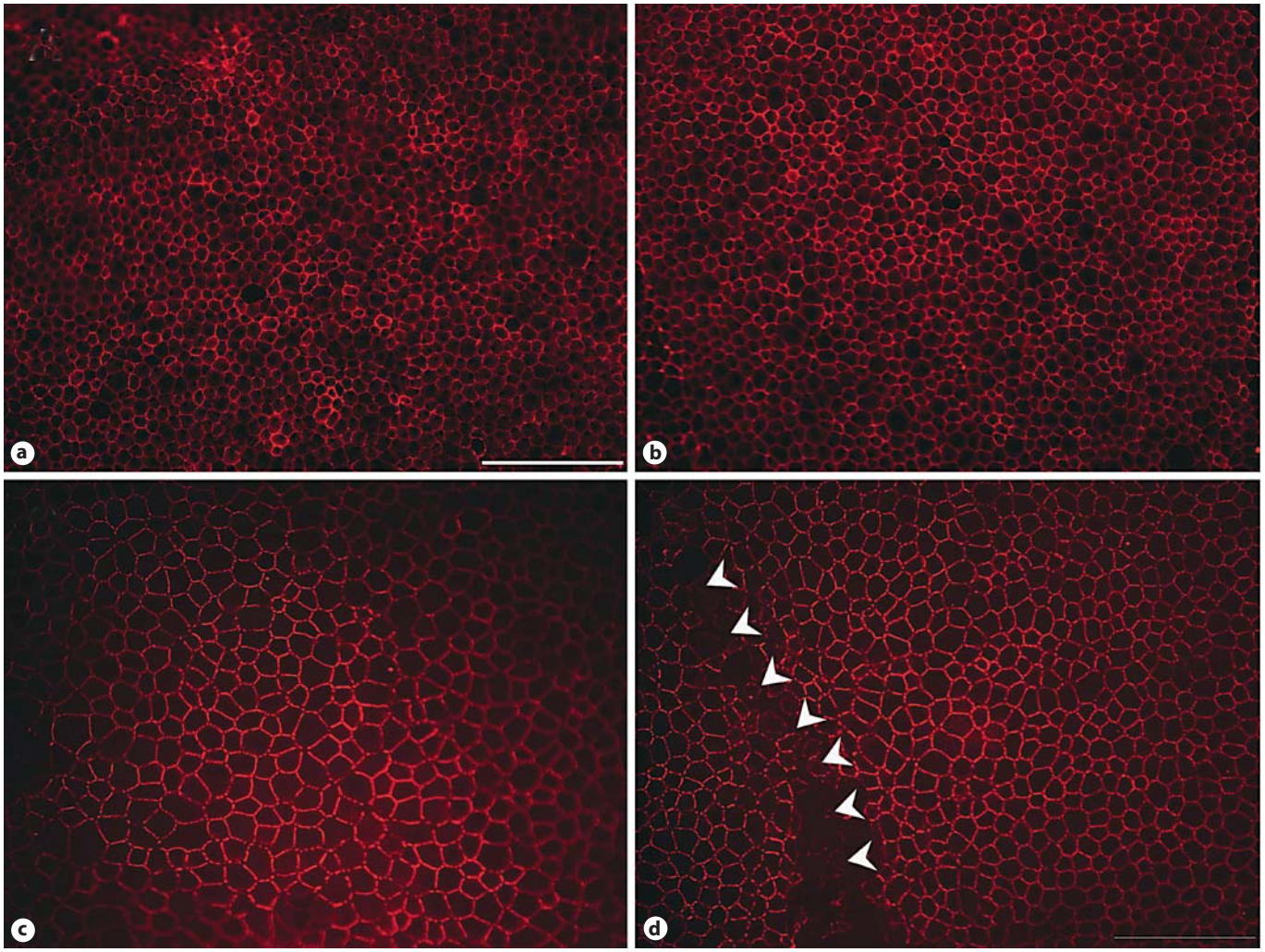

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Fig. 7. Anti ZO-1 immunostaining of 2 pairs of corneas, 1 (**a**, **b**) and 28 (**c**, **d**) days after electroporation: **a** and **c** were electroporated, **b** and **d** were control corneas. The fixation and immunostaining protocol considerably attenuated eGFP fluorescence, which explains why few transfected cells were visible at this magnification (×10). Only minor changes were observed after electroporation, mainly consisting of an increase in cell polymegethism and polymorphism. Folds, seen in both electroporated and control corneas, were only consequences of storage in organ culture. An example is shown in **d** (arrowheads), although this is a control cornea. Scale bar = 200 μm (for all images).

technique of choice for long-term expression. The use of other promoters should allow determination of the maximum expression time possible in the endothelium after electroporation. Nevertheless, we observed longer expression of the eGFP protein, although both genes had the same CMV promoter. The apparently variable expression of both transgenes may be due to the more sensitive detection method (high fluorescence of eGFP vs. enzymatic method for β-gal).

In any case, if short time expression of transgenes restricts its clinical use to acute treatments, this apparent drawback should be regarded with particular interest in the field of organ culture. Indeed, it could turn out to be an advantage, especially if the purpose of the transfection is to alter the proliferative capacity of EC during storage only in order to provide graft with an enriched endothelium without prolonged effect in the recipient eye.

The non-hexagonal appearance of the transfected cells was due to the homogeneous staining of their cytoplasms, which revealed irregular basal cell expansions previously described for normal cells [48]. Similar features were also described in murine corneal ECs with lentiviral transfec-

Table 2. Cytotoxicity of eGFP gene electrotransfer

	ECD before electroporation[a] (Sambacornea analyser), cells/mm^2	ECD after electroporation[a] (counting of nuclei), cells/mm^2	Cell death (ethidium homodimer III), %	Area of viable cells (calcein+ cells), %
Hour 3 (n = 5)				
Control	2,377 ± 186	2,103 ± 349	0.4 ± 0.5	79.1 ± 16.0
Transfected	2,434 ± 196	2,150 ± 597	3.5 ± 3.6	74.5 ± 12.5
p	0.345	0.686	0.144	0.345
Day 3 (n = 5)				
Control	2,818 ± 385	2,128 ± 442	0.7 ± 1.2	86.5 ± 6.4
Transfected	2,795 ± 202	1,831 ± 374	2.1 ± 2.3	77.9 ± 12.9
p	0.500	0.080	0.345	0.225
Day 14 (n = 5)				
Control	1,813 ± 422	1,420 ± 237	1.4 ± 1.4	87.0 ± 7.5
Transfected	1,786 ± 511	1,306 ± 302	0.8 ± 0.9	75.0 ± 8.3
p	0.686	0.345	0.686	0.080

Data assessed using triple labelling with Hoechst 33342, ethidium homodimer III and calcein-AM, on 15 corneas, 3 h, 3 days and 14 days after electroporation. Negative controls were 15 untreated paired corneas. Results presented as means ± SD.

[a] ECDs before and after transfection were not measured by the same method. This likely explains an apparent overestimation of cell loss. However, the same bias existed for all corneas.

tion methods [49]. This appearance naturally differed from the typically hexagonal appearance of ECs when their apical surface is observed after alizarin red staining. Generally speaking, electroporation still causes reticence due to fears of high toxicity, but its toxicity in the human corneal endothelium seems acceptable with our parameters. Epithelial changes may be neglected for the applications envisaged by us (endothelial gene therapy or as a tool for studying corneal endothelial biology). The apical junction integrity of the endothelium is preserved. The endothelial survival rate remains acceptable. Initial cell loss probably occurs, but with no excess mortality thereafter.

Although methods to quantify expression of a reporter gene in the corneal endothelium vary widely in the literature and make comparisons very difficult, our mean transfection efficiency of 7.1% was satisfactory considering the stringent experimental conditions, with minimal changes to the normal course of OC. Notably, we obtained microscopic appearances comparable to that described by Collins et al. [50]. Based on simple image observation, the authors reported that nearly all ECs expressed the transgene in rabbit corneas, whereas our study obtained a maximum score of only 54.4% after image analysis. Our quantification technique, which is fully reproducible, precisely measured the endothelial surface area actually transfected (blue) and thus made it possible, given the ECD, to deduce the number of ECs actually transfected. We chose a particularly 'severe' threshold because it is a well-known fact that β-gal staining tends to overlap cell borders and could have led to overestimation of the number of transfected cells when measuring the blue areas. Despite these reservations about quantification methods, our system achieved transfection efficiency comparable to most non-viral vectors assessed on human corneas to date [20, 51] and close to that of some viral vectors, particularly adeno-associated viruses and herpes [14], but without their drawbacks. The very high levels (up to 54%) occasionally obtained in our study nevertheless remain lower than those obtained in humans using adenoviral vectors [12, 15]. The relatively low mean rate is fully compatible with certain therapeutic applications, in particular the controlled activation of endothelial mitosis intended to increase ECD transiently in an effort to augment the graft reserve before transplantation in the recipient. The fact that even a low transfection rate can be of interest for some applications has been underlined by Dannowski et al. [52]. This type of application is well suited to transient transgene expression because an increase in ECD over a brief period may be sufficient. Moreover, such a transient expression is also likely to facilitate its acceptance by the scientific and medical community with a view to clinical application.

Transfection efficiency varied greatly from cornea to cornea, indicating that the method requires optimization. Three factors may explain the variation: (1) EC area (which is inversely proportional to ECD). The electrical principles governing electroporation mean that efficiency depends on cell size, the smallest cells being harder to permeabilize [53]. In our series, use of identical current parameters for all corneas should have caused lower efficiency in those with the smallest ECs (and highest ECDs). We could not obtain confirmation of this, probably due to the influence of cofactors. However, the good reproducibility for both corneas from a single donor, which typically have comparable endothelia, shows that the intrinsic quality of the endothelium is one of the main factors influencing transfection efficiency. This may be improved by adapting the electrical parameters (increase in intensity and/or number of pulses) to the endothelium. (2) Stromal hydration, which alters total corneal thickness, is also able to affect the passage of the electric current and to cause transfection efficiency to vary. Stromal hydration is itself multifactorial, depending on corneal

surface quality, preservation time, intrinsic quality of the stromal collagen, and composition of the preservation medium. (3) Adaptation of electric parameters to corneal thickness merits assessment. Changes in stromal hydration during preservation ultimately cause endothelial folds that may alter local resistance to current passage (creating paths of least resistance, for example). Reduction of corneal thickness by incubation in hyperosmolar deswelling medium, which is routinely performed at the end of preservation in cornea banks, reduces the extent of stromal folding and may help to assess this hypothesis.

During this first study, we elected to use fixed parameters for the solution containing the plasmid (NaCl 0.9%) and for the electric current. Given the scarcity of human corneas for scientific use (also reported by other authors in the same field of interest [15]) and the multiple parameters relative to donors and storage, it was not possible to obtain a large series of corneas with reproducible EC characteristics. Lack of corneas prevented the use of different combinations of electric parameters in the first attempt. It has been demonstrated for other tissues [53–55] that adapting the buffer and current parameters may considerably increase transfection efficiency. We are continuing our research, having changed the composition of the buffer containing the plasmid (osmolarity, electric charge and ionic species) and the electric pulse train in order to optimize gene electrotransfer.

In any case, gene electrotransfer may enable alteration of graft tissue during preservation with at least 3 aims: (1) immunomodulation, to minimize the endothelial consequences of graft rejection, as has already been proposed with other transfection methods [12, 21, 56–60]; (2) transfection of apoptosis-resistant genes such as Bcl-xL or HO-1 [49, 61] to minimize EC loss during OC preservation in order to deliver more corneas with higher ECD; (3) triggering of transient EC multiplication by altering the cell cycle, or making cells secrete growth factors to create an autoamplification loop [52], in order to repopulate low-ECD corneas or even to provide 'super quality' corneas with very high ECD likely to prolong graft survival. For all these applications, electroporation is also likely to be the less immunogenic method, a drawback often encountered with viral vectors, but also with non-viral ones.

