UNIVERSITÉ LAVAL

Impact des facteurs embryonnaires sur la réceptivité maternelle

Rôles de la Gonadotrophine Chorionique humaine et de l'Interleukine 1

Thèse

Amélie Bourdiec

Doctorat en microbiologie-immunologie
Philosophiae Doctor (Ph.D.)

Québec, Canada

Résumé

La réceptivité de l'endomètre à l'embryon est un point crucial en reproduction. Il semblerait qu'une synchronisation entre l'endomètre maternel et le développement de l'embryon soit nécessaire. Pour se faire, les stéroïdes ovariens préparent le contact de l'endomètre avec l'embryon. De son côté, l'embryon communique avec l'endomètre par l'intermédiaire d'un vaste réseau moléculaire.

Dans certains cas d'infertilité, la réceptivité maternelle est altérée, il en résulte alors un échec d'implantation. D'où l'importance d'explorer le côté maternel de l'implantation. C'est pourquoi, en tenant compte des précédents travaux menés au laboratoire**,** portant sur le système IL (interleukine) 1, la hCG (hormone gonadotrophine chorionique humaine) et les cellules de l'endomètre, nous avons étudié le rôle de ces deux facteurs embryonnaires précoces, dans l'acquisition de la réceptivité endométriale, au début du phénomène d'implantation.

Ce projet de recherche sur la physiologie de l'implantation embryonnaire est strictement fondamental et représente la base pour une meilleure compréhension de la fertilité et l'infertilité féminine. Pour se faire, des approches *in vitro* et *in vivo* ont été utilisées. Nos résultats semblent indiquer que la famille de l'IL1 est ciblée de façon originale par la hCG. Celle-ci semble favoriser l'effet de l'IL1 par le biais d'un déséquilibre pro-fonctionnel touchant les différents récepteurs. En outre, l'effet de la hCG sur les différentes cibles de l'IL1 apparaît sélectif et semble modérer certains aspects qui pourraient s'avérer néfastes pour l'implantation embryonnaire, tandis qu'elle favorise certaines cibles par effet de coopération. Nos travaux démontrent l'existence d'une dynamique dans l'expression des différents récepteurs de l'IL1 en lien avec la présence du chef de file des facteurs embryonnaires, la hCG.

Abstract

The endometrium receptivity for embryo is a crucial feature in reproduction. Overall, it seems that synchronization between the maternal endometrium and the developing embryo is necessary. At the maternal side, the appointment with the embryo is carefully prepared by ovarian steroids' action. An another hand, the embryo communicates with the endometrium through a large molecular network.

In some cases of infertility, maternal receptivity is altered, resulting in implantation failure. Moreover, the maternal side of implantation is in question. Therefore, taking into account our previous studies revealing a close cooperation between major and early embryonic signals, hCG (human chorionic gonadotropin) and interleukin1 (IL1), at the feto-maternal interface, we have studied the role of these embryonic factors in the acquisition of endometrial receptivity. This research on the physiology of embryo implantation is strictly fundamental and the basis for a better understanding of female fertility and infertility.

Using *in vitro* and *in vivo* approaches, our results suggest that the IL1 family is targeted in an original way by hCG. This seems to favor the effect of IL1 through a functional imbalance affecting different IL1 receptors. In addition, the effect of hCG on various targets of IL1 appears to be selective and seems to moderate some aspects that could be detrimental for embryo implantation, while it favors certain targets by synergic cooperation. Our work demonstrates the existence of a dynamic expression of different IL1 receptors in link with the presence of hCG, the leading embryonic factor.

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AMPc Adénosine monophosphate cyclique

BrdU Bromodeoxyuridine

BSA bovine serum albumin

CHUL centre hospitalier mère enfant de l'université Laval

COX-2 cyclooxygénase 2 COX-2

CSF colony stimulating factors

CTEV cellules trophoblastiques extravillositaires

cx3cl-1 Fractalkine

CXCL-8 IL-8

DMEM Dulbecco modified Eagle medium

dNTP deoxyribonucleotide triphosphate

E1 œstrone

E2 œstradiol

EEC cellules épithéliale de l'endomètre

ELISA Enzyme-Linked Immunosorbent Assay

EMT transition épithélio-mésenchymateuse

ESC cellules stromales de l'endomètre

FBS fetal bovine serum

FC Fold changes

FDR false discovery rate

Fig Figure

FIV fécondation in vitro

fN fibronectine

FSH hormone folliculostimulante

GD gestational day

GM-CSF Granulocyte macrophage colony-stimulating factor

GnRH gonadolibérine

GO Genes ontology

GRO Cotton rat growth-regulated protein

HBSS Hanks balanced salt solution

hCG hormone gonadotrophine chorionique humaine

hLHCGR hCG receptor

HMVEC lignée endothéliale microvasculaire humaine

HSFA hôpital Saint François d'Assise

hTERT human telomerase catalytic protein

ICE1 Interleukin-1beta-converting enzyme

IFN interféron

IGFBP-1 l'insulin like growth factor binding protein 1

IL Interleukine

IL1RAcP IL1 protéine accessoire

IP10 interferon inducibe protein 10

IRAK Interleukin 1 receptor-associated kinase

IV intravenous injection

JNK c-Jun N-terminal kinase

LH hormone lutéinisante

LIF Leukemia inhibitory factor

lN laminine

MAPK Mitogen-activated protein kinases

MCDB endothelial cell basal medium

MCP Monocyte chemoattractant protein

MCPIP MCP1-induced protein

MEC matrice extracellulaire

MEKK MAP/ERK kinase kinase

MIP Macrophage inflammatory protein

MMPs métalloprotéinases

MPA medroxyprogestérone

NF-kappa B nuclear factor-kappa B

P4 progestérone

PAGE polyacrylamide gel electrophoresis

PBS phosphate buffered saline

PCA Principal Component Analysis

PCR Réaction en chaîne par polymérase

PKA protéine kinase A

PRL prolactine

RANTES Regulated on activation, normal T cell expressed and secreted

SAM Significance Analysis of Micro-arrays

SDF stromal-derived factor-1

TAK TGF-beta activated kinase

TCM milieu conditionné de cellules trophoblastiques

TGF transforming growth factor

TIMP inhibiteurs naturels tissulaires endogènes des métalloprotéases

TLR les Toll-like receptors

Tm melting temperature

TMB 3,3',5,5'-tetramethylbenzidine

TNF tumor necrosis factor

TRAF TNF receptor associated factor

uNK natural killer utérin

XCL-1 Lymphotactine

Avant-propos

Remerciements

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J'ai réalisé la majeure partie des expériences relatives au projet. De plus, j'ai contribué à l'analyse des tous les résultats et rédigé la première version des manuscrits présentés ci-dessous :

Chapitre 2 : Human chorionic gonadotropin triggers angiogenesis via the modulation of endometrial stromal cell responsiveness to interleukin 1: a new possible mechanism underlying embryo implantation.

Chapitre 3 : Transcriptome analysis reveals new insights into the modulation of endometrial stromal cell receptive phenotype by embryo-derived signals interleukin-1 and human chorionic gonadotropin: possible involvement in early embryo implantation.

Chapitre 4 : Human Chorionic Gonadotropin Regulates Endothelial Cell Responsiveness to Interleukin 1 and Amplifies the Cytokine-Mediated Effect on Cell Proliferation, Migration and the Release of Angiogenic Factors.

« Patience et longueur de temps font plus que force ni que rage. »

Jean de la Fontaine

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Chapitre 1 Introduction à la réceptivité maternelle et à l'implantation embryonnaire

Lors de chaque cycle menstruel, l'endomètre se prépare à soutenir l'implantation, et la croissance d'un embryon. Pour cela, la succession des différentes phases du cycle est requise afin d'assurer un fonctionnement physiologique optimal de l'axe reproducteur féminin. La reproduction requiert une succession d'étapes comprenant l'ovulation d'un ovocyte compétent (1), la production de sperme compétent (2), la fertilisation de l'ovocyte par un spermatozoïde (3), la génération d'un embryon viable (4), son transport dans la cavité utérine (5) et une implantation réussie de celui-ci dans l'endomètre réceptif (6). Un défaut lors de cette dernière étape diminue le potentiel de fertilité, et conduit à un échec de l'implantation embryonnaire.

En Amérique du Nord, l'infertilité affecte environ 15% de la population en âge de procréer. L'infertilité se déclare par une incapacité du couple à concevoir un enfant après un an de rapports sexuels fréquents et non protégés. L'infertilité d'origine féminine représente 65% des cas, l'infertilité masculine 20%, et dans 15% des cas l'origine de l'infertilité est inconnue (Beckman 2013). Aujourd'hui, 85% des couples infertiles qui recevront le traitement approprié pourront espérer avoir un enfant. De nombreuses, et coûteuses thérapies médicales sont envisageables tel que la stimulation ovarienne, l'insémination intra-utérine et la procréation médicalement assisté par les techniques de fécondation *in vitro* (FIV).

Qu'elle soit naturelle ou assistée, le succès de l'implantation embryonnaire est une étape cruciale de la reproduction. Le taux moyen d'implantation des embryons conçus par fécondation *in vitro* reste faible (20-25%).

Même si de nombreux progrès ont été accomplis en procréation médicalement assistée, l'implantation demeure le résultat d'une collaboration réussie, finement régulée et étroitement coordonnée entre tissus maternels et embryonnaires, situé au carrefour de l'endocrinologie et de l'immunologie.

Sur le plan scientifique, cette collaboration reste le mystère de la reproduction humaine. De plus en plus, le côté maternel de l'implantation est perçu comme la dernière frontière en matière de progrès en reproduction (Juan Felipe Velez de la Calle 2012).

1.1 Contrôle endocrinien de l'endomètre

1.1.1 Description de l'endomètre

L'endomètre est la muqueuse qui tapisse l'intérieur de la cavité utérine (Hitschmann 1908; Noyes 1950). La muqueuse utérine peut être divisée en trois parties. La couche adjacente au myomètre, appelée *pars basalis* (couche profonde ou encore couche basale), ne présente aucun changement au cours du cycle menstruel et n'est pas éliminée lors des menstruations. La couche intermédiaire, ou couche spongieuse, se caractérise par un stroma d'aspect spongiforme tandis que la couche superficielle présente un stroma plus compact, d'où son nom de couche compacte. Ces deux dernières couches sont regroupées sous le nom de couche fonctionnelle (*functionalis*). Ce tissu richement vascularisé se développe à chaque cycle (au cours de la vie fertile) à partir de la couche basale pour éventuellement permettre l'implantation de l'embryon s'il y a fécondation(Wheater 2004).

1.1.2 Le système endocrinien

Par la libération séquentielle d'hormones stéroïdiennes, les ovaires induisent les différentes phases du cycle menstruel. Ces hormones ovariennes, œstrogènes (œstrone (E1) et œstradiol (E2)) et progestérone (P4), sont elles-mêmes sous la dépendance des hormones gonadotrophiques, l'hormone folliculostimulante (FSH), l'hormone lutéinisante (LH) et éventuellement la gonadotrophine chorionique humaine (hCG) en cas de grossesse (Fig.1). Les hormones ovariennes ont des effets radicaux sur le devenir de l'endomètre (Thibault 2001; Berferon 2006). Elles orchestrent les changements histologiques et biochimiques de l'environnement utérin. Lors de la phase proliférative, qui est une phase de régénération, les mitoses sont fréquentes, l'épithélium s'épaissit, les glandes se développent. Ces observations sont la manifestation de l'augmentation du taux d'œstrogènes. Les changements les plus caractéristiques opèrent lors de la phase sécrétrice (Dallenbach-Hellweg 2010).

1.1.2.1 Phase proliférative

Lors de la phase proliférative, plusieurs follicules ovariens commencent à croître, mais un seul arrive à maturité, les autres vont dégénérer. À chaque cycle, la FSH stimule la croissance folliculaire. Les cellules de la granulosa entourant l'ovocyte répondent à la FSH par une sécrétion d'œstrogènes. Lorsqu'un des follicules a atteint la maturité (follicule de Graaf), celui-ci libère brusquement de fortes quantités d'œstrogènes, ce qui provoque un rétrocontrôle positif sur la libération de FSH et de LH. À la fin de la phase proliférative la monté d'oestrogènes induit un pic de LH, déclenchant l'ovulation. L'ovocyte est alors recueilli par le pavillon de la trompe tandis que les restes du follicule se transforment en corps jaune, véritable

glande endocrine temporaire productrice d'œstrogènes et de progestérone (Cunningham F.G. 2005).

Sous l'effet des oestrogènes l'endomètre augmente de 0,5 mm à 4-5 mm d'épaisseur en moyenne. La croissance de l'endomètre est la principale caractéristique de la phase proliférative (Fig 1). Cette phase dure habituellement 14 jours, mais peut fluctuer entre 10 et 20 jours dans des conditions physiologiques. La phase proliférative, aussi connu sous le nom de préovulatoire et folliculaire, peut se diviser en stade prolifératif débutant, moyen et avancé (Mazur 2005; Trévoux 2009; Dallenbach-Hellweg 2010).

1.1.2.2 Phase sécrétoire

Lors de la phase sécrétrice, l'endomètre atteint une épaisseur de 7,0 à 8,0 mm. Les glandes et le stroma se développent dans une séquence ordonnée. Les différentes cellules de l'endomètre affichent des caractéristiques histologiques spécifiques de l'activité sécrétoire. Contrairement à la phase proliférative, les changements dans les glandes et le stroma varient fortement d'un jour à l'autre, ce qui permet une datation précise des jours de cycle. La montée de l'œstradiol en phase proliférative stimule l'expression des récepteurs à la progestérone. La progestérone agit sur l'activité sécrétoire de l'endomètre dans le but d'assurer un milieu favorable pour l'embryon. La phase sécrétoire, également appelée phase lutéale, peut se diviser en stade débutant, moyen et avancé (Mazur 2005).

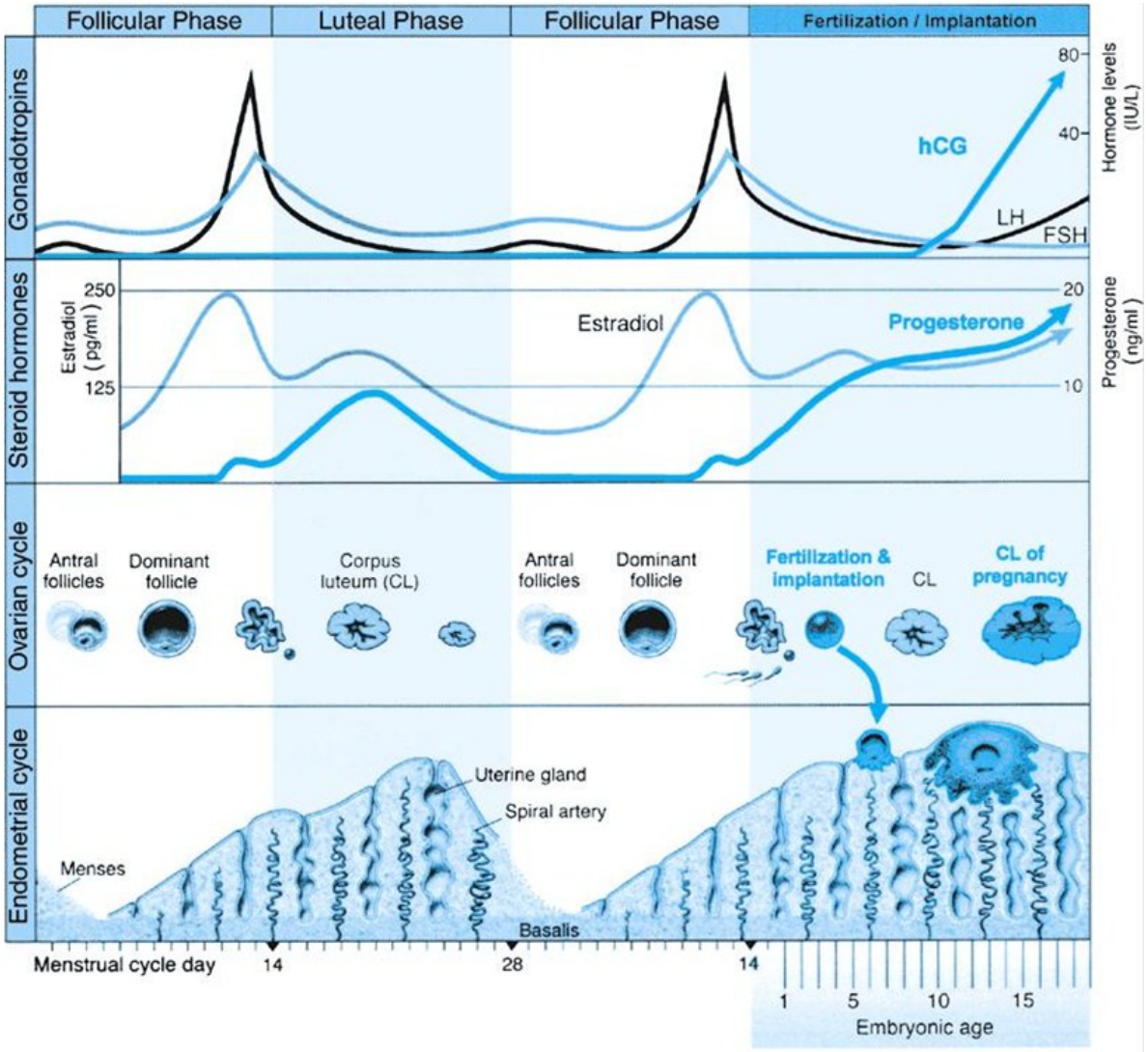

Figure 1 Régulation hormonale du cycle menstruel. Adaptée de Williams Obstetric (Cunningham F.G. 2005).

En absence d'implantation embryonnaire, le corps jaune dégénère et régresse. Ceci aura pour conséquence une chute drastique des taux d'œstrogènes et de progestérone. Au niveau de l'endomètre cette chute hormonale se traduit par le tassement de la muqueuse. L'épithélium glandulaire commence à être difficile à observer en raison d'une autolyse débutante. L'endomètre se nécrosera et se détachera de l'utérus pour être expulsé par la voie génitale (Trévoux 2009). Au dernier jour du cycle, une thrombose débutera dans les petits vaisseaux et une hémorragie suivra avec extravasation d'érythrocytes dans le stroma (Mazur 2005; Trévoux 2009). Le premier jour des menstruations est considéré comme le début d'un nouveau cycle. Il faut noter que les menstruations sont décrites comme un phénomène physiologique témoignant d'une fonction utérine parfaitement ordonnée, mais paradoxale (De Brux 1971). C'est une destruction tissulaire récidivante. Aucun autre tissu du corps humain ne démontre, de façon orchestrale et rapide, une fonction de réparation et de récupération fonctionnelle.

1.2 Prélude de la grossesse : le défi de la synchronisation embryo-maternelle

1.2.1 L'endomètre et la réceptivité endométriale

1.2.1.1 La fenêtre d'implantation

Le développement embryonnaire est directement soumis à l'environnement utérin. De même, les signaux embryonnaires modulent la différenciation de l'utérus adjacent en décidue. C'est cette synchronisation embryo-maternelle qui définit la phase d'implantation (Ray, Gigarel et al. 2000). Plus spécifiquement, l'embryon doit

avoir atteint un stade blastocyste, être compétent à l'implantation, et de l'autre côté l'utérus doit être dans un état dit « réceptif » (Psychoyos 1973).

Au cours de l'implantation, l'endomètre offre une réceptivité maximale à l'embryon. La durée de la fenêtre d'implantation est une caractéristique d'espèce. Chez la femme, on pense que cette fenêtre dure à peu près 4 à 5 jours, du jour 19-20 au jour 24-25 d'un cycle menstruel normal (Wilcox, Baird et al. 1999).

Durant les dix dernières années, il a été montré que cette fenêtre d'implantation dépend en grande partie, sinon de façon quasi exclusive, de l'expression coordonnée de chimiokines, métalloprotéinases (MMPs), et de molécules d'adhésion. De plus, l'expression utérine et placentaire de facteurs de croissance précoces, ainsi que de cytokines pro-inflammatoires, permettent les stades d'apposition puis d'adhésion (1), régulent l'invasion de la couche basale de l'endomètre par les cellules placentaires (2), et enfin permettent et régulent l'invasion des cellules trophoblastiques dans l'endomètre (3) (Chaouat, Ledee-Bataille et al. 2003). Cette expression coordonnée est, pour certains médiateurs, totalement ou partiellement hormono-dépendante et débute à l'ovulation quand la sécrétion œstrogénique pure devient une sécrétion mixte estroprogestative. Dès lors, l'endomètre subit des modifications structurelles et moléculaires, permettant à un embryon compétent de s'implanter au cours de cette fenêtre. Ce remodelage endométrial inclue la transformation sécrétoire des glandes, l'arrivée de cellules utérines *natural killer* (uNK), et un remodelage de la paroi des artères spiralées. Sous l'effet de la progestérone et de l'AMP cyclique (AMPc), les cellules stromales de l'endomètre se décidualisent et acquièrent la propriété unique de réguler l'invasion trophoblastique, de résister aux agressions du stress oxydatif et de développer un environnement de tolérance immunitaire locale et programmé (Gellersen, Brosens et al. 2007). Enfin, la nidation induit la décidualisation complète du stroma de

l'endomètre dont les cellules constituent la composante maternelle du placenta (Barry 2005).

1.2.1.2 Transformation deciduale de l'endomètre

La transformation prédéciduale du stroma devient la caractéristique histologique principale datant de la fin de phase sécrétoire (Fig.2) (Mazur 2005; Trévoux 2009). Elle débute autour des artères spiralées. Les cellules stromales s'élargissent, deviennent ovales en forme de polygone. Les glandes, sous la couche superficielle, prennent un aspect très ramifié. Les cellules épithéliales sont cylindriques et ont un cytoplasme vacuolaire.

Le changement prédécidual est vaste. Le stroma montre un infiltrat lymphoïde. Un nombre croissant de cellules immunitaires envahit le chorion depuis la couche basale. Cet infiltrat lymphoïde aura une importance particulière pour la tolérance locale de l'œuf implanté dans l'endomètre. Au début de la fenêtre d'implantation, des pinopodes (petites protrusions dont la demi-vie est inférieure à 48h) apparaissent à la surface des cellules épithéliales. L'apparition des pinopodes paraît liée au phénomène d'apposition du blastocyste, et coïncider avec l'apparition des intégrines spécifiques αVβ3 et de la sécrétion d'HB-EGF (Heparin-binding Epithelial Growth Factor) qui caractérise la phase sécrétrice moyenne (Damario, Lesnick et al. 2001; Stavreus-Evers, Aghajanova et al. 2002).

A la lumière utérine, la composition du glycocalix (mucus à la surface épithéliale), diffère pour rendre le site implantable. La nature non adhésive de l'épithélium de surface utérin est en partie attribuée à l'expression dans le glycocalix de mucines anti adhésives tel que MUC-1. Par ailleurs, son pic d'expression correspond au moment de l'implantation, mais celle-ci peut être localement clivée par le bastocyste compétent en phase d'adhésion (Meseguer, Aplin et al. 2001).