Although far less efficient than adenoviral vectors, our electroporation device permits simple ex vivo transfection of the human corneal endothelium with a transfection rate comparable with most non-viral vectors, and is thus useful for selected applications. Furthermore, optimizations may be possible to enhance its efficacy and reproducibility.

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Publication 5 (soumis à Stem Cells, IF 7,87) Revisited microanatomy of the corneal endothelial periphery: new evidences for a continuous centripetal migration of endothelial cells in human

TITLE PAGE

Revisited Microanatomy of the Corneal Endothelial Periphery: New Evidence for Continuous Centripetal Migration of Endothelial Cells in Humans

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ABSTRACT

The control of corneal transparency depends on the integrity of its endothelial monolayer, which is considered nonregenerative in adult humans. In pathological situations, endothelial cell (EC) loss, not offset by mitosis, can lead to irreversible corneal edema and blindness. However, the hypothesis of a slow, clinically insufficient regeneration starting from the corneal periphery remains debatable. The authors have re-evaluated the microanatomy of the endothelium in order to identify structures likely to support this homeostasis model. Whole endothelia of 88 human corneas (not stored, and stored in organ-culture) with mean donor age of 80±12 years were analyzed using an original flat-mounting technique. In 61% of corneas, cells located at the extreme periphery (last 200 µm of the endothelium) were organized in small clusters with two to three cell layers around Hassall-Henle bodies. In 68% of corneas, peripheral ECs formed centripetal rows 830+/-295 µm long, with Descemet membrane furrows visible by scanning electron microscopy. EC density was significantly higher in zones with cell rows. When immunostained, ECs in the extreme periphery exhibited lesser differentiation (ZO-1, Actin, Na/K ATPase, CoxIV) than ECs in the center of the cornea, but preferentially expressed “stem cell” markers (Nestin, Telomerase and occasionally BCRP) and, in rare cases, the proliferation marker Ki67. Stored corneas had fewer cell clusters but more Ki67-positive ECs. We identified a novel anatomic organization in the periphery of the human corneal endothelium, suggesting a continuous slow centripetal migration, throughout life, of ECs from specific niches.

INTRODUCTION

Corneal endothelial cells (ECs) are crucial for vision because they control movement of ions and water into the hydrophilic stroma and thus regulate corneal transparency [1]. Derived from the cranial neural crest [2], they form a monolayer of hexagonal cells organized in a tessellated mosaic on their basal membrane, the Descemet membrane (DM). They uniformly cover the whole posterior corneal surface, and at its periphery reach the trabecular meshwork (TM) in the angle between the cornea and iris root. Central endothelial cell density (ECD, expressed in cells/mm^2) physiologically decreases by 0.6% per year during adulthood [3, 4] without impairing transparency, even in centenarians. However, several endothelial dystrophies and certain traumatisms dramatically accelerate this pace: ECD falls below the threshold of 300-500 cells/mm^2, where irreversible corneal edema occurs, causing permanent vision loss. Such endothelial dysfunctions represent one third of the indications for corneal grafts performed yearly worldwide.

In vivo, corneal endothelium has limited wound-healing capacity, using residual ECs which, by enlargement and migration, cover the defects left by lost cells [5] without cell division. Joyce and colleagues have demonstrated that human corneal ECs in vivo are arrested in the G1 phase of the cell cycle [6, 7]. They express negative regulators of the cell cycle belonging to the CIP/KIP family (p21 and p27), to the INK4 family (p16, p15 and p19), and to the p53 family of proteins (p53, TAp63) [8]. Mitotic inhibition may be caused by the presence of transforming growth factor-beta (TGF-β) in aqueous humor, by contact inhibition of densely-packed ECs in a joint mosaic [9], and by stress-induced premature senescence [10-12].

However, a series of clinical and experimental arguments suggest the existence of endothelial regeneration in the human corneal periphery. Higher ECD in the endothelial periphery was described more than 25 years ago [13-15]. The possibility of centripetal migration of ECs from the periphery is suggested by at last two observations: the reported colonization of donors' DM by recipients' ECs after full-thickness corneal graft [16, 17]; and the demonstrated longer survival of corneal grafts in recipients with high ECD in the endothelial periphery [18, 19], namely in keratoconus, a noninflammatory corneal disease affecting young people and characterized by progressive stromal thinning and ectasy but an endothelium that remains normal. This second observation suggests that the central endothelium is continuously sustained by the peripheral reserve. Furthermore, the continuous insidious destruction of peripheral ECs by certain artificial lenses implanted during cataract surgery in the 1980s, in contact with the corneal periphery, led 10-15 years later to edematous decompensation of the cornea, thus constituting an iatrogenic demonstration of the importance of peripheral ECs in endothelial and corneal homeostasis. Similarly, there is undoubtedly a rapid decrease in central ECD after corneal graft when no ECs persist in the recipient's corneal periphery, typically in edematous decompensation of the cornea. This suggests that central ECs that die cannot be replaced by peripheral ECs.

In experimental conditions, human corneal ECs retain residual proliferative capacity, even in very old donors [20]. In vitro, peripheral ECs have higher mitotic activity than central ones, as their morphology in primary culture is less typically hexagonal and their intercellular junctions loosen [21]. These differences may be due partly to the higher density of precursors in the endothelial periphery [22]. Whikehart [23] and McGowan [24] suggested that human corneal endothelial stem cells (SCs) may be sequestered in niches located deep in the TM or in the so-called transition zone (TZ) between the endothelial edge and the TM, and supply new cells to heal corneal endothelial wounds, at least during in vitro experiments.

In this study, we present a large series of corroborating observations that combine optical and electron microscopy with immunolocalization done on flat-mounts of whole human endothelia. This provides evidence that EC proliferation exists in the extreme periphery of human corneas in vivo. Cell organization in clusters and radial rows, first described in this paper, progresses by one step the hypothesis that ECs continuously migrate centripetically from the extreme periphery, where stem cells may nest, to the centre of the cornea.

MATERIALS AND METHODS

Cornea characteristics

Three groups of corneas were studied. Group 1 (non-stored corneas) comprised 39 corneas obtained from the Anatomy Department of the Faculty of Medicine of Saint-Etienne thanks to body donations to science, and processed immediately after procurement with no immersion in storage medium. Group 2 (stored corneas) comprised 45 corneas studied after storage in our eye bank. These were normal tissues procured after relatives had been informed, as authorized by French bioethics law. They were not suitable for transplantation owing to inconclusive donor serology despite normal endothelial characteristics, in particular central ECD>2000 cells/mm^2, the conventional cut-off value for corneal graft qualification. They were stored in organ culture, the prevalent method used in European eye banks, which maintains cell metabolic activity for at least 5 weeks [25]. Corneas were immersed in a closed vial of 100 mL of a commercial medium containing 2% fetal calf serum (CorneaMax, Eurobio, Les Ulis, France) at 31°C, renewed after 2 weeks. Forty corneas were studied just before medium renewal, and five 3 days after. Corneas in groups 1 and 2 were procured from donors placed in 4°C mortuary chambers, by in situ excision with 18 mm diameter trephination, thus comprising the whole cornea and a rim of adjacent sclera with TM and iris root, as per the procedure recommended in France for corneas intended for transplantation. Group 3 (fresh corneas) comprised four central corneal buttons (8.25 mm in diameter) obtained, after patients' informed consent, directly in the operating theatre during full-thickness graft for keratoconus in Saint-Etienne University Hospital. Time from procurement to fixation was only 15-30 minutes.

In group 1, donor age was (mean+/-standard deviation) 84+/-8 years and death-to-procurement time 20+/-16 hours. In group 2, donor age was 77+/-10 years. In group 3, donor ages were 23, 28, 30 and 30 years. Handling of the donor tissues adhered to the tenets of the Declaration of Helsinki of 1975 and its 1983 revision in protecting donor confidentiality.

Observation of flat-mounted corneas (*en face* observation)

Cell morphology

ECs were stained using Alizarin red to visualize cell borders of the whole endothelial mosaic. Prior to staining, the endothelial side was rinsed with balanced salt solution (BSS, Alcon, Rueil Malmaison, France). The dye (0.5% Alizarin red (Sigma, St Louis, MO) dissolved in 0.9% sodium chloride with a pH adjusted to 5.2) was filtered through a 0.2 µm filter and placed in the corneal concavity for 90 seconds. After removal of excess dye, corneas were fixed for 2 minutes in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) of pH 7.45. Corneas were then immersed in PBS and shaken until detachment of the dark red deposit that had formed on the endothelial surface. Nuclei were counterstained either with 0.4% trypan blue solution (Sigma) for 2 minutes or Hoechst 33342 (Sigma) 10µg/ml in PBS for 1 minute. After a brief rinse in PBS, corneas were flat-mounted with four radial cuts. A large coverslip was maintained using adhesive tape to ensure mount stability. In a few corneas, the endothelium was observed using phase contrast combined with fluorescence imaging of nuclei stained with Hoechst but not Alizarin red.

Cell count

ECD was determined in various endothelial zones (see below) using image analysis with the Cell^ P software (Olympus Soft Imaging Solutions, Berlin, Germany). Briefly, cell nuclei were automatically isolated after Hoechst image thresholding. Systematic manual controls prevented false-negative and false-positive cases.

Nuclei were then numbered in large regions of interest (ROIs) comprising at least 1000 nuclei. Half of the nuclei truncated by the ROI edges were taken into account.

Cell mortality assessment

In order to assess local differences on the whole endothelium in susceptibility to death after stresses caused by donor death and/or corneal storage, EC viability was determined using trypan blue staining in a subset of corneas randomly chosen from groups 1 and 2. For group 1, corneas were studied within minutes of procurement. This vital staining is routinely used in eye banks during organ culture. The endothelial side was exposed for 1 minute to 0.4% trypan blue solution (Sigma). After two rinses in BSS to remove excess dye, the cornea was fixed for 2 minutes in 4% PFA. After a brief rinse in PBS, nuclei were counterstained with Hoechst 33342 (Sigma) 10µg/ml in PBS for 1 minute, and corneas were flat-mounted as previously described.

Histology on cross-sections

To compare the *en face* view and conventional histology on cross-sections, corneas were fixed in 4% PFA for 24 hours, then dehydrated in successive ethanol baths and embedded in paraffin. Serial 6-micron-thick sections were cut on a microtome, deparafinized, and stained with hematoxylin eosin safran (HES).

Immunostaining on flat-mounted corneas: proliferation, differentiation, stem cell markers

EC differentiation was studied using the expression pattern of four proteins: hexagonal apical organization of the tight junction protein ZO-1; hexagonal organization of the cytoplasmic F-actin belt; basolateral localization of Na^+/K^+ ATPase that participates in EC pump functions; and cytoplasmic expression of Cytochrome C Oxidase (COX) IV, which stains mitochondriae. Stemcellness was studied using Nestin, Telomerase, Breast Cancer Resistance Protein (BCRP/ABCG2), Oct-4, and P63-α antibodies. EC proliferation was studied with the ubiquitous proliferation marker Ki67 already validated for human corneal ECs [26].