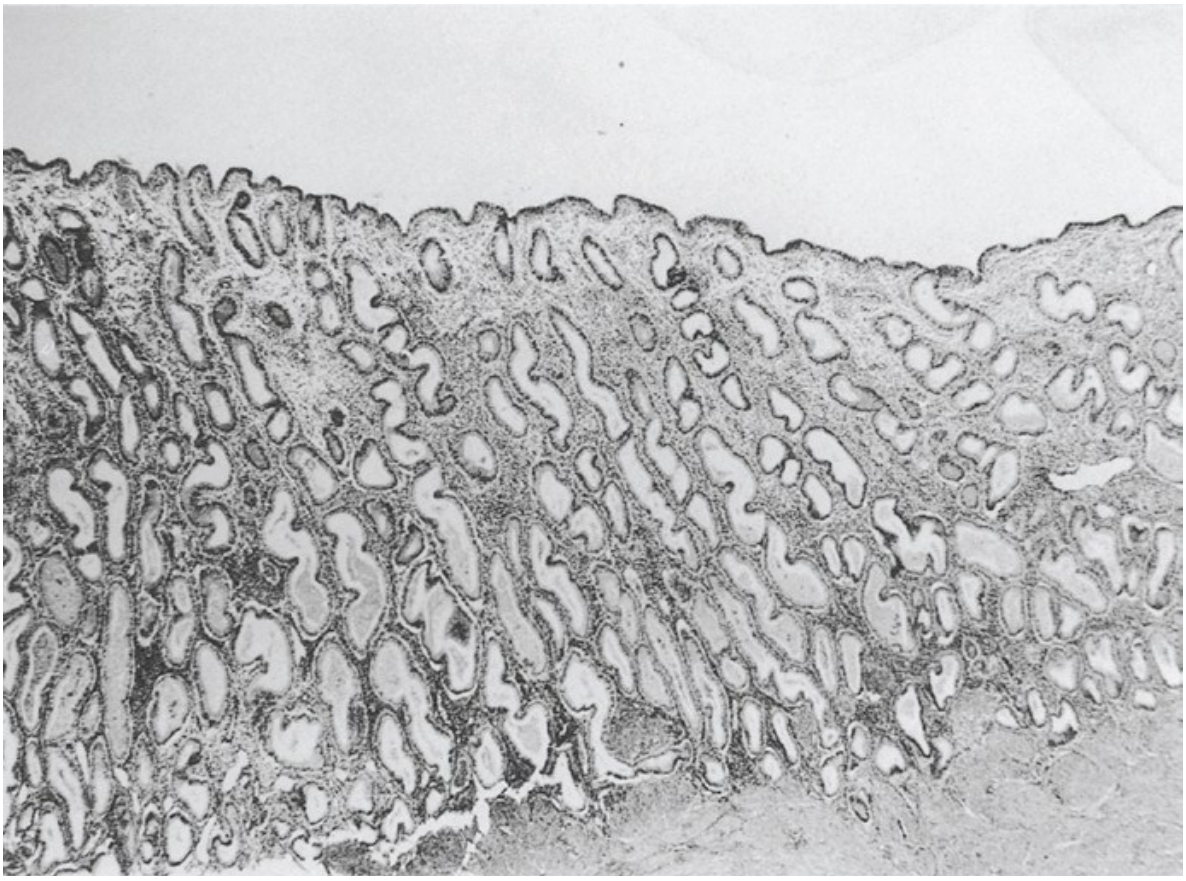

Figure 2 Phase sécrétrice, 4 jours après l'ovulation, HE 25X. Les modifications post-ovulatoires sont présentes. L'épithélium de surface apparaît en dent de scie, tandis que les glandes sont tortueuses et actives. Tiré de Atlas of endometrial histopathology (Dallenbach-Hellweg 2010).

La semaine suivant la nidation, l'endomètre subit des modifications sous forme de régression progressive du compartiment épithélial et de décidualisation complète du stroma. Les artérioles spiralées se transforment progressivement en artère utéro-placentaire.

La décidualisation représente un processus de différenciation morphologique et biochimique initié par l'augmentation de la progestérone. Chez l'espèce humaine, la décidualisation complète est dépendante de la présence d'un embryon implanté. Les cellules stromales s'arrondissent, acquièrent des caractéristiques myofibroblastiques, sécrètent une panoplie de molécules spécifiques telles que la prolactine (PRL) et l'insulin like growth factor binding protein 1 (IGFBP-1). Cependant bien que la progestérone semble indispensable à l'initiation et au maintient de la décidualisation, il apparaît que l'expression de la prolactine est surtout liée à la voie AMPc-PKA (Gellersen and Brosens 2003). En effet, la présence, seule, d'acétate de medroxyprogestérone (MPA) à un effet minime sur la sécrétion de prolactine *in vitro*, alors que l'ajout d'AMPc l'augmente fortement comme illustré à la figure 3.

La transformation décidual implique une modification profonde du stroma incluant une expression modifiée des récepteurs aux stéroïdes ovariens, un remodelage de la matrice extra cellulaire et du cytosquelette. Lors de la gestation, la transformation décidual s'étend jusqu'à la couche basale de l'endomètre. Ce phénomène est critique pour l'invasion du trophoblaste et la formation du placenta (Gellersen and Brosens 2003).

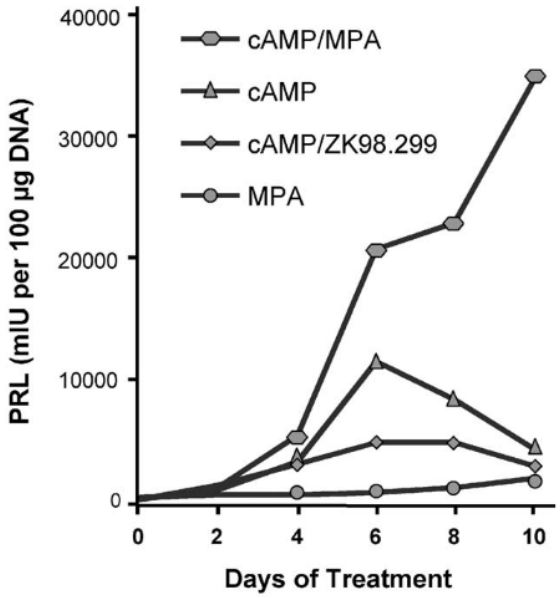

Figure 3 Induction de la prolactine par les cellules stromales de l'endomètre en réponse à l'AMPc, progestatifs (MPA) et antiprogestines (ZK98.299).Tirée de (Gellersen and Brosens 2003).

1.2.2 Développement synchrone de l'embryon

1.2.2.1 Naissance de l'oeuf et migration vers la cavité utérine

L'ovule, expulsé dans les trompes de Fallope, est fécondé dans l'ampoule tubaire environ 12 à 24h suivant l'ovulation. La première mitose de la segmentation se termine alors que l'embryon atteint la portion moyenne de la trompe, environ 30h après la fécondation. Au 3^{e} jour, la morula, formée de 12 à 16 cellules pluripotentes, atteint la portion intramurale de la trompe. Après 4 ou 5 jours, le blastocyste entre dans la cavité utérine où l'implantation a lieu le jour suivant (Fig.4).

L'implantation se déroule selon une succession d'étapes caractérisées par le degré de contacts entre les cellules épithéliales utérines et trophoblastiques. Lorsque le blastocyste entre dans la cavité utérine, il s'oriente et se positionne grâce à des contractions du myomètre. Puis, la perte de la zone pellucide permet au blastocyste de s'implanter. L'apparition des premiers contacts entre les cellules trophoblastiques et utérines correspond à la phase d'apposition. Au cours de cette étape le blastocyste est plaqué contre l'épithélium luminal, les contacts cellulaires sont assurés par l'interdigitation des microvillosités apicales. Ces contacts étroits induisent l'adhérence totale du blastocyste à la membrane plasmique de l'épithélium utérin. Enfin le trophoblaste pénètre et envahi l'endomètre (Fig.5). Au 8^{e} jour l'embryon est complètement enfoui dans l'endomètre (Barry 2005).

1.2.2.1 L'embryon « compétent »

Pour s'implanter et se développer de façon adéquate, le blastocyste nécessite la fécondation d'un ovule mature par un spermatozoïde de morphologie normale à l'ADN de bonne qualité (peu fragmenté). Ces dernières années, dans le cadre de la procréation médicalement assistée, des critères se basant sur la morphologie et le développement de l'embryon ont permis d'identifier les embryons à haut potentiel implantatoire (Bavister 1995; Van Royen, Mangelschots et al. 1999; Ebner, Moser et al. 2003).

Déterminé sur le nombre de cellules qui compose l'embryon (les blastomères), leur taille, la fragmentation de l'embryon et sa symétrie, le biologiste établit un score pour sélectionner les embryons les plus viables et les plus susceptibles de s'implanter. Dans ce cas 49% de ces embryons implantés ont donné lieu à une grossesse menée à terme (Van Royen, Mangelschots et al. 1999). Cependant, ce score ne permet pas de prédire le potentiel de développement ultérieur. Des critères additionnels sont donc nécessaires. L'analyse des fluides comme les milieux de cultures des embryons ou du liquide folliculaire ont permis de doser des protéines précocement produites tel que l'IL1, l'IL10, la hCG et HLA-G.

Le nouveau défi pour augmenter l'efficacité du transfert de blastocyste *in utero* repose sur l'amélioration de la synchronicité avec l'endomètre. Ainsi une alternative consisterait en l'utilisation de cocultures prolongées avec des cellules de l'endomètre maternel (prélevé lors du précédent cycle) comme support (étude Endocell) (Velez de la Calle JF 2012).

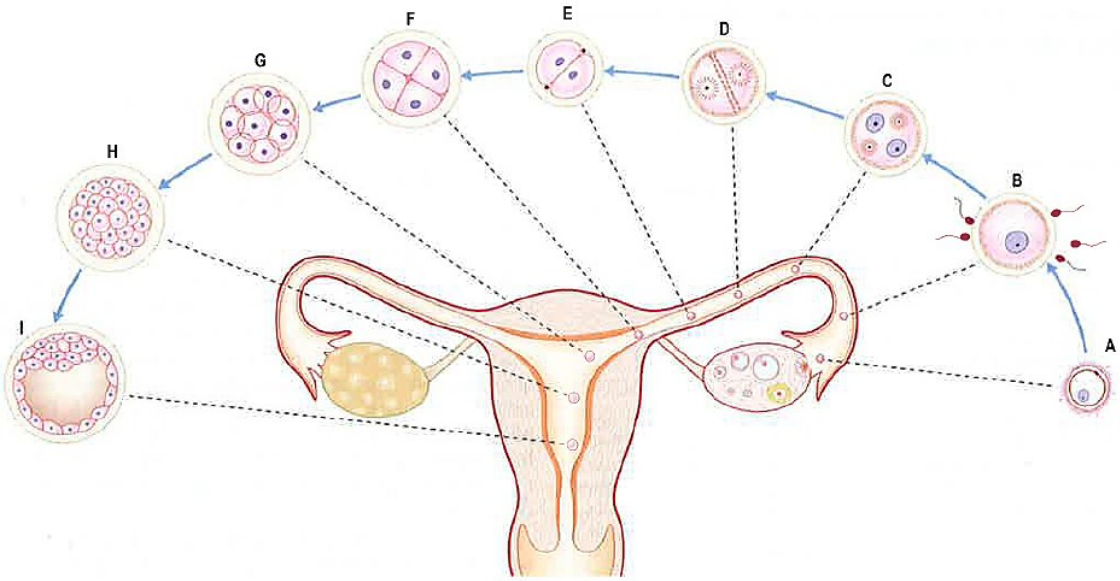

Figure 4 Étapes du développement pré-embryonnaire lors de la première semaine. A, Ovulation. B, Fécondation. C, Formation de pronucléus. D Première division du zygote. E-G, Segmentation du zygote. H, Morula. I Blastocyste. Tiré d'Embryologie (Barry 2005).

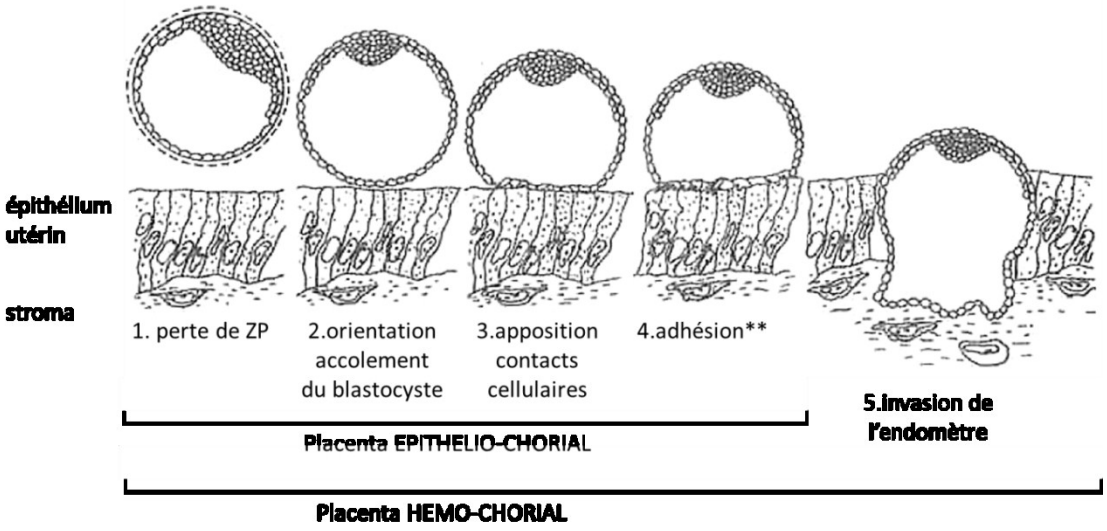

*Figure 5 Schéma des différentes phases de l'implantation. Le disque embryonnaire est arbitrairement orienté au pôle opposé au site d'implantation. **Phase ultime de l'implantation dans les espèces à placentation épithélio-choriale. Tirée de Reproduction chez les mammifères et l'homme (Thibault 2001).*

1.2.2.2 Implantations ectopiques

L'œuf peut parfois s'implanter en situation anormale si sa migration est ralentie le long de la trompe utérine. Cette implantation ectopique a lieu le plus souvent dans la trompe, mais d'autres localisations comme la cavité péritonéale ou l'ovaire peuvent être observées. Dans ces cas, la grossesse n'arrive pas à terme car cette implantation anormale ne permet pas un développement embryonnaire satisfaisant. De plus, l'infiltration de ces structures par le trophoblaste est susceptible de provoquer une hémorragie mettant en jeu la vie de la mère (Barry 2005). D'ailleurs, il est paradoxal de constater que, alors que l'implantation peut se produire dans n'importe quel tissu du corps humain (cas des grossesses extra-utérines spontanées, ou expérimentales), la plupart du temps sans aucune transformation préalable de ce dernier, l'endomètre, lui, est un des rares tissus dans lequel l'embryon ne peut pratiquement pas s'implanter excepté durant cette fenêtre implantatoire.

1.2.3 Immunotolérance

En phase sécrétoire, la cellule épithéliale de l'endomètre apparaît polarisée, avec une sécrétion apicale en direction de la lumière, différente de la sécrétion du stroma (Fahey, Schaefer et al. 2005). En étudiant les lavages utérins, recueillis pour analyser le fluide endoluminal de femmes fertiles et de femmes dont la procréation est médicalement assistée, il a été possible de documenter l'expression protéique. Les résultats ont mis en évidence que l'expression endoluminale était ciblée sur les principaux chémo-attractants des cellules uNK, MCP1 (Monocyte chemoattractant protein 1) et IP10 (interferon inducibe protein 10), ainsi que certaines cytokines comme l'IL (interleukine) 15, le GM-CSF (Granulocyte macrophage colony-stimulating factor), RANTES (Regulated on activation, normal T cell expressed and secreted), MIP (Macrophage inflammatory protein) 1β, MIPα, le TNF (tumor necrosis

factor) α, l'IL1Ra et l'IL1β (Boomsma, Kavelaars et al. 2009). Ces résultats soulignent l'importance d'une réactivité locale nécessaire au processus d'implantation embryonnaire. En cas d'échec d'implantation, des sécrétions anormales pour des cytokines clef comme le LIF (Leukemia inhibitory factor) ont été observées (Ledee-Bataille, Lapree-Delage et al. 2002).

Aussi plusieurs cytokines et leurs récepteurs ont une expression dans l'endomètre, qui varie au cours du cycle, avec un pic au cours de la fenêtre d'implantation. Parmi l'ensemble de ces nombreuses et redondantes molécules, certaines pourraient présenter un bon potentiel de marqueurs de la réceptivité utérine tel que : le système LIF, le système IL1, l'IL12, L'IL15, l'IL18 et le CSF. Sous le prisme de l'immunologie de la reproduction nous pouvons diviser la gestation précoce en deux phases : Un stade simili inflammatoire, consécutif à la fécondation et à la présence du liquide séminal dans l'endomètre; suivi d'un stade anti-inflammatoire. Les cytokines de l'inflammation seront à l'origine d'un dialogue continu entre l'embryon et le système immunitaire de la mère. L'équilibre qui en résulte compose les bases de la tolérance materno-fœtale (Medawar 1953). Cependant, la question demeure, comment les dangers potentiels d'une réaction inflammatoire se transforment-ils en facteurs de croissance placentaire ?

Le premier contact embryon-mère est un paradoxe biologique, car de façon générale deux épithéliums ne s'apposent pas. La manière dont va se présenter l'épithélium de surface de l'endomètre est essentielle. De plus, un autre paradoxe est la réaction inflammatoire classique qui a lieu contre les spermatozoïdes, et qui permet de recruter des macrophages et des lymphocytes qui peuvent être néfastes pour le futur embryon (McMaster, Newton et al. 1992). Par la suite, une réaction pseudo-inflammatoire aura pour rôle d'attirer l'embryon, faciliter son éclosion, puis permettre l'apposition et l'adhésion. Enfin, une poussée anti-inflammatoire permet

la mise en place des acteurs qui assureront l'angiogenèse, la placentation et la croissance (Ledee-Bataille, Lapree-Delage et al. 2002). Ce sont les bases de l'immunotropisme « des cytokines qui nourrissent et fortifient le futur placenta » (Chard 1995).

Théoriquement un embryon peut être décrit comme une greffe semi-allogénique, partageant des propriétés similaires à une tumeur envahissante. Par conséquent, le fœtus pourrait être la cible de l'immunité à médiation cellulaire. Citée à de nombreuses reprises dans ce mémoire, la cellule immunitaire qui semblerait soutenir l'immunotropisme est la cellule tueuse uNK. L'environnement cytokinique dans lequel évolueront les uNK au contact des cellules du trophoblaste est essentiel. Déjà présentes en grand nombre lors de la fenêtre d'implantation, ces cellules immunitaires sont indispensables pour la conduite jusqu'au terme de la grossesse Guimond, Wang et al. 1998). *In utero* les activités lytique des uNK seraient inhibées par la liaison de leurs récepteurs KIR à l'antigène HLA-G, sécrété par les cellules maternelles (Le Bouteiller and Tabiasco 2006). De plus, présent au cœur d'une mer de cytokine, des facteurs immunosuppresseurs tel que le TGFβ2, PIBF (progesterone induced blocking factor) et le TJ6 (encoding ATPase, H+ transporting, lysosomal V0 subunit a2) interviendraient également dans la tolérance à l'embryon. Les NK utérins sécrètent aussi des cytokines immunotrophiques et de l'IL-10 (Chaouat, Tranchot Diallo et al. 1997; Zhang, Croy et al. 2005). . Enfin, il ne faut pas oublier que la décidue reste une muqueuse capable de se défendre contre les agressions. Ainsi dans un contexte d'échec d'implantation, l'activation des uNK par de fortes quantités d'IL18 et d'IL12 ou par l'IL2, devient nocive pour l'embryon (Ledee-Bataille, Bonnet-Chea et al. 2005). À l'inverse, une absence totale de réactivité de la muqueuse endométriale inhibe l'apposition et l'adhésion du blastocyste. C'est l'équilibre entre les cytokines pléiotropiques pro et anti-inflammatoires qui viendra nourrir et soutenir la croissance de l'embryon.

1.3 Les facteurs embryonnaires

1.3.1 L'hormone Gonadotrophine Chorionique Humaine

L'hormone de grossesse (la hCG), est le chef de file des facteurs embryonnaires. En tant que tel, l'expression de la hCG fut investigué dans les cellules embryonnaires en culture (Lopata and Hay 1989). C'est au stade blastocyste qu'apparaîtrait l'ARNm de la hCG dans les cellules trophoblastiques ainsi que la protéine sécrétée, et ceci avant son implantation. Récemment, des faibles quantités d'hCG maternel auraient été détectées à la surface des cellules épithéliales de l'endomètre en phase sécrétoire, suggérant que celle-ci interviendrait dans la réceptivité endométriale par des effets paracriens ou juxtacriniens (figure 6) (Zimmermann, Ackermann et al. 2012). Le terme d'hCG réfère à quatre molécules indépendantes, produites par différents types cellulaires. L'hétérodimère est composé de deux sous unités (hCGα et hCGβ), liées de façon non covalente par des forces de faible énergie (interactions ionique et hydrophobe).Le poids moléculaire est approximativement de 36 KDa. La séquence de la sous unité alpha est identique aux séquences de la sous unité alpha de la LH, FSH et TSH, seule la séquence de la chaîne bêta est unique à la hCG (Bellisario, Carlsen et al. 1973; Carlsen, Bahl et al. 1973; Morgan, Birken et al. 1975). Après la combinaison des deux sous unités, des modifications post-traductionnelles ont lieu dans le réticulum endoplasmique et l'appareil de Golgi (Ruddon, Hanson et al. 1979). Enfin, l'hormone est rapidement sécrétée depuis des granules hCG spécifiques, formées à partir du Golgi (Morrish, Marusyk et al. 1987). Le point isoélectrique de la hCG est de 3.5 grâce à l'ajout d'oligosaccharides et de résidus acides, ce qui confère à la hCG une demi-vie plus longue (36h) qu'à sa molécule sœur, la LH (0.43h) (Ryan, Charlesworth et al. 1988; Moyle, Matzuk et al. 1990).

Durant la gestation, le placenta produit deux variants, la hCG sécrétée par les cellules différentiées du syncytiotrophoblates et la hCG hyperglycosylé (hCG-H) sécrétée par les cellules cytotrophoblastiques (Kovalevskaya, Genbacev et al. 2002). Cette dernière possède une chaîne d'oligosaccharides plus longue, modifiant le poids moléculaire finale à 40-41 KDa. Les sucres modifient également la structure 3D de la protéine en limitant son repliement, ce qui expose de nouveaux sites et laisse suggérer que ce variant peut lier différents récepteurs.

Lors du cycle menstruel, une forme sulfatée d'hCG qui agit comme la LH pour stimuler l'ovulation (Birken, Maydelman et al. 1996), produite à un niveau extrêmement bas (<2mUI/ml) (Cole and Gutierrez 2009). Lors de ménopause ou d'aménorrhée, l'ovaire ne parvient plus à maintenir le rétrocontrôle des œstrogènes qui inhibaient la libération de gonadotrophine par l'hypothalamus. Ceci conduit à l'augmentation de la sécrétion de LH et d'hCG jusqu'à 33.6 mUI/mL. C'est également le cas des femmes ayant subi une salpingectomie bilatérale (Snyder, Haymond et al. 2005).

Enfin, dans un contexte tumoral, les cellules cancéreuses dédifférenciées peuvent sécréter la sous unité bêta libre et hyperglyosylé (Butler, Ikram et al. 2000). Plusieurs rapports traitent la question qui est assez controversée. Des recherches ont montré que la plupart des cancers produisent une substance hCGβ immunoréactive (Acevedo, Tong et al. 1995; Acevedo and Hartsock 1996). Alors que d'autres publications estiment que 50% environ des cancers produisent la sous unité libre bêta de la hCG (Cole 2009; Muller and Cole 2009).

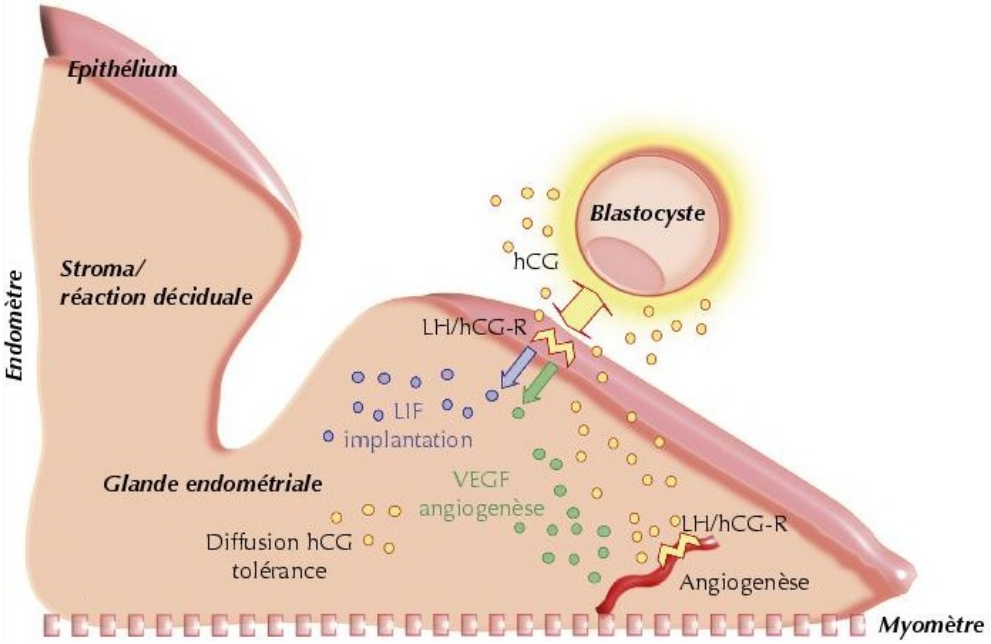

Figure 6 Effets paracrines de l'hGC sur l'endomètre. Tirée de médecine de la reproduction, Sophie Perrier d'Hauterive (Sophie Perrier d'Hauterive 2007).