Specific primary antibodies were IgG from either mouse or rabbit (Table 1). Nonspecific rabbit IgG (Zymed, Carlsbad, CA; 02-6102) and mouse IgG (Zymed, Carlsbad, CA; 02-6502) were used for negative controls. Secondary antibodies used at 1/500° were Alexa Fluor488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA; A11001) and Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen, A21428). Each cornea was cut radially into four or eight pie-shaped wedges. Fixation was done at RT for 45 minutes either in 0.5% PFA in PBS pH 7.45 or using methanol, depending on the antigen. Fixation was followed by antigen retrieval with 0.5% sodium dodecyl sulfate (SDS) in water for 5 minutes at RT to reveal telomerase. Membrane permeabilization with 1% Triton X-100 in PBS was necessary for PFA-fixed corneas except the corneal wedges treated with SDS. These experimental conditions were optimized after previous experiments (data not shown). Heat-inactivated goat serum was used to block the nonspecific binding sites for 30 minutes at 37°C. Corneal pieces were immersed in primary antibodies diluted in 2% inactivated goat serum and 2% bovine serum albumin in PBS for 1 hour at 37°C. After three rinses in PBS, pieces were incubated with the secondary antibody for 45 minutes at 37°C. The counterstain was done with Hoechst 33342 10µg/ml in PBS for 1 minute at RT. Lastly, the corneas were flat-mounted in PBS just before observation. All images were captured using an IX81 fluorescence microscope (Olympus, Tokyo, Japan) with Cell^P imaging software.

Table 1. List of primary antibodies

Target protein	Animal source	Clonality	Laboratory	Reference	Dilution	Fixative
ZO-1	mouse	monoclonal	Zymed, Carlsbad,CA	33-9100	1/200	methanol
Actin	mouse	monoclonal	Sigma, Saint Louis, MO	A4700	1/200	methanol
Na^+/K^+ ATPase	mouse	monoclonal	Millipore, Billerica , MA	#05-369	1/200	methanol
COX IV	rabbit	monoclonal	Cell Signaling, Danvers, MA	#4850	1/400	methanol
Telomerase	rabbit	monoclonal	Abcam, Cambridge, UK	ab32020	1/200	0.5% PFA
Nestin	rabbit	polyclonal	Abcam, Cambridge, UK	ab92391	1/200	0.5% PFA
BCRP/ABCG2	mouse	monoclonal	Chemicon, Temecula , CA	MAB4145	1/200	0.5% PFA
Oct-4	rabbit	polyclonal	Cell Signaling, Danvers, MA	#2750	1/200	0.5% PFA or methanol
P63-α	rabbit	polyclonal	Cell signaling, Danvers, MA	#4892	1/200	0.5% PFA or methanol
Ki67	mouse	monoclonal	Dako, Glostrup, Denmark	M 7240	1/200	0.5% PFA

Scanning electron microscopy of Descemet membrane

Five corneas (two from group 1 with death-to-procurement time of 11-66 hours, and three from group 2 stored for 10, 20, and 23 days) with mean donor age of 85+/-19 years (53-100 years) were observed after removal of ECs, to give access to the bare DM. ECs were removed by incubation with 1% SDS in water followed by vigorous washing in distilled water. The corneas were fixed in 0.1N cacodylate-buffered 2% glutaraldehyde pH 7.4 at 4°C for 24 hours. They were washed with 0.2N cacodylate and then distilled water. The fixed specimens were dehydrated through ascending concentrations of acetone up to pure acetone. They were then dried using a critical point dryer (E3000, Quarum Technologies, East Sussex, UK). After coating with gold-palladium in a mini sputter coater (Polaron SC 7620, Quarum Technologies) and mounting on metal stubs, the specimens were observed using a Hitachi S-3000N SEM at an electron accelerating voltage of 5 kV.

Definition of observation zones

Cell characteristics were studied systematically in the surgical or anatomical regions of the posterior surface of the cornea, which were defined as follows, from center to periphery (Fig. 1): (1) endothelial center with a 4.0 mm radius, corresponding to the disk usually trephined by the surgeon for corneal graft; (2) endothelial periphery (P) of approximately 1.5-2 mm width (depending on corneal diameter), stopping at the anatomic endothelium edge i.e. the end of the DM, also called Schwalbe's line; (3) for our work, we had to define a supplementary zone inside the periphery corresponding to the last 0.2 mm of the endothelial periphery, which we named endothelial extreme periphery (EP), a zone comprising most of the Hassal-Henle bodies that correspond to age-related Descemetic mushroom-shaped expansions; (4) transition zone (TZ), 0.15 mm wide, characterized by absence of DM and of fibers typical of the TM. The zone is often confused in the literature with Schwalbe's ring, which is only prominent in 15% of corneas [27]; (5) and finally TM, approximately 0.5 mm wide. Outside these structures, residues of the iris root, gently detached during procurement, remained as a pigmented ring, followed by bare sclera cut at a 9 mm radius.

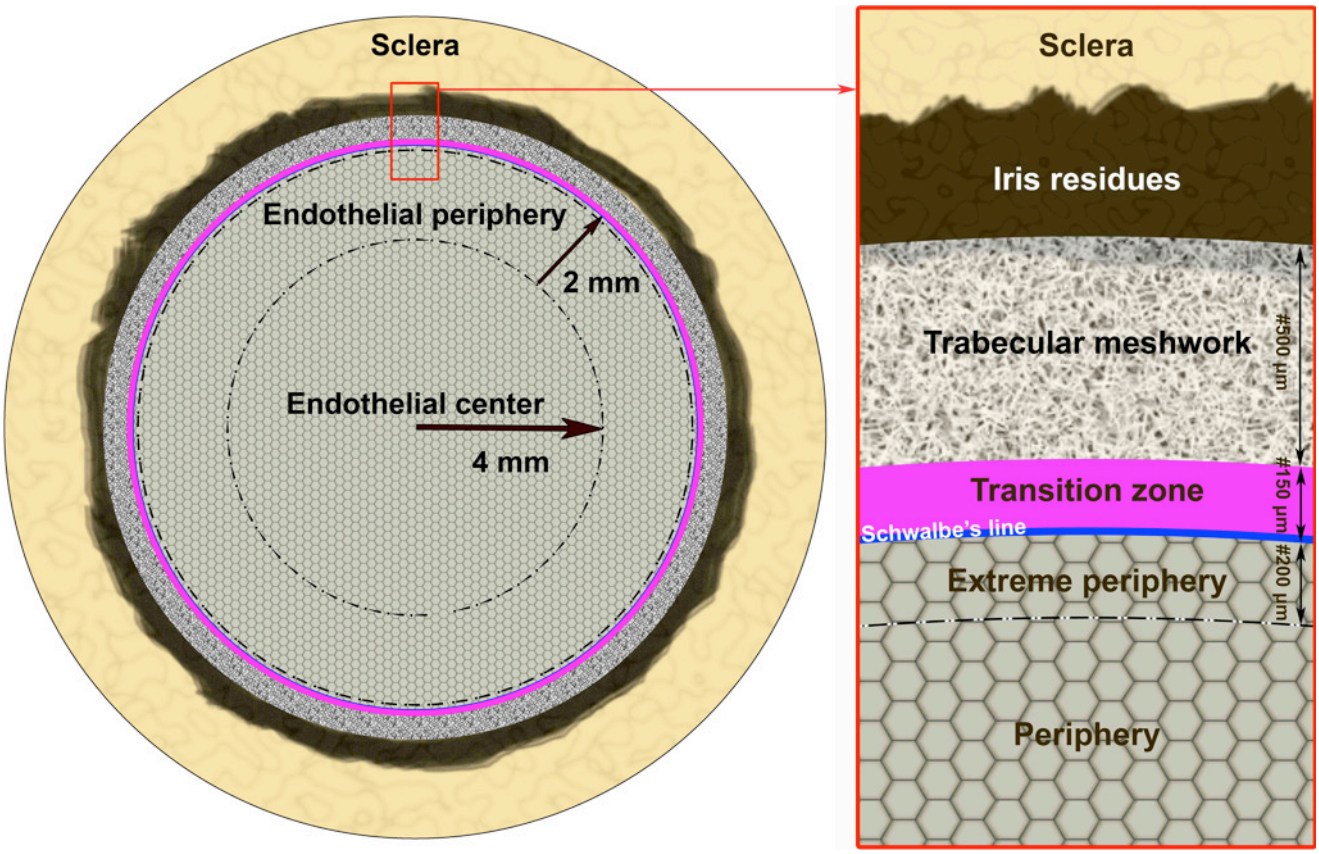

Figure 1. Zones of interest on the posterior surface of a human cornea. The sclera has been trephined to a diameter of 18 mm and the iris removed.

RESULTS

En face and cross-section observations

Cell clusters in the extreme periphery (Fig. 2)

On the flat-mounts, cell clusters were observed in the extreme periphery in nearly one third of cases (61% (n=23/38): 80% of group 1 corneas (n=16/20) and 39% of those in group 2 (n=7/18) (P=0,01 (Khi^2)). These clusters were organized between the Hassall-Henle bodies and were not clearly separate, and so impossible to count precisely. However, we estimate that on average they occupied one third of the circumference of each cornea. On the cross-sections, they always comprised two or three cell layers 7-12 µm deep. The cells in the clusters had spherical or ovoid nuclei with smaller diameters than those of the central ECs, which had flat, strictly round nuclei of larger diameter.

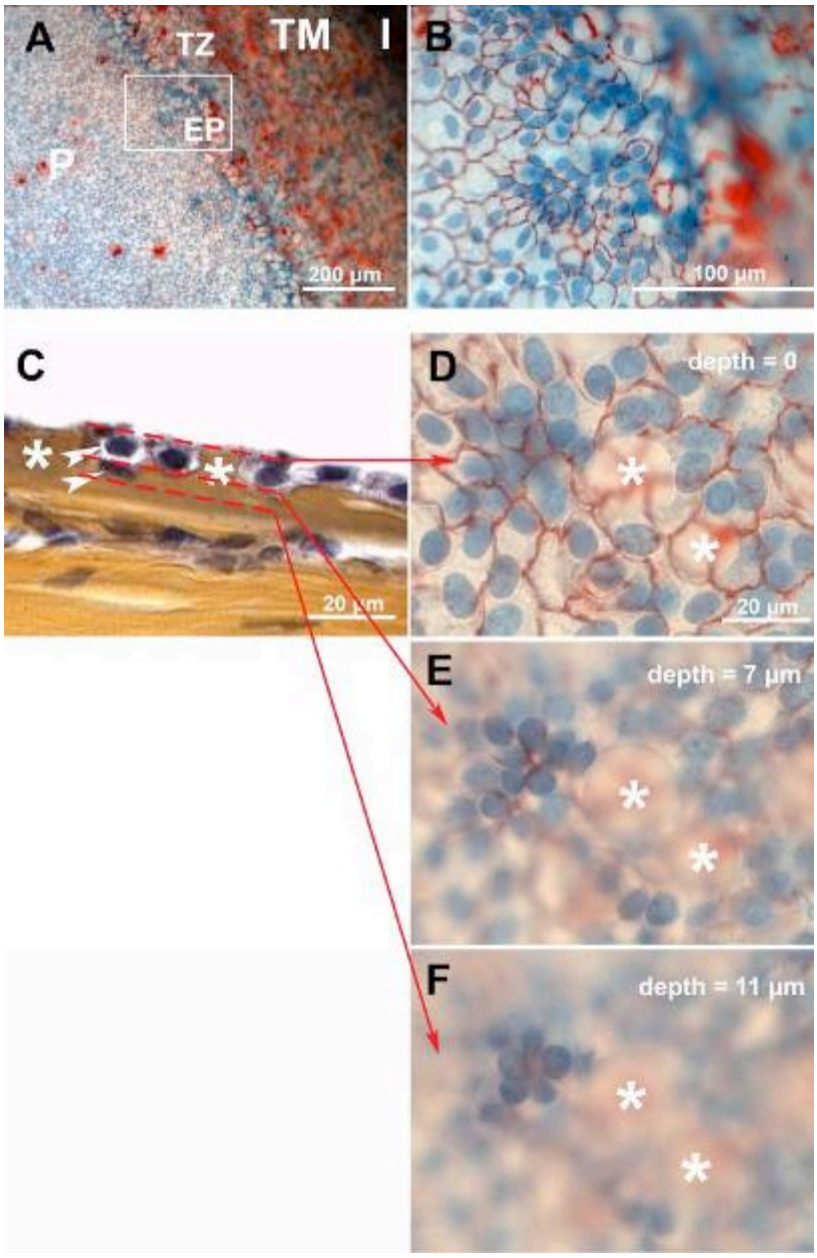

Figure 2. Cell clusters. (**A, B):** En face view of the corneal endothelial periphery (P). Cell clusters were localized in the extreme periphery (EP) in contact with the transition zone (TZ). Trabecular meshwork (TM) and iris root residues (I) are indicated. A x10, B: x40 Alizarin red and trypan blue on fixed tissue. (**C-F)** (x96): Multilayered

organization (arrowheads) of cell clusters, always localized in immediate vicinity of Hassall-Henle bodies (**asterisk**). **(C):** Cross-section (hematoxylin eosin safran). (**D, E, F):** En face view (Alizarin + trypan blue).