Cependant sa détection est contrariée par le fait qu'elle peut être rapidement dégradée par une élastase leucocytaire produite par les macrophages (Kardana and Cole 1994). Il faut noter que le clivage enzymatique de la sous-unité bêta libre est plus rapide que celle des variants hétérodimériques. Les produits résultants de la dégradation sont ensuite éliminés de la circulation par le foie et les reins. Ainsi, le taux de détection de hCGβ sanguin en cas de cancer approche les 30% (Cole, Tanaka et al. 1996).

1.3.1.1 Fonctions biologiques

L'ensemble des différentes fonctions biologiques associées aux variants de la hCG est résumé dans le tableau 1.

1.3.1.1 Le récepteur LHCGR

La hCG partage un récepteur commun avec la LH, le LHCGR. Il s'agit d'un récepteur transmembranaire couplé aux protéines G (GPCR). Le récepteur LHCGR est composé de 699 acides aminés, il est codé par un gène de plus de 80000 paires de bases localisé sur le chromosome 2p21 (Dufau 1998). Le domaine extracellulaire lie les deux hormones avec une haute affinité. Les sept domaines transmembranaires conduisent les signaux vers les différentes protéines couplées au récepteur qui activent l'adénylate cyclase. En réponse la concentration du messager secondaire cytosolique AMPc augmente, ce qui active la protéine kinase A (PKA). Celle-ci va ensuite phosphoryler les différents effecteurs subséquents menant à une réponse cellulaire spécifique. Il est clair que ce n'est pas la seule voie activée par le LHCGR, des voies supplémentaires peuvent être impliquées dans d'autres conditions LHCGR-dépendantes tels que la prolifération et / ou la différenciation des cellules cibles (Ascoli, Fanelli et al. 2002).

Fonctions	Références
hCG	
Signal implantatoire	(Perrier d'Hauterive, Berndt et al. 2007)
Modification matricielle lors de l'invasion	(Fluhr, Bischof-Islami et al. 2008)
Maintien du corps jaune et production de progestérone	(S Aschheim 1927; Strott, Yoshimi et al. 1969)
Interaction entre le sperme et les trompes : communication pré-grossesse?	(Lei, Toth et al. 1993; Eblen, Bao et al. 2001)
Angiogenèse de la vasculature utérine	(Lei, Reshef et al. 1992; Toth, Li et al. 1994; Zygmunt, Herr et al. 2002; Berndt, Blacher et al. 2009)
Différentiation du cytotrophoblast	(Shi, Lei et al. 1993)
Immunosuppression, blocage de la phagocytose	(Noonan, Halliday et al. 1979; Majumdar, Bapna et al. 1982; Akoum, Metz et al. 2005; Tsampalas, Gridelet et al. 2010)
Grandissement utérin en lien avec la croissance du fœtus	(Reshef, Lei et al. 1990; Zuo, Lei et al. 1994)
Relaxation musculaire utérine	(Eta, Ambrus et al. 1994; Doheny, Houlihan et al. 2003)
Croissance et différentiation des organes fœtaux	(Goldsmith, McGregor et al. 1983; Abdallah, Lei et al. 2004; Rao and Lei 2007)
Développement du cordon ombilical	(Rao, Li et al. 1993)
Effet sur le cerveau adulte	(Lei, Rao et al. 1993)
hCG-H	
Stimule l'implantation et l'invasion, bloque l'apoptose lors de gestation et de chorioncarcinomes	(Kamijo, Rajabi et al. 1998; Cole, Dai et al. 2006; Sasaki, Ladner et al. 2008)
Stimule la formation du placenta et favorise l'apparition de chorioncarcinomes par la croissance des cellules cytotrophoblastique	(Cole, Butler et al. 2006; Sasaki, Ladner et al. 2008)
hCG-β	
Bloque l'apoptose, favorise la malignité	(Butler, Ikram et al. 2000; Hamada, Nakabayashi et al. 2005)
hCG p	
Fonctions similaires à la LH	(Birken, Maydelman et al. 1996)

Tableau 1 Effets biologiques des hCG ; Adapté de (Cole 2010)

1.3.1.2 Rôle du système IL1 dans l'implantation

L'interleukine 1 est impliquée dans de nombreux processus physiologiques dont l'angiogenèse et le remodelage tissulaire (Rosenkilde and Schwartz 2004; Naldini and Carraro 2005). Sa présence et son rôle indispensable à l'établissement d'une grossesse permettent de considérer localement cette cytokine comme un signal embryonnaire précoce. Son rôle dans l'implantation est bien décrit (Krussel, Bielfeld et al. 2003; Fazleabas, Kim et al. 2004).

Plus que toute autre famille de cytokines, la famille de l'IL1 est étroitement liée à la réponse immunitaire innée. Bien que les Toll-like receptors (TLR) et les membres de la famille de l'IL1 aient évolué pour contribuer à la défense de l'hôte contre les infections, la famille IL1 comprend des membres qui suppriment l'inflammation, contrairement aux TLR, à la fois spécifiquement au sein de la famille IL1, mais également de façon non spécifique dirigée contre la réponse immunitaire innée *via* les TLR (Dinarello 2009).

La présence de l'IL1β à l'interface embryo-maternelle suggèrerait que cette cytokine pourrait jouer un rôle dans la tolérance immunitaire de l'embryon qui est considéré comme une greffe semi-allogénique dans l'utérus maternel (Haig 1993; Billington 2003). De nombreuses études, notamment sur le placenta humain, ont permis d'établir des mécanismes fondamentaux de ce phénomène, mais de nombreux aspects sont encore mal connus et le processus complexe du paradoxe immunologique n'a pas encore été complètement défini (Matzinger 2002; Moffett and Loke 2006; Blois, Kammerer et al. 2007).

La sécrétion et l'action de nombreux médiateurs comme les cytokines à l'interface embryo-maternelle sont reconnues comme jouant un rôle central dans l'immunotropisme (Loke, King et al. 1995). Les cytokines régulent l'activité des

cellules immunitaires ainsi que la croissance et la prolifération cellulaire (Vilcek 1996), par conséquent, la présence de l'IL1, produite très tôt par les cellules embryonnaires, à l'interface materno-fœtal, pourrait moduler la réponse immunitaire de la mère et contribuer à l'expansion des tissus fœtaux dans l'utérus maternel (Saito 2001; Paria, Reese et al. 2002; Schafer-Somi 2003).

1.3.1.3 Pro et anti inflammatoire, description des membres de la famille IL1

La famille IL-1 est composée de 11 ligands, 8 récepteurs et 2 corécepteurs. Le terme d'interleukine 1 réfère à 2 agonistes, l'IL-1α et l'IL-1β. Ce sont les chefs de file du système IL1.

La forme α est principalement localisée dans le cytoplasme, tandis que la forme β est sécrétée. Synthétisés sous forme de précurseurs, les pro-formes α et β sont clivés par une enzyme spécifique. Soit par ICE1 (Interleukin-1beta-converting enzyme), encore appelé caspase 1, pour l'IL1β, soit par les calpaïnes pour l'IL1α. Contrairement au précurseur IL1α, le précurseur IL1β est inactif. Il existe un antagoniste de l'IL1, l'IL1RN et deux récepteurs IL-1 R1 et IL-1 R2. Seul l'IL1 R1 est capable de transmettre un signal en réponse à l'IL-1. Cependant cette action nécessite le recrutement d'un corécepteur IL1R3, ou encore appelée protéine accessoire (IL1RAcP) (Greenfeder, Nunes et al. 1995). L'IL1 R2 est un inhibiteur naturel de l'IL1 puisqu'il agit comme un récepteur leurre (Colotta, Re et al. 1993), incapable d'initier la transduction du signal, car il n'y a pas de domaine TIR dans sa queue cytoplasmique trop courte.

Nouvelle nomenclature	Ancienne nomenclature	Récepteur	Corécepteur	Propriété
IL-1F1	IL-1α	IL-1RI, IL-1RII	IL-1RacP	Agoniste : IL-1RI+IL-1RacP Antagoniste : sIL-1RI, IL-1RII, IL-1RII+IL-1RacP,sIL-1RII, sIL-1RII+sIL-1RacP
IL-1F2	IL-1β	IL-1RI, IL-1RII	IL-1RAcP	Agoniste : IL-1RI+IL-1RacP Antagoniste : sIL-1RI, IL-1RII, IL-1RII+IL-1RacP,sIL-1RII, sIL-1RII+sIL-1RacP
IL-1F3	IL-1RN	IL-1RI	NA	Antagoniste pour IL-1α, IL-1β
IL-1F4	IL-18	IL-18Rα	IL-18Rβ	Agoniste
IL-1F5	IL-36Ra	IL-1Rrp2	NA	Antagoniste pour IL-36α, IL-36β, IL-36γ
IL-1F6	IL-36α	IL-1Rrp2	IL-1RAcP	Agoniste
IL-1F7	IL-37	?IL-18Rα*	inconnue	Anti-inflammatoire
IL-1F8	IL-36β	IL-1Rrp2	IL-1RAcP	Agoniste
IL-1F9	IL-36γ	IL-1Rrp2	IL-1RAcP	Agoniste
IL-1F10	IL-38	inconnue	inconnue	inconnue
IL-1F11	IL-33	ST2	IL-1RAcP	Agoniste réponse Th2
inconnue	inconnue	TIGIRR-2	inconnue	Anti-inflammatoire
inconnue	inconnue	TIGIRR-1	inconnue	Anti-inflammatoire

Tableau 2 La grande famille de l'IL1. Adapté de Charles Dinarello (Dinarello 2009; Dinarello 2011)

Autour de l'IL1, plusieurs autres stratégies d'inhibitions et de contrôles sont mises en place. En plus du récepteur leurre, l'IL1RN se lie à l'IL1R1 avec une plus grande affinité que l'IL1, réduisant les interactions de l'IL1α et l'IL1β avec leur récepteur. Des formes solubles du récepteur IL1R1, R2 et R3 interviennent également pour neutraliser l'IL1 comme illustré dans la figure 7. Ainsi, l'IL18 (Bazan, Timans et al. 1996), l'IL33 (Schmitz, Owyang et al. 2005) et l'IL36αβγ/ra (Debets, Timans et al. 2001) peuvent être classés en tant que membre de la famille IL-1. Ces derniers se trouvent être les ligands de récepteurs dit orphelin dont leur homologie de séquence (avec des motifs spécifiques Toll et Ig) avec l'IL1RI avait été prédite. L'IL18 se lie au récepteur fonctionnel IL18Rα (alias IL18R1, IL1R5). La transduction du signal a lieu après le recrutement du corécepteur IL18Rβ, similaire à l'IL-1RAcP. L'IL18BP (IL18 binding protein) agit comme un régulateur négatif pour neutraliser l'IL18 grâce à une affinité supérieure (Novick, Kim et al. 1999). Comme l'IL1β, l'IL33 est produite sous forme de précurseurs clivés par la caspase 1 pour libérer une forme active qui pourra lier son récepteur fonctionnel et induire un signal après le recrutement de la même protéine accessoire IL1RAcP (Schmitz, Owyang et al. 2005). IL-1α et l'IL-33 sont des cytokines bi fonctionnelle parce qu'en plus de la liaison à leurs récepteurs à la membrane cellulaire, les formes précurseurs intracellulaires transloquent vers le noyau et influencent la transcription des gènes pro inflammatoires tel que l'IL8 (Reznikov, Waksman et al. 2004; Carriere, Roussel et al. 2007).

De cette façon, les ligands et les récepteurs de la famille IL1 ont évolué vers un double rôle dans la défense de l'hôte, avec des protagonistes pro et anti inflammatoires (Fig.7).