Radial endothelial cell rows in the periphery (Fig. 3)

General description

In the periphery, EC organization in radial rows was observed in more than two thirds of cases (68% (n=26/38)) : 67% (n=12/18) of group 1 corneas and 70% (14/20) of those in group 2 (non-significant difference). These rows on average occupied 86+/-24% of the corneal circumference, leaving some zones with none. These rows were variable in length in the same cornea and also between corneas, varying from 350-1465 µm (mean 827+/-295 µm). There was no difference in donor age or in central ECD between corneas presenting rows and the other corneas (data not shown). ECs of rows consistently had elongated nuclei (compared with those of the central ECs, which were always strictly round), the longitudinal axis of which most often coincided with the axis of the rows. Cells in rows had increased polymorphism and weaker Alizarin red staining than central ECs.

Endothelial cell density in radial rows

Of the seven corneas in group 1 (age=83±13 years, death-to-procurement time=19±20 hours) picked randomly from among those organized in rows, ECD was significantly higher (by 69%±41%) in the cell rows than in the immediately adjacent zone not organized thus, respectively 3483±558 versus 2141±507 EC/mm² ($P<0.05$). The highest maximum difference observed was 4344 versus 1786 cells/mm^2, or +143% in a group 1 cornea (donor aged 72 years, death-to-procurement time of 4.5 hours). This difference was less marked in the 10 group-2 corneas (age=80±14 years, death-to-procurement time=17±11 hours, time in organ culture 14±6 day): 23%±27% higher in the cell rows, respectively 1993±698 versus 1705±750 ECs/mm^2.

Descemet membrane furrows in radial rows

Viewed by light microscopy on flat-mounts, the DM contained imprints of the linear organization of the ECs, in the form of furrows at the base of which EC nuclei were lodged. On the cross-sections, the DM periphery was thicker and more irregular than its center and the furrows defined by DM excrescences covered by EC cytoplasm (**Fig. 4)**. In SEM, the furrows appeared as the dotted line formed by DM plots (**Fig. 3**).

Relationship between cell clusters and radial rows (Fig. 3, 5)

In all corneas, cell rows were separated from cell clusters by a narrow 150-200 µm band comprising polymorph ECs with an ECD lower than in cell rows (see above). Alterations to EC morphology were greater in group-2 corneas. In cross-sections, the DM of this band presented warts more elongated than typical Hassal-Henle bodies.

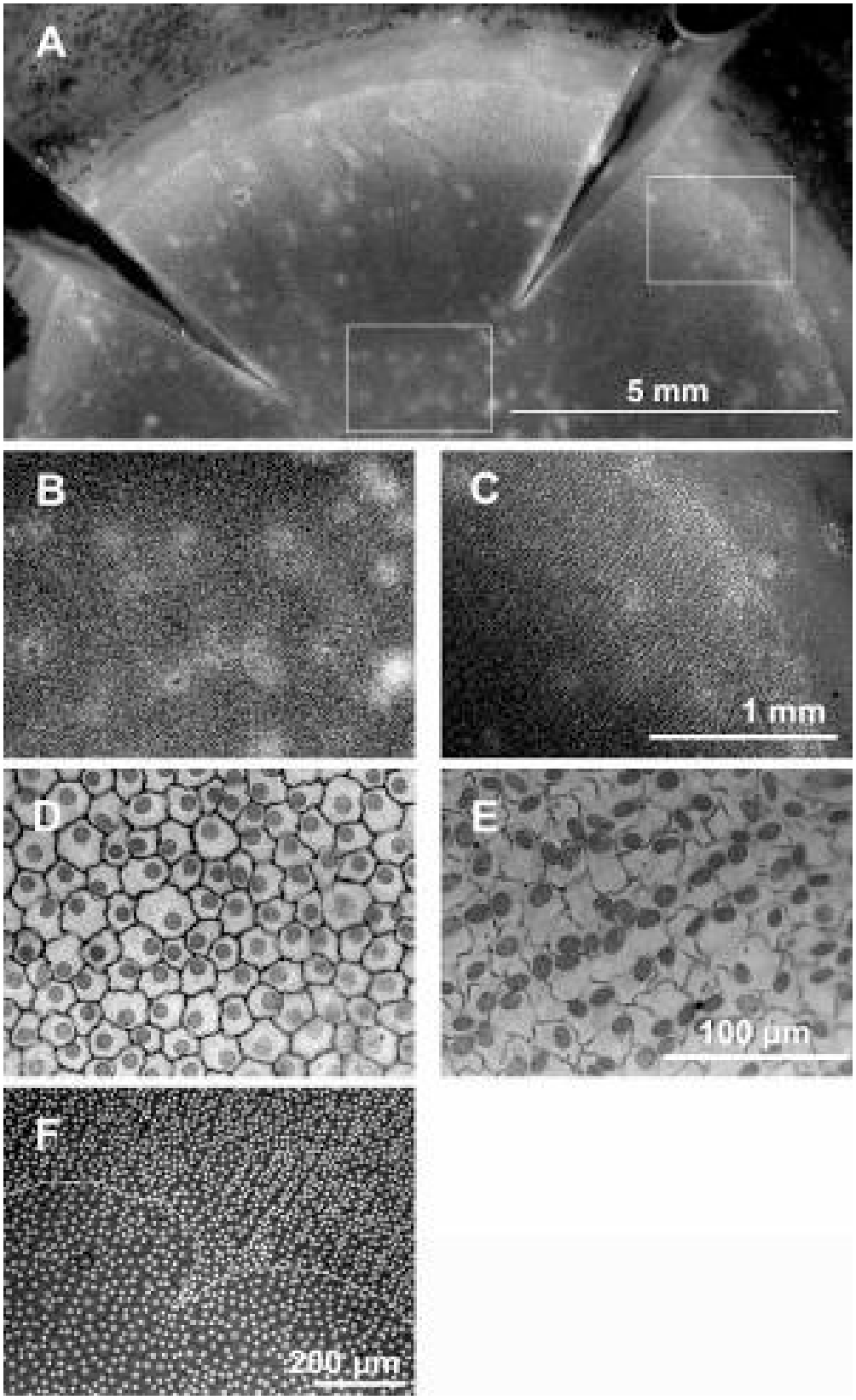

Figure 3. Radial endothelial rows in the corneal periphery. **(A):** General view of half cornea flat-mounted thanks to radial cuts. Hoechst 33342 staining, x4. (**B and C):** Zoom with x10 objective on center **(B)** and periphery **(C)** where cell rows were clearly present. **(D and E):** Difference of endothelial cell morphology between center **(D)** and periphery **(E)**. Alizarine red and Hoechst staining, x40. (**F):** Transition between cell rows and more central endothelium in a non-stored cornea. The white line indicates an artificial boundary between the zones. Hoechst staining, x10.

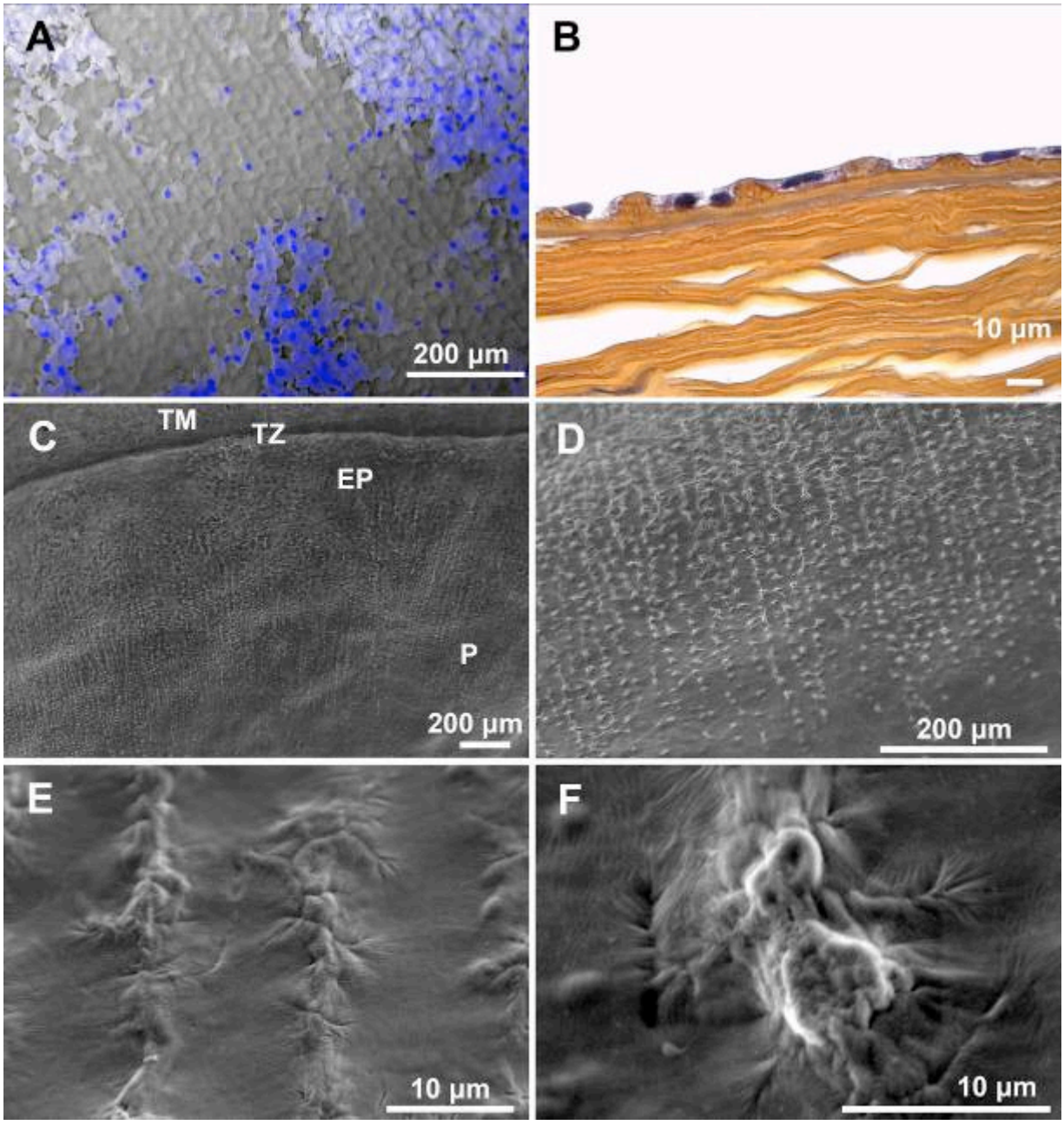

Figure 4. Radial Descemet membrane furrows in the corneal periphery. Furrows were made visible after partial **(A)** or complete **(C,D,E,F)** removal of endothelial cells. (**A):** Flat-mount observed with phase contrast to highlight DM excrescences, and with Hoechst 33342 to locate cell nuclei at the base of the furrows. **(B):** Cross-sections orthogonal to the cell rows, stained with hematoxylin eosin safran. **(C,D,E,F):** Scanning electron microscopy of the bare Descemet membrane, showing furrows defined by real plots and starting near the transition zone (TZ). Abbreviations: TM, trabecular meshwork; EP, extreme periphery; P, periphery.

Endothelial cell mortality

In all corneas (groups 1 and 2), high cell mortality (>50% of cells) was systematically observed in the extreme periphery, with mortality decreasing very rapidly towards the center (**Fig. 5**).

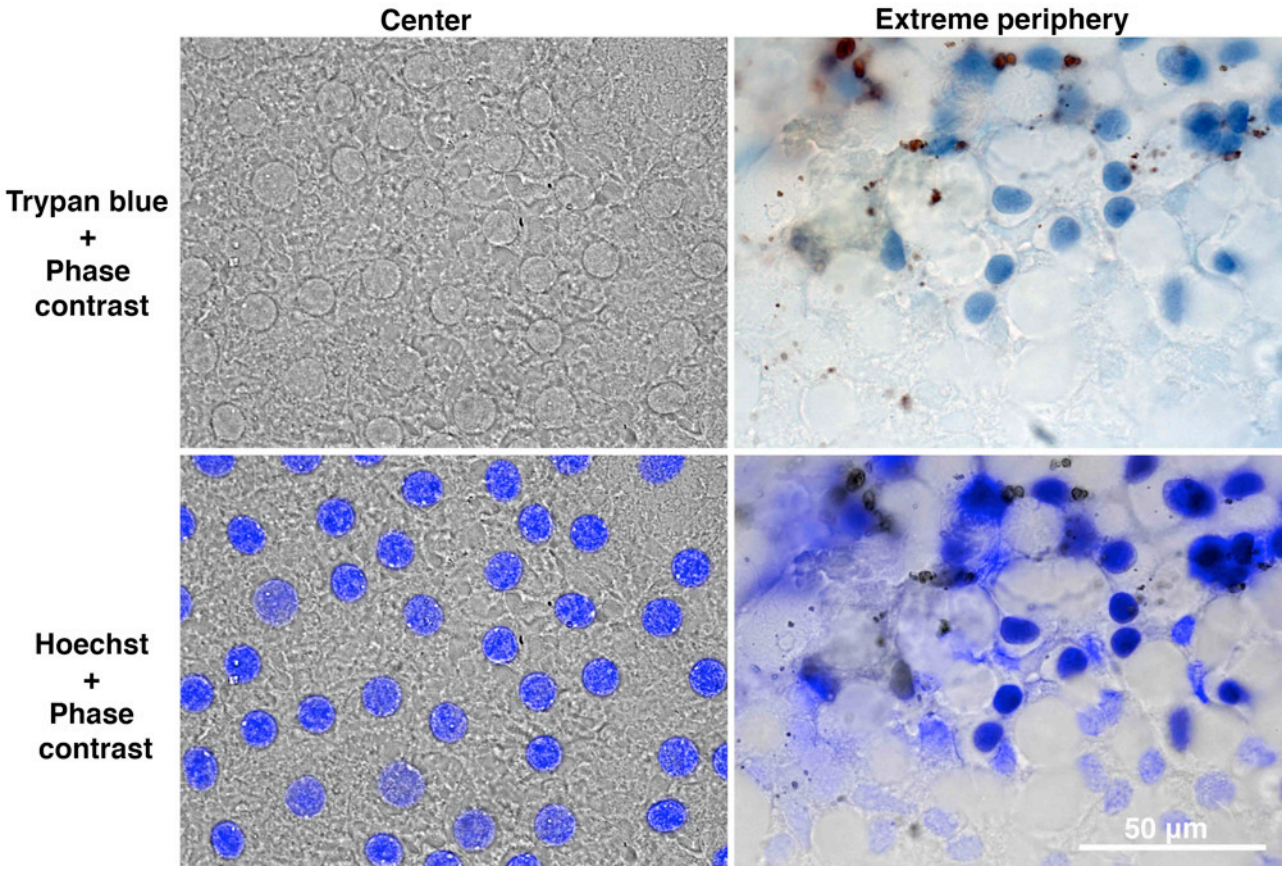

Figure 5. Cell mortality in the extreme periphery compared to the center. Representative example of a non-stored cornea (age 69 years, death-to-procurement time 20 hours). Trypan blue and Hoechst staining. x40

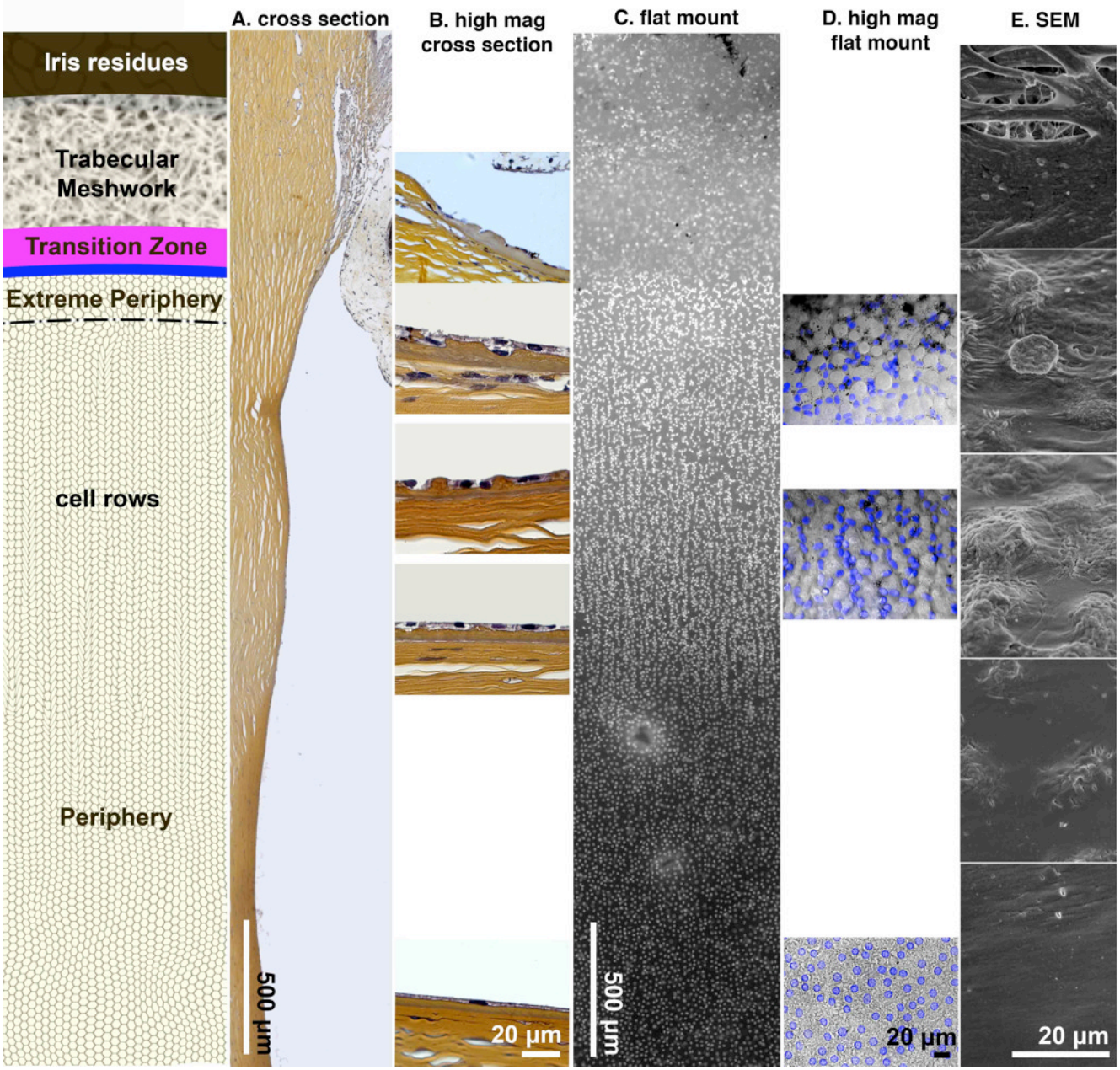

Figure 6. Summary of endothelial and Descemetic centripetal modifications. (**A, B):** Cross-sections. Hematoxylin eosin safran staining demonstrating progressive centripetal alterations of the Descemet membrane (DM). **(C):** En face view after Hoechst staining of nuclei. **(D):** Merge Hoechst and phase contrast to highlight DM excrescences and nuclei preferential orientation. **(E):** Scanning electron microscopy of DM surface structures after endothelial cell removal. All images have been aligned.

Immunolocalization

Differentiation markers

The cells in the extreme periphery did not have the expression patterns typical of central ECs for ZO-1 (absence of apical organization tight-junctions, hexagonal aspect), Actin (absence of submembranal apical belt, hexagonal aspect), Na^+/K^+ ATPase (absence of enzyme activity associated with ionic-pump function, located on the basolateral membranes on cell interdigitations, giving a distinctive star aspect), and COX IV (absence of dense mitochondrial network) **(Fig. 7)**.