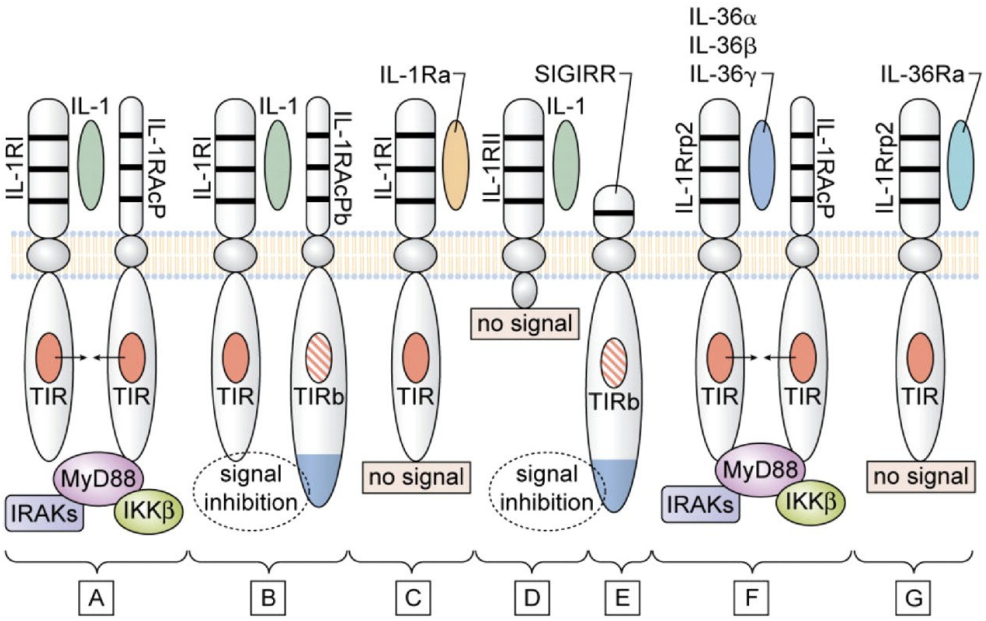

Figure 7 Activation et inhibition des récepteurs membranaires de la famille IL1. Tiré de Dinarello (Dinarello 2011)

1.3.1.4 Transduction du signal IL1β

La première étape de transduction du signal de l'IL1 est le changement de conformation de l'IL-1RI qui facilite le recrutement d'IL-1RAcP. *Via* les domaines intracellulaires TIR, le complexe trimérique (IL1R1, IL1, IL1RAcP) assemble rapidement deux protéines de signalisations intracellulaires, MYD88 et IRAK 4. Ce premier module de signalisation phosphoryle ensuite IRAK (Interleukin 1 receptor-associated kinase) 1 et IRAK2. Puis le signal est suivi par le recrutement et l'oligomérisation de TRAF (TNF receptor associated factor) 6 et la formation subséquente des complexes de signalisation TAK (TGF-beta activated kinase) 1 et MEKK (MAP/ERK kinase kinase) 3 qui activent les facteurs de transcriptions NF-kappa B (nuclear factor-kappa B), JNK (c-Jun N-terminal kinase) et p38 MAPK (Mitogen-activated protein kinases) (Fig.8) (Weber, Wasiliew et al. 2010). La translocation nucléaire de NF-kappa B permet sa liaison à un motif d'ADN conservé que l'on retrouve dans de nombreux gènes cibles de l'IL1, en particulier ceux de IL-6(Saccani, Pantano et al. 2002), de l'IL-8 (Hoffmann, Dittrich-Breiholz et al. 2002), de la protéine chimiotactique des monocytes (MCP1/CCL2) (Wolter, Doerrie et al. 2008), et de la cyclooxygénase 2 (COX-2) Nakao, Ogata et al. 2000). De nombreuses études menées sur les cellules stromales prélevées en phase sécrétrice moyenne, et stimulées par l'IL1β montrent que cette cytokine affecte plusieurs catégories fonctionnelles, reflétant les multiples actions de recouvrement de cette cytokine pléiotropique. Publié par Rossi en 2005, le groupe de gènes le plus ciblé par l'IL1 dans les cultures primaires de cellules stromales humaine, en phase sécrétoire, contient des modulateurs immunitaires et des cytokines.

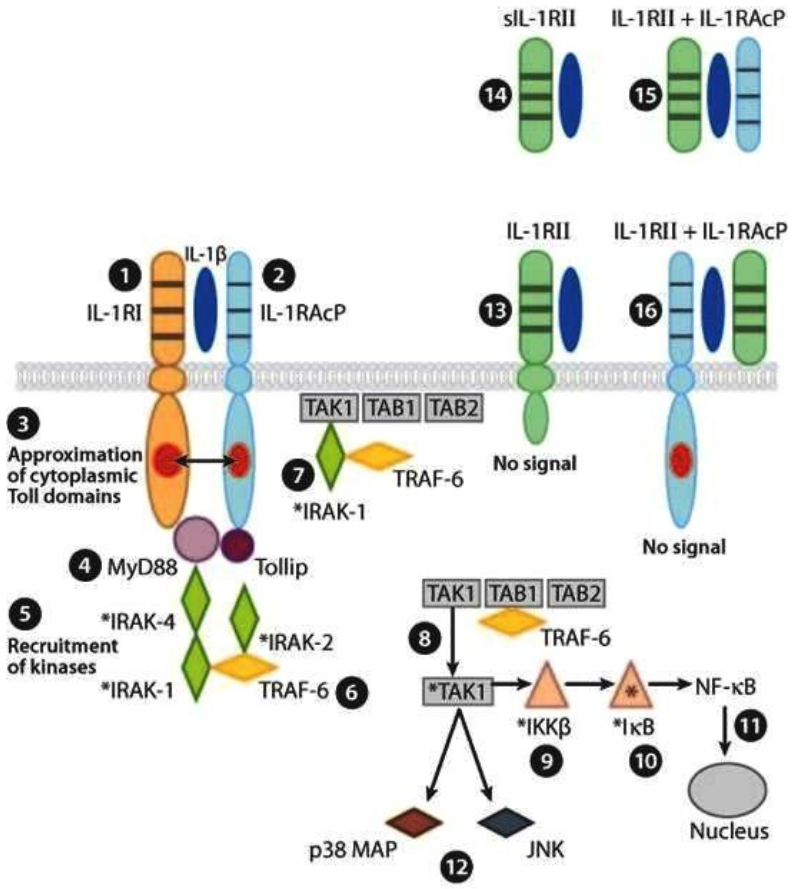

Figure 8 Activation de l'IL1R1 via IL1ß et sa neutralisation. Tiré de Dinarello (Dinarello 2009).

1 à 12, étapes de transduction du signal; 13 à 16, neutralisation de l'IL1ß.

Plusieurs cibles d'entre elles avaient déjà été reportées être régulées positivement par l'IL-1β tel que l'IL-8, IL-6, CXCL1 (GRO (Cotton rat growth-regulated protein) a), CXCL2 (GROß), CCL2 (MCP-1), CSF (colony-stimulating factor) 2 et CSF3 Rossi, Sharkey et al. 2005). En parallèle, l'IL1α et l'IL1β induisent aussi l'expression de leurs gènes, qui sert de boucle de rétroaction positive qui amplifie la réponse à l'IL-1 de manière autocrine ou paracrine (Dinarello, Ikejima et al. 1987; Granowitz, Clark et al. 1992; Gaestel, Kotlyarov et al. 2009). Au laboratoire, des travaux antérieurs menés sur une lignée épithéliale KLE et des cultures primaires de l'endomètre suggèrent que l'IL1β peut moduler la réceptivité des cellules endométriales à sa propre action. Les données ont montré une augmentation significative de IL1R1, IL1R2 et de l'IL1RN au niveau de l'ARNm et de la protéine (Bellehumeur, Blanchet et al. 2009).

1.3.1.5 Des membres de la famille de l'interleukine 1 comme marqueurs de l'implantation embryonnaire ?

La présence des récepteurs à l'IL1 a été rapportée tant sur la surface des cellules stromales et épithéliales de l'endomètre, que sur la surface des cellules du trophoblaste, prouvant ainsi que ces tissus pourraient répondre à la présence de cette cytokine (Simon, Frances et al. 1994; Bischof and Campana 2000; Boucher, Kharfi et al. 2001). Dans le cas d'une fécondation *in vitro*, la présence de l'IL-1 (fortement exprimé par le blastocyste) est corrélée avec le succès de l'implantation de l'embryon après son transfert dans la cavité utérine (Sheth, Roca et al. 1991). Aussi, chez les femmes, l'avortement spontané est corrélé avec une diminution de l'IL1β et de l'IL6 plasmatique (von Wolff, Thaler et al. 2000).

Il a été démontré que l'IL1β induit l'expression de LIF (marqueur de la réceptivité et de l'implantation embryonnaire) par les cellules endométriales (Sawai, Matsuzaki et al. 1997).

Dans un modèle *in vivo*, l'injection du récepteur antagoniste à l'IL1 (IL1RN), réduit considérablement le nombre d'embryons implantés en altérant la réceptivité de l'endomètre à ces derniers (Simon, Frances et al. 1994; Simon, Valbuena et al. 1998). Chez la femme, au cours de l'implantation, l'embryon augmente l'expression d'intégrine spécifique (β3), qui est partiellement induit *via* l'IL1β (Simon, Gimeno et al. 1997). Chez le singe, l'infusion d'hCG et d'IL1β mime les changements qui ont lieux lors de la grossesse et conduisent à l'immunotropisme (Strakova, Mavrogianis et al. 2005).

D'après des études, il semblerait que la présence d'IL18 dans l'environnement cytokinique utérin serait associée à un faible pronostique d'implantation embryonnaire, d'accouchement prématuré ou de prééclampsie (Chaouat, Ledee-bataille et al. 2003; Chaouat, Ledee-Bataille et al. 2003; Ledee-Bataille, Olivennes et al. 2004). L'IL18 est physiologiquement présente tout au long du cycle menstruel sous forme inactive. La détection de celle-ci dans la lumière utérine témoigne d'une activation endométriale, dont la cause reste à déterminer s'il ne s'agit pas d'une infection. L'IL18 aurait des effets inverses en fonction de l'environnement cytokinique. Lorsque l'IL12 est indétectable ou faiblement présente, l'IL18 est exprimé au niveau des glandes et des artères spiralées et la présence de lymphocytes (NK) est modérée. Cependant en présence d'IL12, l'IL18 est activé et sécrété et aurait un effet anti-implantatoire *via* l'activation des NK.

Ainsi, une fine régulation du système et de la famille IL-1 est essentielle pour le bon déroulement du processus implantatoire. Cependant, les souris déficientes en IL-1RI, IL-1RAcP, l'IL-1α, IL-1β ou doublement déficientes en IL-1α/β ne présentent pas de différences phénotypiques comparativement aux souris sauvages. On pourrait conclure de ces modèles soit que l'IL-1α ou IL-1β ne sont pas essentiels au développement embryonnaire normal, à la croissance postnatale, à l'homéostasie, à

la reproduction, ou à la résistance à la flore microbienne, soit qu'il existe des phénomènes de compensations.

1.4 Remodelage tissulaire et angiogenèse endométriale

1.4.1 Régulation cyclique de la matrice extra cellulaire

Sous l'action des stéroïdes ovariens, l'endomètre subit des modifications communes à un grand nombre d'espèces tel que l'augmentation de la perméabilité des capillaires, de l'œdème et le remaniement des constituants de la matrice extracellulaire (MEC). La dynamique de l'endomètre pour préparer l'implantation nécessite un remodelage tissulaire constant. Ceci suggère une action coordonnée, non seulement entre différents facteurs, mais également entre les cellules du stroma (Trévoux 2009). Visible à l'échographie, l'angiogenèse en fenêtre implantatoire est capable de renseigner sur l'état de la réceptivité utérine. Les métalloprotéinases (MMPs) sont les chefs de fil impliqués dans la régulation de la matrice extra cellulaire et lors de l'adaptation angiogénique.

Dans l'endomètre, l'expression des MMPs est principalement sous le contrôle de la progestérone, mais de nombreux facteurs interviennent pour réguler leurs activités, depuis leur expression jusqu'à leur dégradation. Au cours du cycle menstruel les vaisseaux utérins vont subirent un remodelage vasculaire en vue du développement d'un plexus capillaire sous-épithélial permettant l'implantation de l'embryon. Comme tout équilibre, une absence ou un excès de vascularisation est inadapté aux conditions menant à une implantation réussie. La réaction vasculaire locale corrèle avec le trafic immunologique lors de la fenêtre d'implantation. Il existe un équilibre subtil entre l'angiogenèse et la présence des uNK. La présence de ces cellules immunitaires dans l'endomètre conditionne l'angiogenèse placentaire et

l'immunotropisme local aboutissant à la croissance de l'embryon (Kwak-Kim and Gilman-Sachs 2008).