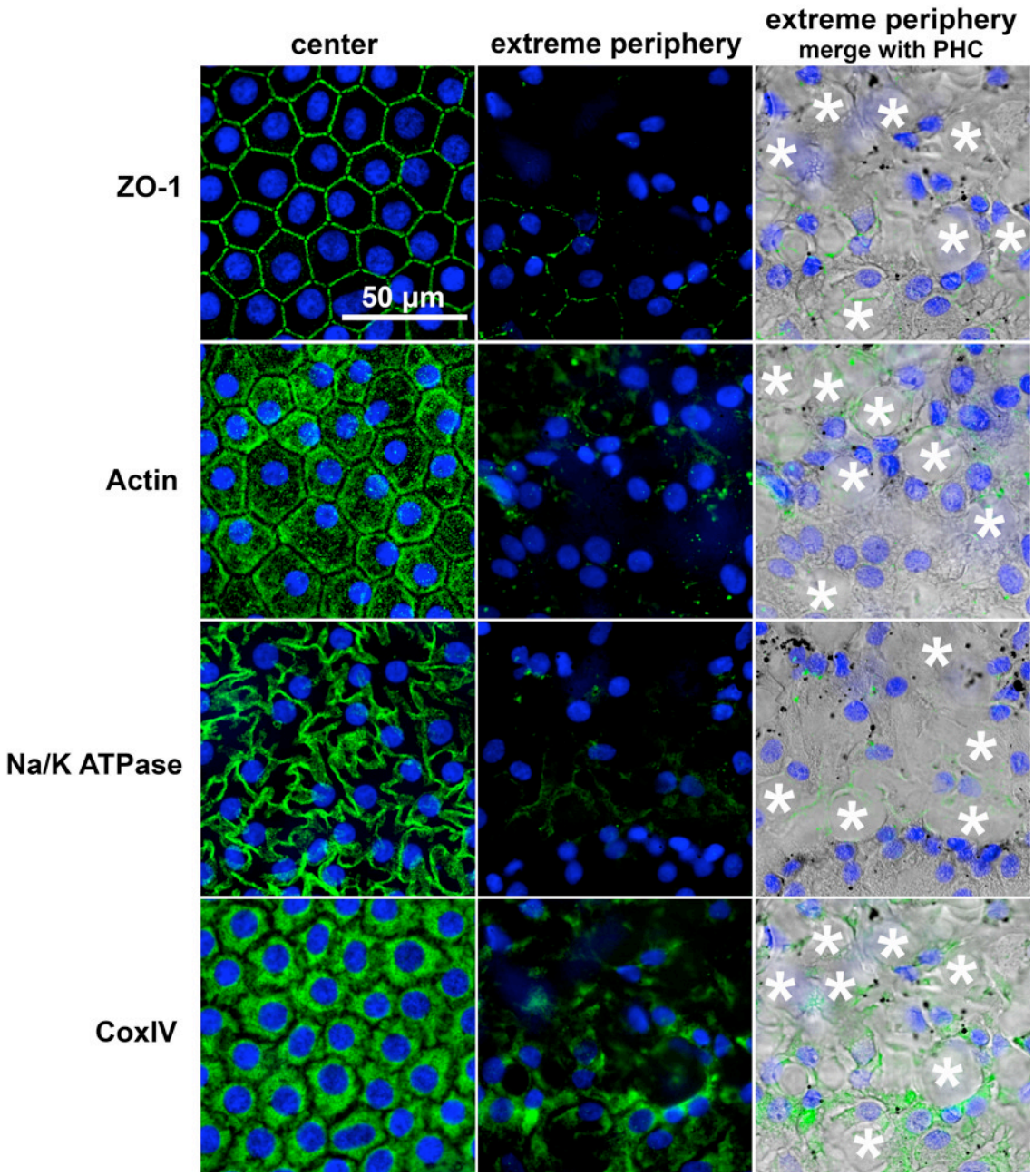

Figure 7. Immunolocalization of differentiation markers in endothelial cells. The typical expression patterns of ZO-1, Actin, Na/K ATPase and CoxIV observed in the center were absent in the extreme periphery, in the cell-cluster zone. Phase contrast (PHC) was used only for the extreme periphery to allow visualization of Hassal Henle bodies (asterisk), which by definition were absent in the center. x40

Stem cell markers (Fig. 8 and Table 2)

Expression of Nestin (nuclear and/or cytoplasmic) and Telomerase (cytoplasmic) was found in ECs in the extreme periphery. Weak expression of Nestin was observed in the cytoplasm of the scarce isolated ECs elsewhere in the endothelium. Expression of BCRP/ABCG2 was detected in only a few ECs of the extreme periphery, but also in the center of both corneas from one group-1 donor, procured very shortly after death (4.5 hours). There was no difference of expression of any of the five markers between corneas of groups 1 and 2 (data not shown).

Table 2. Expression of five stem cell markers in endothelial cells according to their localization on corneas.

	Telomerase (n=12)	Nestin (n=10)	BCRP/ ABCG2 (n=11)	Oct-4 (n=10)	P63 (n=6)
Extreme periphery	9++	8++	1	0	0
Center	0	8	2	0	0

n = number of corneas assessed

++ brightly expressed in a majority of cells whereas other staining concerned only few cells with weaker expression.

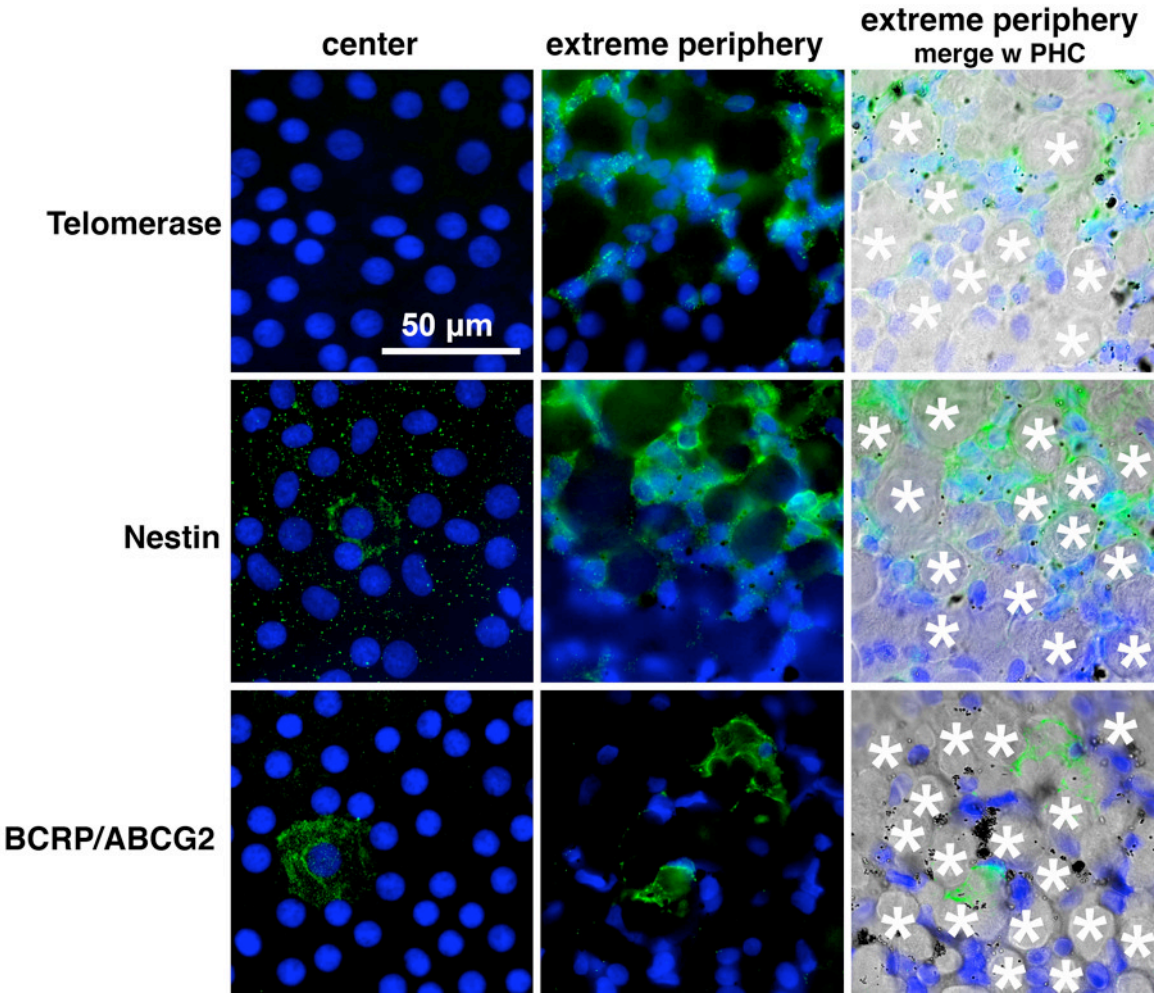

Figure 8. Immunolocalization of stem cells markers in endothelial cells. Telomerase and Nestin were largely expressed in the cell-cluster zone of the extreme periphery of almost all corneas. By contrast, BCRP/ABCG2 was found only in two non-stored corneas. Phase contrast (PHC) was used only for the extreme periphery to allow visualization of Hassal Henle bodies (asterisk). x40

Proliferation marker Ki67 (Fig. 9)

In the non-stored corneas of group 1, scarce Ki67-positive ECs were present, but only in clusters in the extreme periphery of the endothelium of two corneas with very short death-to-procurement times (4.5 and 5 hours). In the stored corneas of group 2, many Ki67-positive ECs were observed in the periphery and slightly beyond, but only in corneas of which the organ-culture medium had been renewed 3 days before observation. In the group-3 corneas, no marking was observed.

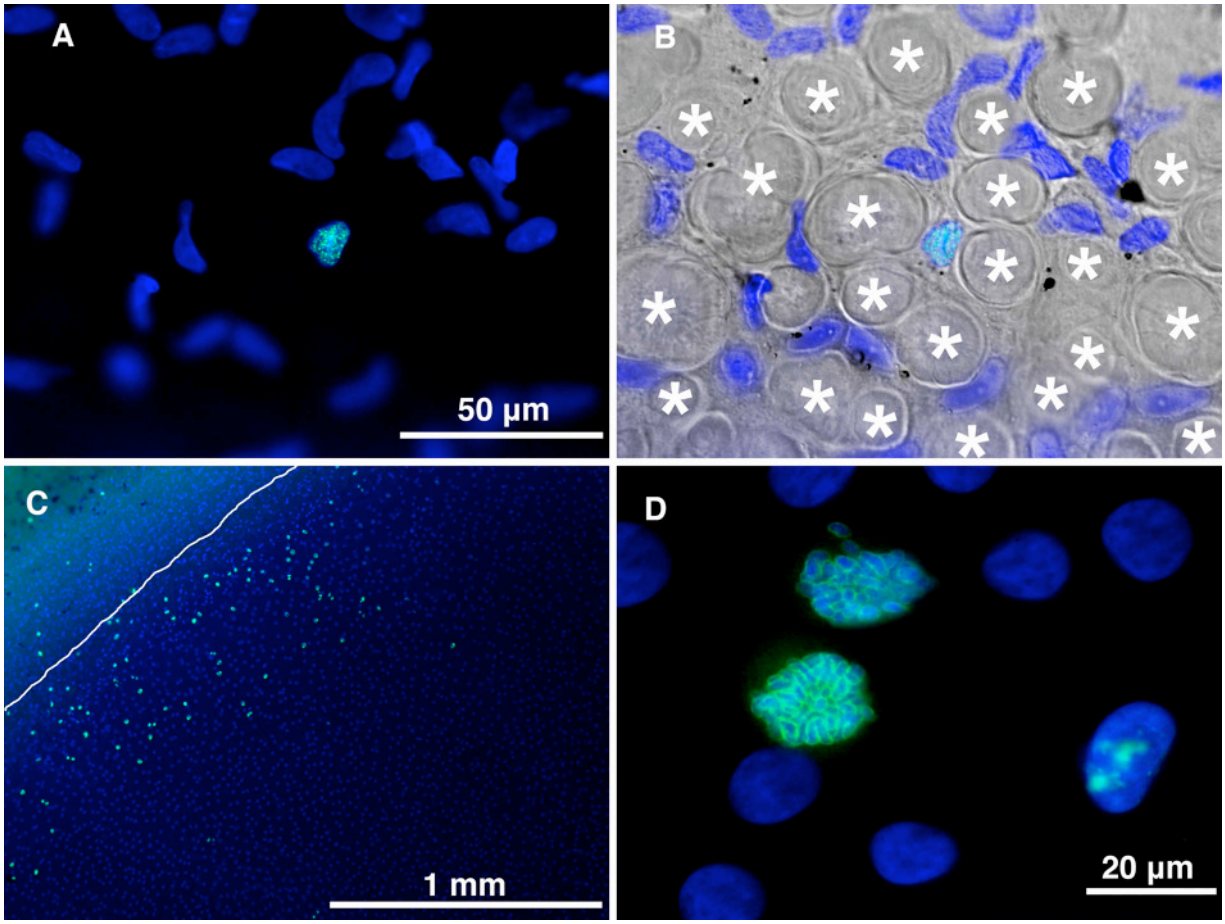

Figure 9. Immunolocalization of the proliferation marker Ki67. **(A, B):** Non-stored cornea with death-to-procurement time of 4.5 hours. Only a few isolated endothelial cells (ECs) located in the extreme periphery were positive. **(A):** Hoechst + Ki67, **(B):** Merge with phase contrast to highlight Hassall-Henle bodies (asterisk). (**C, D):** Stored cornea observed 3 days after storage medium renewal. Multiple Ki67-positive ECs were visible, with metaphases indicating active cell cycling. The line in C delineated the transition zone.