1.4.1.1 Mode d'action des MMPs

Le remodelage du stroma de l'endomètre nécessite à la fois la dégradation et la reconstruction des composants de la MEC. Ces effets sont orchestrés par les hormones ovariennes et sont souvent médiés par des facteurs de croissance et des cytokines locales telles que l'IL-1 et le transforming growth factor (TGF). La matrice extra cellulaire est le point d'ancrage des cellules qui composent le tissu et présente des compétences pour la migration cellulaire, la division et la différenciation cellulaire (Birkedal-Hansen, Moore et al. 1993; McIntush and Smith 1998). La dégradation des protéines de la MEC peut être effectuée par une variété d'activités enzymatiques, mais les métalloprotéinases matricielles ou MMPs, sont considérées comme les principaux contributeurs à ce processus (Hulboy, Rudolph et al. 1997). Par leur liaison à la membrane plasmique les MMPs peuvent focaliser leur activité enzymatique et facilité le processus de migration cellulaire et le renouvellement tissulaire. Pour être efficace et sans conséquence pathologique, la protéolyse matricielle se doit d'être focalisée. À cette fin, plusieurs stratégies sont développées au niveau membranaire (Werb 1997). Celles-ci sont schématiquement résumées dans la figure 9.

Leurs inhibiteurs naturels tissulaires endogènes (TIMP) et l'α_2-macroglobuline, également exprimés dans l'endomètre, interviennent pour réguler l'activité enzymatique des MMPs (Hampton and Salamonsen 1994; Sayegh, Awwad et al. 1995). Toutes les circonstances susceptibles d'interrompre l'équilibre délicat entre les MMPs et leurs inhibiteurs naturels conduisent à un certain nombre de complexités pathologiques liés à la grossesse et l'infertilité, tel que l'endométriose (Curry and Osteen 2003).

1.4.2 Remodelage et implantation embryonnaire : un phénomène pseudotumoral

L'invasion et la migration des cellules trophoblastiques extravillositaires (CTEV) dans l'endomètre partage des similitudes avec l'invasion des cellules malignes et métastatiques (Fig.10). Cependant il existe deux grandes différences : La pénétration des CTEV dans la paroi utérine par protéolyse de la MEC et la colonisation des vaisseaux utérins est un processus physiologique strictement localisé dans l'endomètre ainsi que la couche interne du myomètre, et est défini dans le temps (début de gestation) (Wallace, Fraser et al. 2012). La différenciation des CTEV vers un phénotype invasif a été comparé au processus non contrôlé de la transition épithélio-mésenchymateuse (EMT) observé dans le cancer (Kalluri and Weinberg 2009). Cette transition implique le passage d'une colonne de cellules, en contact par une série de molécules d'adhésion, en cellules individuelles capables de migrer sur de longues distances.

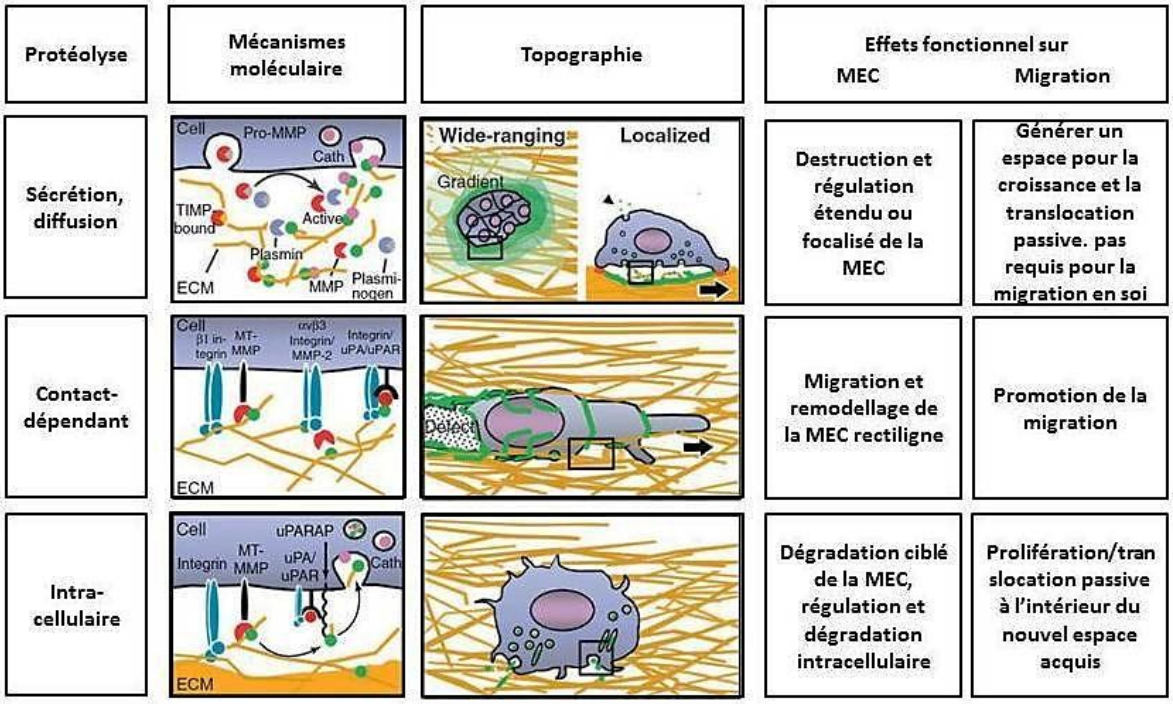

Figure 9 Diagramme explicatif de la protéolyse matricielle focalisé. Adapté de (Wolf and Friedl 2011)

Ce phénomène est rendu possible par des changements dans les protéines du cytosquelette et la production de protéases initiée par des facteurs du microenvironnement tumoral (TGF-β, HGF et EGF). Ces nouvelles propriétés permettent aux cellules cancéreuses de migrer dans différentes régions du corps. Cependant, la transition subie par les CTEV pourrait être un phénotype intermédiaire de l'EMT. Il se caractérise principalement par l'expression d'intégrine $\alpha_5\beta_1$, puis $\alpha_1\beta_1$ et $\alpha_V\beta3$. La première étape de la différenciation s'accompagne d'une régulation positive du récepteur de la fibronectine (Fn) $\alpha_5\beta_1$. Lorsque les CTEV individuels quittent le feuillet cellulaire pour envahir la paroi utérine, ils expriment le récepteur à la laminine (Ln) $\alpha_1\beta_1$, tout en continuant à exprimer le récepteur $\alpha_5\beta_1$. L'expression des intégrines $\alpha_6\beta_4$ et de la E-cadhérine disparaît, ce qui favorise le processus invasif. L'ensemble de ce phénomène est appelé « integrin switch » (Damsky, Librach et al. 1994).

De nombreuses molécules d'adhésion exprimées dans la décidue et par les CTEV sont modulées au cours de l'invasion par différents facteurs tels que les hormones ovariennes, les cytokines IL-1, MCSF, TNF, les facteurs de croissance EGF, hGF TGF-β, LIF, hCG rendant compte de la plasticité phénotypique de l'endomètre et du trophoblaste (Fluhr, Bischof-Islami et al. 2008). Nombre de ces mêmes facteurs sont responsables de la production de MMPs par les cellules tumorales et les CTEV. D'autres protéases telles que la plasmine (sérine protéase) et les calpaïnes (protéases calcium dépendant) jouent un rôle dans la migration des cellules trophoblastiques et métastatiques (Salamonsen 1999; Franco and Huttenlocher 2005).

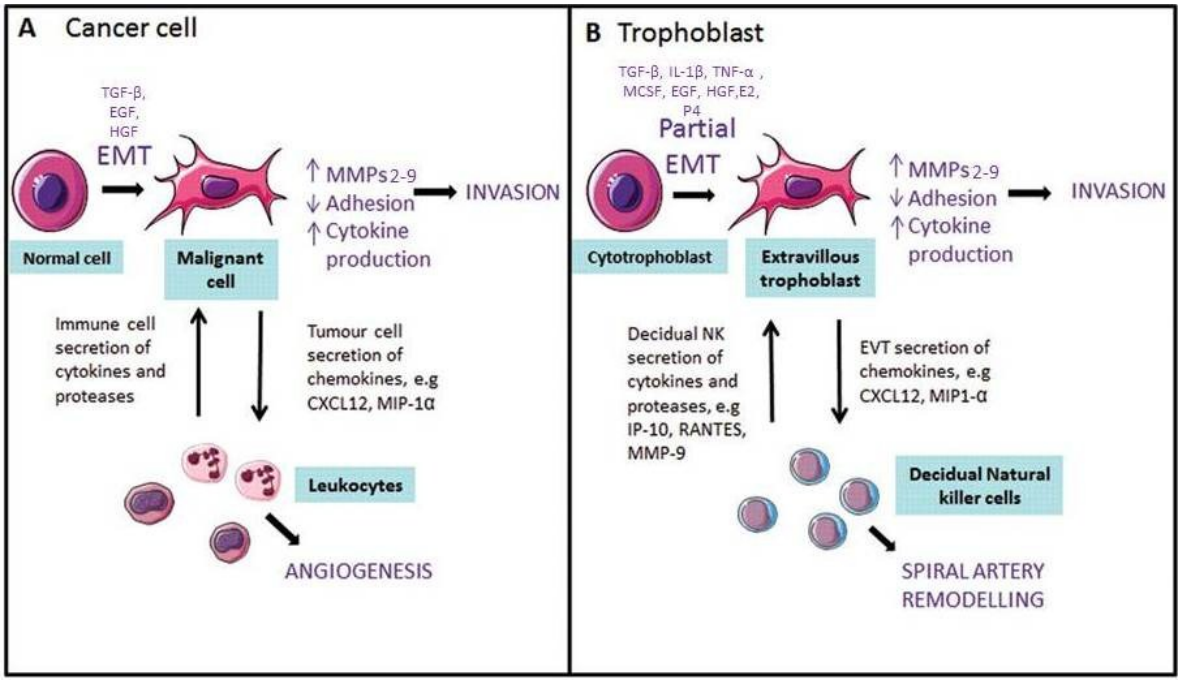

*Figure 10 Similitudes entre l'EMT des cellules cancéreuses et la différenciation des cytotrophoblastes en CTEV. (**A**) Suite à l'EMT, les cellules malignes invasives sécrètent de fortes quantités de protéases et cytokines qui favorisent l'invasion des tissus. La sécrétion de chimiokines comme CXCL-12 et MIP-1α attire les leucocytes, ils joueront un rôle dans l'angiogenèse tumorale. (**B**) Lors de la transformation en CTEV invasive, la forte sécrétion de protéases et cytokines, en interaction avec les NK de la decidue contribue au remodelage des artères utérine. Adapté de (Wallace, Fraser et al. 2012).*

Les CTEV invasives sécrètent de nombreuse pro-MMP, mais les deux formes majoritaires sont la pro-MMP-2 et la pro-MMP-9. En réponse, les cellules de la décidue produisent des fortes quantités de TIMPs (Cohen, Meisser et al. 2006). Les caractéristiques invasives des CTEV sont contrôlées de façon spatiotemporelle par de nombreux facteurs. Le TGF-β stimule la synthèse de TIMP-1, l'inhibiteur associé à MMP-9, et de TIMP-2. L'EGF, sécrété par la décidue permet la synthèse et l'activation des MMP-2 et 9. D'autres facteurs de croissance comme le VEGF et le FGF stimulent l'activité du plasminogène et de MMP-9. En 2008 une étude a montré que la hCG augmentait la sécrétion de MMP2 et 9 des CTEV, mais diminuait l'expression des TIMP1-2 et 3 (Fluhr, Bischof-Islami et al. 2008). De façon générale les cytokines pro inflammatoires 1; 6; 11; 15 stimulent les MMPs et l'invasion, alors que les cytokines anti-inflammatoires (IL-10) à l'inverse inhibent le processus.