DISCUSSION

The corneal periphery is a complex region that is essential for ocular embryogenesis and for homeostasis of the adult cornea. Superficially, the limbus, a heavily vascularized junction zone between the cornea and sclera, contains epithelial SCs located in specific niches called limbal epithelial crypts (LECs) [28, 29] as well as many transient amplifying cells (TACs) [30]. The epithelium is a multilayered tissue with regular turnover and capable of rapid *ad integrum* healing even after total mechanical destruction, providing the limbus is intact. Epithelial SCs seem to be activated in case of massive epithelial damage, whereas physiological turnover is thought to be provided by TACs [30, 31]. While the existence of epithelial SCs in the limbus is an old premise, the anatomic description of their specific niches is more recent [28, 32]. These LECs are located at the ends of the palisades of Vogt, which are solid radial cords of cells in the extreme periphery of the corneal epithelium. The periphery of the corneal stroma probably also houses keratocyte progenitors, at greater depth [33]. Lastly, the posterior, i.e. truly intra-ocular, face of the cornea is even more complex than its surface, as it contains the posterior limbus (TM, Schlemms'canal, TZ) implicated in the regulation of intraocular pressure and the edges of the corneal endothelium. It may house corneal endothelial TACs or SCs [22-24]. In the hypothesis of slow endothelial renewal, however, their proliferative capacity remains very limited and in particular, unlike the epithelium, they cannot respond to massive endothelial damage, even when the posterior-limbus structures remain intact. This limitation has yet to be explained.

For the first time, using a large number of human corneas, some of which were studied without storage, and complementary methods of observation, we were able to describe a new microanatomic organization of ECs in the endothelial periphery, which we termed cell clusters and radial cell rows, and which suggests that ECs slowly and continuously migrate from periphery to center throughout life.

EC organization in the extreme periphery of the human cornea, in small clusters of 2-3 layers between Hassall-Henle bodies, had never been described. Hassall-Henle bodies were described in the late 19th century, and are associated with aging of the normal cornea [34]. These mushroom-shaped Descemetic structures are solely observed in the endothelial periphery. They are absent in children and may progressively appear after 30 years, initially near Schwalbe's line and extending centripetically over 1 mm [35]. They are constant in humans, but their number varies among individuals. Two hypotheses may explain their association with the peripheral cell clusters we describe. Firstly, that Hassall-Henle bodies are synthesized throughout life by the cells of the clusters that perpetually reside in the periphery. Secondly, that clusters do not initially exist and Hassall-Henle bodies progressively develop during the continuous centripetal migration of cells that emerge from the posterior limbus and synthesize collagen at their emerging site. Secondarily, Hassall-Henle bodies, with aging, become obstacles that progressively slow cell migration and are consequently responsible for cell accumulation in clusters. Further studies on younger donors are necessary to ascertain whether cell clusters are present independently of Hassall-Henle bodies.

In 1957, Vrabec noted peculiar fan-shaped groups of ECs in the corneal periphery that were closely related to the anterior anchorage of the ciliary muscle tendons [36], but did not describe multilayered clusters. As it is very unlikely that endothelial SCs nest at the surface, the finding of a deeper structure, closer to the vascularization, may indicate a potential niche. The apparent smallness and round nuclei of these cells is also consistent with SCs or stem-like cells. These clusters are not directly connected with the cell rows we describe, remaining separated by an irregular zone where Descemet warts do not have the round shape typical of Hassall-Henle bodies. Cells in this zone are not fully differentiated, as we demonstrated using immunostaining, as they do not have structural and functional proteins typical of mature ECs, but they nevertheless already form

a monolayer which is one feature of ECs. Our staining pattern for SC and TAC markers is consistent with previous reports by Whikehart and McGowan, but with certain differences. They described telomerase activity only in ECs located in a zone 4-12 mm from the center and not in the centre, TM or TZ [23], whereas protein expression was described in the TM, TZ and endothelial periphery on cross-sections and only in the TM on flat-mounted whole corneas [24]. In our series, with immunostaining of flat-mounted whole corneas, telomerase expression seems restricted to the extreme periphery and TM. We found Nestin predominantly in the extreme periphery, whereas it was present only in the TM and TZ (similar on cross-sections and flat-mounts) for Whikehart [23]. BCRP/ABCG2 has been described as a marker for hematopoietic [37] and corneal epithelial SCs [38], among other cell types. We found it expressed occasionally in isolated ECs. These results, associated with negativity for Oct-4 [39, 40] and P63 [41], both stem cell markers, suggest that cells in clusters may be TACs rather than SCs. The absence of immunostaining for cells located more deeply in the clusters may be due to our choice of staining only on flat-mounts in order to screen for rare cells among an intact endothelial population.

Unlike the LECs described for the corneal epithelial SCs in the limbus [28] and which meet all niche criteria (anatomic protection, specific niche cells, proximity to vascularization, specific microenvironment, adhesion molecules, extracellular matrix components) [42, 43], we did not expect to find similar structures for the endothelium, owing to the absence of clinically significant proliferative capacity. Nevertheless, our microanatomic observations supply fresh arguments in favour of niche-like structures in the extreme periphery of the cornea.

We found overmortality in the extreme periphery, in the zone of the presumed TACs, in non-stored corneas very soon after donor death. Post-mortem ischaemia of this heavily vascularized zone probably plays an important role. Increased susceptibility to post-mortem oxidative stress is a hypothesis for these poorly differentiated cells [44], which may have limited protective systems such as, for example, the heat shock proteins in the more central ECs [45]. The literature does not report any particular fragility of SCs or TACs, but, except in corneal-epithelium studies, the other SC types were always procured from beating-heart donors. For the corneal epithelium however, it has been shown that longer death-to-procurement time reduces the proliferative capacity of TACs [46, 47], which may indicate specific mortality of limbic epithelial SCs due to postmortem ischaemia. In our study, this apparent higher sensitivity of peripheral cells to cell death soon after donor death may also partly explain the extreme scarcity of Ki67-positive cells in non-stored corneas.

Notably, we also observed overmortality in the extreme periphery of stored corneas, accompanied by lesser frequency of cell clusters and higher EC polymorphism. Organ culture is a storage method derived from cell culture and employed since the early 1970s [48]. Contrary to short-term 4°C storage (5-8 days), it lasts up to 5 weeks. Storage-induced cell alterations have been extensively studied in the central cornea, the area of interest for corneal graft. Despite generally satisfactory preservation of corneal viability, attested by constant good clinical results identical to 4°C short-term storage [49, 50], death of central ECs increases dramatically during OC, with approximately 1% cell loss per day [51, 52] compared with 0.6% per year throughout life [3, 4]. However, organ culture-specific alterations of ECs in the extreme periphery have never been described. As organ culture is known to promote endothelial wound healing [53], we suppose that dead cells in the cell clusters rapidly desquamate and are replaced by neighboring ECs, explaining both why EC clusters were less frequent and why EC polymorphism increased. Moreover, the continuous overmortality that we observed in the extreme periphery may be explained by a specific sensitivity to ischemia reperfusion-like syndrome, as organ-culture conditions restore metabolic activity after prolonged postmortem ischemia. This specific overmortality in corneas of non-stored or stored corneas, not previously reported, may seriously limit further study of EC

proliferation ex vivo. The possibility, independently of donor age, of triggering EC division after intercellular junction loosening with EDTA and incubation at 37°C in mitogen-rich medium was demonstrated by Senoo [54]. Cell division was nevertheless not reported to be restricted in the extreme periphery, even if proliferation capacity was higher in the periphery regardless of donor age [55]. In a similar study on thirteen corneas (from donors aged 15-68 years) stored for 7 days at 4°C in OptisolGS, subsequently exposed to EDTA, and cultured at 37°C in a cell-culture medium with 10% fetal calf serum (FCS) plus growth factors for 48 and 96 hours to promote cell proliferation, more KI67-positive ECs were found in the center than in the periphery [56]. The number of Ki67-positive cells was inversely proportional to ECD. No proliferation was observed when initial ECD exceeded 2000 cells/mm^2. Having showed that cell death occurs preferentially in the extreme periphery where TACs may reside, we suppose that, in most previous studies, the initial 4°C storage time may have been deleterious to the peripheral TACs already weakened by donor death-induced cellular stress, precluding observation of a higher number of Ki67-positive ECs in the extreme periphery, even after mitotic stimulation.

Interestingly, we observed proliferating ECs in the periphery of organ-cultured corneas, but only shortly after medium renewal. We suppose that proliferation was not higher in the extreme periphery for the reasons set out above. Organ-culture media, typically containing 2% FCS, are not known to promote EC division. EC mitosis has only been described in corneas with numerous endothelial lesions due to extended death-to-procurement time and subsequently stored in a specific organ-culture medium supplemented with 8% FCS [57]. In our study, we suppose that the initial storage time triggered cell loss sufficient to locally loosen cell-cell contact and that the rapid increase in nutrients and growth factors during medium renewal stimulated division of several ECs near the local lesions, in a way similar to the experiments reported above with EDTA followed by incubation with mitogens.

In our study, the occasional expression of Ki67 in only one non-stored cornea with very short death-to-procurement time (probably with limited ischemia) may indicate that in vivo cell division in this zone concerns only a limited number of cells. Considering cell cycle duration, Ki67 staining of only a few cells is consistent with clinical experience.

To our knowledge, the EC organization in radial rows observed in two thirds of corneas has never previously been described. In vivo, neither the biomicroscopy used for clinical corneal observation nor the specular microscopy used for endothelial observation allows analysis of the periphery of the posterior face of the cornea. Using SEM, Svedbergh exhaustively described the natural history of the human corneal endothelium of 14 subjects aged 13-88 years without reporting radial rows [58]. His study was done on intact endothelia, whereas Descemetic furrows are only visible after EC removal. Also with SEM, and this time after EC removal by sonication, Inaba studied the periphery of the DM of 87 corneoscleral rims (from donors aged 2-100 years), which are postoperative residues of full-thickness corneal graft after central trephination. However, he only examined the TZ and the Hassall-Henle bodies, i.e. a zone slightly more peripheral that that of the radial cell rows, and so neither could he describe organization in rows [35]. Indeed, it is likely that the zone comprising the rows damaged by trepanation. Lastly, when the endothelium is observed on histological cross-sections, the most common technique in the literature, rows are not visible and clusters are very hard to see, in the absence of serial cross-sections. Cell clusters and radial rows were frequent but not constant in our series, suggesting inter-individual variability. This is not surprising, and can be compared with the variability of healing capacity clinically observed at the ocular surface, as well as with the success rate of epithelial culture from limbal explants [46]. Organization in radial cell rows is associated with much higher local ECD than in zones not organized thus. This strongly suggests that continuous proliferation in the periphery regularly “pushes” ECs

centripetally. The radial cell rows occupy a narrow peripheral band (mean 827+/-295 µm), which may be relatively well protected by a cornea that is naturally less transparent in this zone, particularly due to the presence of an arcus senilis, frequent in elderly subjects and in our donor population. We also observed by SEM that this likely cell migration throughout life leaves a highly visible imprint on the DM. In particular, we observed plot structures that perfectly match the images obtained by third harmonic multiphoton microscopy which Aptel and colleagues recently published and titled “Descemetic fibrous patches connecting EC to the stroma” [59]. The DM contains a nonbanded layer that is synthesized by ECs throughout life, and gradually thickens from 2-10 µm between 10-80 years [60]. The largest mean thickness found in the periphery, and the association with the plots, indicate increased collagen synthesis by peripheral ECs, linked to their greater number and/or more intense activity. However, the linear organization of the plots, which form clear furrows, reflect the deposit of collagen along the EC path throughout life. The function of these plots, located between the ECs and potentially at the intercellular junctions, has yet to be determined. The heterogeneous distributions of cell-line length indicate that cell migration is heterogeneous in the same cornea and between individuals. Cell lines have never been found in the central endothelium of normal corneas. This indicates that corneal-endothelium regeneration is a very slow process and limited to the peripheral endothelium of normal corneas. The age-related decrease in ECD in the central endothelium indicates that this regenerative mechanism may only partially offset cell loss in the central endothelium during adulthood. Again, the absence of young donors in our series prevents us from verifying at what point in life this organization appears.

In the radial cell row zone of non-stored corneas, we found weaker Alizarin red staining and higher polymorphism than in central ECs. These characteristics had already been described years ago using silver cell border staining in ECs of the TZ (although a linear organization was not described) [36]. This may indicate looser intercellular junctions and therefore higher cell-mobility potential than in the center, where the intensity of alizarin red staining and the regular morphometry correlate with the stability of the cell mosaic. Note that both modifications are actually observed in the center during wound healing, when cell migration becomes necessary to cover a defect left by dead cells. The possibility for ECs to easily move in from the extreme periphery is a supplementary argument for slow centripetal migration from stem/progenitor cell niches, and may explain why these seem to accumulate in a zone where cells are more uniform and probably more stable, as shown in Fig. 3F. Cell nuclei in radial cell rows were always elongated and radially oriented, whereas nuclei of central ECs were perfectly round. This morphological difference between peripheral and central ECs suggests that the endothelium is more stable around the optical axis, and is supplied by a peripheral store of cells that are less differentiated and liable to migrate slowly towards the central zone. In this peripheral zone, which is not needed for vision, the pump function of the endothelium for deturgescence of the corneal stroma, which incidentally is thicker in its periphery than in its center, is probably less efficient.

To summarize, we propose a unifying model of human corneal endothelial homeostasis. This model comprises, in the “non-visual” periphery of the cornea, a renewal zone where ECs divide very slowly and migrate towards the center but probably desquame, while the central ECD in the optical axis remains stable. Cell clusters located in the extreme periphery may be SC niches or at least emerging points for TACs migrating from deeper niches. Once they make contact with aqueous humor, their dividing capacity, but not their migrating potential, may be partly repressed by cell cycle inhibition by TGF beta and by cell contact inhibition as soon as they form a monolayer. These cells may then be continuously pushed towards the center and retain some proliferative capacity in experimental conditions, but as they never divide in vivo after reaching the endothelial monolayer, they do not shorten their telomeres [10, 61]. During migration, they acquire full differentiation and slowly lose their residual proliferative capacity owing to environmental stresses (UV light, temperature changes,

aqueous humor soluble factors) [11, 62] that are probably more pronounced in the corneal center, far from limbal vascularisation. These mechanisms may contribute to the stability of the central endothelium, which, as an ionic pump, is devoted solely to maintaining a transparent optical axis.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Tables

Table 1. List of primary antibodies

Target protein	Animal source	Clonality	Laboratory	Reference	Dilution	Fixative
ZO-1	mouse	monoclonal	Zymed, Carlsbad,CA	33-9100	1/200	methanol
Actin	mouse	monoclonal	Sigma, Saint Louis, MO	A4700	1/200	methanol
Na^+/K^+ ATPase	mouse	monoclonal	Millipore, Billerica , MA	#05-369	1/200	methanol
COX IV	rabbit	monoclonal	Cell Signaling, Danvers, MA	#4850	1/400	methanol
Telomerase	rabbit	monoclonal	Abcam, Cambridge, UK	ab32020	1/200	0.5% PFA
Nestin	rabbit	polyclonal	Abcam, Cambridge, UK	ab92391	1/200	0.5% PFA
BCRP/ABCG2	mouse	monoclonal	Chemicon, Temecula , CA	MAB4145	1/200	0.5% PFA
Oct-4	rabbit	polyclonal	Cell Signaling, Danvers, MA	#2750	1/200	0.5% PFA or methanol
P63-α	rabbit	polyclonal	Cell signaling, Danvers, MA	#4892	1/200	0.5% PFA or methanol
Ki67	mouse	monoclonal	Dako, Glostrup, Denmark	M 7240	1/200	0.5% PFA

Table 2. Expression of five stem cell markers in endothelial cells according to their localization on corneas.

	Telomerase (n=12)	Nestin (n=10)	BCRP/ ABCG2 (n=11)	Oct-4 (n=10)	P63 (n=6)
Extreme periphery	9++	8++	1	0	0
Center	0	8	2	0	0

n = number of corneas assessed

++ brightly expressed in a majority of cells whereas other staining concerned only few cells with weaker expression.

Figures and figure legends

Figure 1. Zones of interest on the posterior surface of a human cornea. The sclera has been trephined to a diameter of 18 mm and the iris removed.

Figure 2. Cell clusters. (**A, B):** En face view of the corneal endothelial periphery (P). Cell clusters were localized in the extreme periphery (EP) in contact with the transition zone (TZ). Trabecular meshwork (TM) and iris root residues (I) are indicated. A x10, B: x40 Alizarin red and trypan blue on fixed tissue. (**C-F)** (x96): Multilayered organization (arrowheads) of cell clusters, always localized in immediate vicinity of Hassall-Henle bodies (**asterisk**). **(C):** Cross-section (hematoxylin eosin safran). (**D, E, F):** En face view (Alizarin + trypan blue).

Figure 3. Radial endothelial rows in the corneal periphery. **(A):** General view of half cornea flat-mounted thanks to radial cuts. Hoechst 33342 staining, x4. (**B and C):** Zoom with x10 objective on center **(B)** and periphery **(C)** where cell rows were clearly present. **(D and E):** Difference of endothelial cell morphology between center **(D)** and periphery **(E)**. Alizarine red and Hoechst staining, x40. (**F):** Transition between cell rows and more central endothelium in a non-stored cornea. The white line indicates an artificial boundary between the zones. Hoechst staining, x10.

Figure 4. Radial Descemet membrane furrows in the corneal periphery. Furrows were made visible after partial **(A)** or complete **(C,D,E,F)** removal of endothelial cells. (**A):** Flat-mount observed with phase contrast to highlight DM excrescences, and with Hoechst 33342 to locate cell nuclei at the base of the furrows. **(B):** Cross-sections orthogonal to the cell rows, stained with hematoxylin eosin safran. **(C,D,E,F):** Scanning electron microscopy of the bare Descemet membrane, showing furrows defined by real plots and starting near the transition zone (TZ). Abbreviations: TM, trabecular meshwork; EP, extreme periphery; P, periphery.

Figure 5. Cell mortality in the extreme periphery compared to the center. Representative example of a non-stored cornea (age 69 years, death-to-procurement time 20 hours). Trypan blue and Hoechst staining. x40

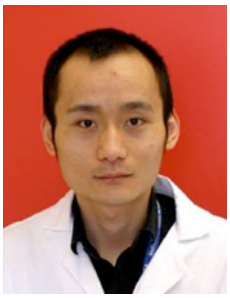
Figure 6. Summary of endothelial and Descemetic centripetal modifications. (**A, B):** Cross-sections. Hematoxylin eosin safran staining demonstrating progressive centripetal alterations of the Descemet membrane (DM). **(C):** En face view after Hoechst staining of nuclei. **(D):** Merge Hoechst and phase contrast to highlight DM excrescences and nuclei preferential orientation. **(E):** Scanning electron microscopy of DM surface structures after endothelial cell removal. All images have been aligned.

Figure 7. Immunolocalization of differentiation markers in endothelial cells. The typical expression patterns of ZO-1, Actin, Na/K ATPase and CoxIV observed in the center were absent in the extreme periphery, in the cell-cluster zone. Phase contrast (PHC) was used only for the extreme periphery to allow visualization of Hassal Henle bodies (asterisk), which by definition were absent in the center. x40

Figure 8. Immunolocalization of stem cells markers in endothelial cells. Telomerase and Nestin were largely expressed in the cell-cluster zone of the extreme periphery of almost all corneas. By contrast, BCRP/ABCG2 was found only in two non-stored corneas. Phase contrast (PHC) was used only for the extreme periphery to allow visualization of Hassal Henle bodies (asterisk). x40

Figure 9. Immunolocalization of the proliferation marker Ki67. **(A, B):** Non-stored cornea with death-to-procurement time of 4.5 hours. Only a few isolated endothelial cells (ECs) located in the extreme periphery were positive. **(A):** Hoechst + Ki67, **(B):** Merge with phase contrast to highlight Hassall-Henle bodies (asterisk). (**C, D):** Stored cornea observed 3 days after storage medium renewal. Multiple Ki67-positive ECs were visible, with metaphases indicating active cell cycling. The line in C delineated the transition zone.

CURRICULUM VITAE



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PUBLICATIONS INTERNATIONALES EN COURS

He Z, Campolmi N, Gain P, Dumollard J.M, Peoc'h M, Piselli S, Garraud O, Thuret G. «Radial columns of endothelial cells at corneal periphery: new evidences for a continuous centripetal migration from cell niches in human corneas». Submit to Stem Cells

He Z, Campolmi N, Ha Thi B.M, Dumollard J.M, Peoc'h M, Garraud O, Piselli S, Gain P, Thuret G. «Investigation of the proliferative status of human corneal endothelial cells using optimized immunolocalization on flat-mounted whole cornea». Submit to Molecular Vision

He Z, Dumollard J.M, Peoc'h M, Gain P, Thuret G. Effects of fixatives temperature on immunolocalization in flat mounted human corneal endothelium. In preparation for J Histochem Cytochem

Flury M, **He Z,** Campolmi N, Gain P, Kress B, Thuret G. «Fabrication of optical mosaics to optimize corneal endothelial cell quality determination in eye banks». Submit to Optics Letter

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  -114ème Congrès de la Société Française d'ophtalmologie (SFO), Paris, France, 2008. **He Z**, Pipparelli A, Piselli S, Dumollard J.M, Peoc'h M, Thuret G, Gain P. *« Development of immunolocalization on flat-mounted whole cornea »*

- ***Poster*** *(en premier auteur) :*

  He Z, Pipparelli A, Piselli S, Dumollard J.M, Peoc'h M, Gain P, Thuret G. *« Investigation of the proliferative status of ex vivo human corneal endothelial cells by developing immunolocalization on flat-mounted whole cornea ». Ce travail a été communiqué dans deux congrès européens:*

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  -Annual meeting of the European Association for Vision and Eye Research (EVER). Portoroz, Slovenia, 2008

- ***Prix***

  He Z, Pipparelli A, Piselli S, Dumollard J.M, Peoc'h M, Gain P, Thuret G. *« Investigation of the proliferative status of ex vivo human corneal endothelial cells by developing immunolocalization on flat-mounted whole cornea. Deux prix européen:*

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