1.4.3 Angiogenèse endométriale

L'angiogenèse se définit par l'extension de l'arbre vasculaire à partir de vaisseaux préexistants. Cette dernière se produit aussi bien au cours du développement qu'au cours de l'âge adulte dans des circonstances physiologiques, telles que l'adaptation à l'exercice musculaire, la placentation, qu'en pathologie lors des cancers, par exemple (Otrock, Mahfouz et al. 2007). Lors de l'angiogenèse, la protéolyse extracellulaire est essentielle au désassemblage et au réassemblage des cellules endothéliales de leur environnement matriciel afin de permettre leur migration. Elle est assurée par le système de la coagulation et de la fibrinolyse, les métalloprotéinases et les molécules d'adhérence. Enfin, un équilibre constant entre les facteurs pro et anti-angiogéniques contrôle l'angiogenèse. Les mêmes facteurs de croissance ainsi que leurs récepteurs sont impliqués dans ces différents aspects

de la vasculogenèse et de l'angiogenèse au cours du développement et à l'âge l'adulte (Papetti and Herman 2002).

L'angiogenèse dépend de la coordination de plusieurs processus qui comprend la dégradation de la membrane basale vasculaire; la migration des cellules endothéliales en direction des stimuli pro angiogénique; la prolifération endothéliale; la formation d'une lumière au sein de cordons de cellules et enfin l'anastomose par raccordement des tubes endothéliaux néo-formés. Par la suite il y a une étape de maturation qui comprend également plusieurs étapes telles que la reconstruction de la membrane basale, le recrutement de cellules mésenchymateuses qui deviendront des cellules musculaires lisse ou des péricytes, et enfin l'action de ces différents constituants va inhiber la prolifération des cellules endothéliales (qui deviennent quiescentes) pour former un vaisseau différencié (Moses 1997; Klagsbrun and Moses 1999). Dans l'endomètre on peut distinguer trois phases pendant lesquelles l'angiogenèse intervient : lors des menstruations dans le but de permettre la régulation du lit vasculaire à partir de la couche basale de l'endomètre; au cours de la phase proliférative afin de promouvoir la croissance rapide de l'endomètre; pendant la phase sécrétoire au cours de laquelle apparaît le remodelage vasculaire et le développement d'un plexus sous épithélial permettant l'implantation (Gargett and Rogers 2001).

1.4.3.1 Imprégnation hormonale

Il est admis que les hormones stéroïdes modulent l'angiogenèse dans l'endomètre. La présence des récepteurs aux œstrogènes et à la progestérone retrouvée dans la paroi des artères spiralées, laisse envisager un rôle direct des hormones stéroïde dans l'angiogenèse endométriale. Seules les artères de l'endomètre fonctionnel subissent des modifications structurelles hormono-dépendante. Peu abondantes en phase proliférative en cours d'imprégnation œstrogénique, les modifications de

l'arbre vasculaire endométrial sont plus importantes en phase sécrétrice sous imprégnation œstroprogestative. Les artères deviennent fortement spiralées et leurs parois s'épaississent. Ceci implique la prolifération des cellules musculaires lisses et des cellules endothéliales. Les principaux acteurs pro-angiogéniques tel que le VEGF qui participent au remodelage vasculaire sont sécrétés par les cellules déciduales en réponse aux variations cycliques des stéroïdes ovariens (Ancelin, Buteau-Lozano et al. 2002; Mueller, Pritts et al. 2003. S'il y a implantation, la transformation des artères spiralées va se poursuivre sous l'effet de l'invasion des trophoblastes, pour aboutir à la formation des artères utéroplacentaires.

Après l'implantation, l'angiogenèse intervient dans la zone mésométriale. Cette angiogenèse est induite par l'hypoxie locale, et fait intervenir le facteur HIF (hypoxia inducible factor) qui permet la stimulation du VEGF et des angiopoïétines ainsi que leurs récepteurs. De nouvelles données suggèrent un rôle central de l'hCG dans l'adaptation de la vascularisation utérine aux besoins croissants du fœtus. Elle se fait par une vasodilatation, une augmentation de la perméabilité des vaisseaux existants et le développement de nouveaux vaisseaux (Reisinger, Baal et al. 2007). *In vitro*, des concentrations physiologiques d'hCG augmentent la formation des capillaires et la migration des cellules endothéliales de façon dose-dépendante ; *ex vivo,* la hCG induit une néovascularisation de la membrane chorio-allantoïde de poulet de façon comparable au VEGF (Zygmunt, Herr et al. 2002). Ces données, ainsi que d'autres (voir Chapitre 4), soulignent l'importance de considérer l'hormone de grossesse comme un facteur pro-angionénique.

1.4.3.2 Implication des cellules immunitaires dans la production des cytokines pro-angiogéniques

Au début de la gestation, l'endomètre est enrichi en cellules immunitaires, principalement en macrophages/monocytes et lymphocytes de type NK (natural killer). Comme illustré en figure 10, lors de l'invasion trophoblastique les cellules immunitaires vont coopérer et participer au processus angiogénique par le biais de fortes sécrétions de chimiokines qui vont augmenter la croissance vasculaire de la décidue (Kane, Kelly et al. 2009). La coordination entre l'angiogenèse et l'inflammation est rendue possible par le fait que les cellules immunitaires et endothéliales répondent aux mêmes stimuli tel que les chimiokines. Ces petites molécules ont d'abord été décrites pour leur rôle dans le recrutement des leucocytes au site d'inflammation, mais celles-ci, ainsi que leurs récepteurs sont désormais reconnues comme des médiateurs de l'angiogenèse.

Les chimiokines sont séparées en différents groupes en fonction de leur séquence en acide aminé qui forme des motifs conservés (CC et CXC). Plusieurs membres de la famille des CXC chimiokines ont été les premiers identifiés comme des régulateurs de l'angiogenèse en agissant soit comme molécule pro-angiogénique soit comme molécule angiostatique (Strieter, Polverini et al. 1995). Il est intéressant de noter que la présence ou l'absence d'un motif ELR dans leur séquence en acide aminé semble être en corrélation avec une activité pro-angiogénique ou angiostatique, respectivement.

Rôle dans l'angiogenèse	Récepteurs de chimiokines	Ligands
Angiogénique	CXCR1	IL-8 (CXCL-8), GCP-2 (CXCL6)
	CXCR2	IL-8, GCP-2 (CXCL6), Gro-α-β-γ (CXCL-1, 2, 3), ENA-78 (CXCL-5), PBP (CXCL-7)
	CXCR4	SDF-1 (CXCL12)
	CCR1	MIP-1α (CCL-3), MIP-1β (CCL4), RANTES (CCL5), HCC-1/3, 2, 4 (CCL-14, 15, 16)
	CCR2	MCP-1 (CCL2), MCP-2 (CCL8), MCP-3 (CCL7), MCP-4 (CCL13)
	CCR3	eotaxin-1, 2 (CCL-11, 24), MCP3 (CCL7)
	CCR4	TARC (CCL-17), MDC (CCL-22), MIP-1α (CCL-3), RANTES (CCL5)
	CCR5	MIP-1α (CCL-3), MIP-1β (CCL4), RANTES (CCL5)
	CCR6	MIP-3α(CCL20)
	CCR7	MIP-3β (CCL19)
	CCR8	I-309 (CCL-1)
	CCR9	TECK (CCL25)
	XCR1	Lymphotactine (XCL-1)
	CX3CR1	Fractalkine (CX3CL-1)
Angiostatique	CXCR3	PF-4 (CXCL4), MIG (CXCL-9), IP-10 (CXCL-10), I-TAC (CXCL-11)
	CXCR5	BLC (CXCL-13)

Tableau 3 Résumé des chimiokines et de leurs rôles dans l'angiogenèse. Adapté de (Rossi and Zlotnik 2000; Bernardini, Ribatti et al. 2003; Rosenkilde and Schwartz 2004; Keeley, Mehrad et al. 2008)

En outre, l'expression des chimiokines pro-angiogénique (ELR^+), comme l'IL-8 / CXCL8, GRO-α/CXCL-1 et ENA-78/CXCL5, est régulée négativement par des inhibiteurs de l'angiogenèse, telles que l'interféron (IFN)-α,-β,-γ, tandis que les interférons stimulent l'expression des chimiokines ELR^- angiostatiques, comme IP-10/CXCL10, Mig/CXCL9 et I-TAC/CXCL11 (Miller et Krangel, 1992;. Cole et al, 1998).

En dépit de ces évidences, la « règle ELR » comporte quelques exceptions comme le stromal-derived factor-1 (SDF-1) / CXCL12 et GRO-β/CXCL2) CXCL2 (Martins-Green and Hanafusa 1997; Salcedo, Wasserman et al. 1999). De plus, le nombre de chimiokines régulatrices de l'angiogenèse augmente avec les membres des CC et CX3C chimiokines qui ne possèdent pas de motif ELR dans leur séquence. CCL2 (MCP-1) est le médiateur CC chimiokine de la néovascularisation le mieux décrit dans la littérature (Roy and Kolattukudy 2012). Enfin, plusieurs groupes ont récemment rapportés que les cellules endothéliales expriment des récepteurs de chimiokines fonctionnels montrant que CXCR2 et CXCR4 sont des récepteurs angiogéniques, tandis que CXCR3 est un récepteur angiostatique (Addison, Daniel et al. 2000; Romagnani, Annunziato et al. 2001).

1.5 Modèle animal de l'implantation embryonnaire : la souris

L'intérêt des modèles animaux réside dans la création de situations expérimentales non envisageables chez l'humain. En matière de reproduction, au-delà des différences majeures entre la souris et l'humain, certains points coïncident tels que l'intervention des mêmes hormones stéroïdiennes, l'implantation hémochoriale (Fig.4), une courte fenêtre de réceptivité, la décidualisation du stroma et l'agressivité du trophoblaste (Trévoux 2009).

C’est grâce à différentes études, menées sur des animaux ovariectomisés, que l’on a pu comprendre que le développement embryonnaire préimplantatoire complet nécessite des facteurs paracriniens venant du tractus reproducteur, sous l’influence des hormones stéroïdes (Psychoyos 1973).

1.5.1 Le modèle murin

1.5.1.1 Le système endocrinien

En moyenne, la durée du cycle œstral chez la souris est de 4-5 jours, mais il est très variable. Les souris ovulent spontanément au cours de chaque cycle œstral. Les femelles deviennent cyclées quand elles atteignent la puberté à l’âge de 4 semaines. Les différentes phases du cycle œstral chez les femelles adultes sont régies par l’axe hypothalamo-hypophyso-ovarien fonctionnel.

La maturité sexuelle coïncide avec la libération pulsatile de gonadolibérine (GnRH) de l'hypothalamus et à la hausse des niveaux de gonadotrophines circulantes, hormone folliculo-stimulante (FSH) et l'hormone lutéinisante (LH) par l'hypophyse. Si la hausse des taux de FSH déclenche la croissance folliculaire et la maturation, l'ovulation se produit sous l'influence des niveaux croissants de LH. Ces changements sont reflétés par la production dans l'ovaire des hormones oestrogéniques puis progestatives au cours de chaque cycle.

1.5.1.2 L’implantation embryonnaire

La gestation commence avec la fécondation de l'ovocyte par un spermatozoïde. La préparation utérine pour l'implantation requiert la présence de progestérone et d’oestradiol. En phase préovulatoire (proestrus), l’épithélium endométrial prolifère sous l’influence des oestrogènes, puis pendant la phase oestro-progestative

(metaoestrus, dieostrus) les cellules du stroma prolifèrent. Le stade oestrus représente une période où les femelles montrent des signes de comportement d'accouplement. Chez la souris, comme chez l'humain, la phase pendant laquelle l'utérus est réceptif à un embryon est nommée fenêtre d'implantation (Fig.11) (Paria, Huet-Hudson et al. 1993; Carson, Bagchi et al. 2000). Lors d'un cycle oestrien fécond, l'ouverture de la fenêtre d'implantation est consécutive à un pic d'oestradiol au quatrième jour après l'accouplement.

Le premier signe de contact entre le blastocyste et l'utérus peut être détecté expérimentalement par une injection intraveineuse de colorant bleu « Chicago Blue Sky » dès la fin de la journée 4 ou le début de la journée 5 de la gestation (Deb, Reese et al. 2006). L'adhésion du blastocyste à la muqueuse utérine conduit à la réaction déciduale du stroma seulement aux sites d'implantation. Le processus d'implantation peut être reporté et rétabli expérimentalement par la manipulation de la sécrétion d'œstrogènes (Hou, Paria et al. 1996). La décidualisation du stroma peut aussi être induite expérimentalement en réponse à des stimuli autres que l'embryon (Abrahamsohn and Zorn 1993).

Cependant, une bonne préparation de l'utérus par les stéroïdes sexuels est nécessaire à cet effet (Fig.11). L'utérus d'une femelle pseudogestante est un modèle de choix pour l'étude de la décidualisation.

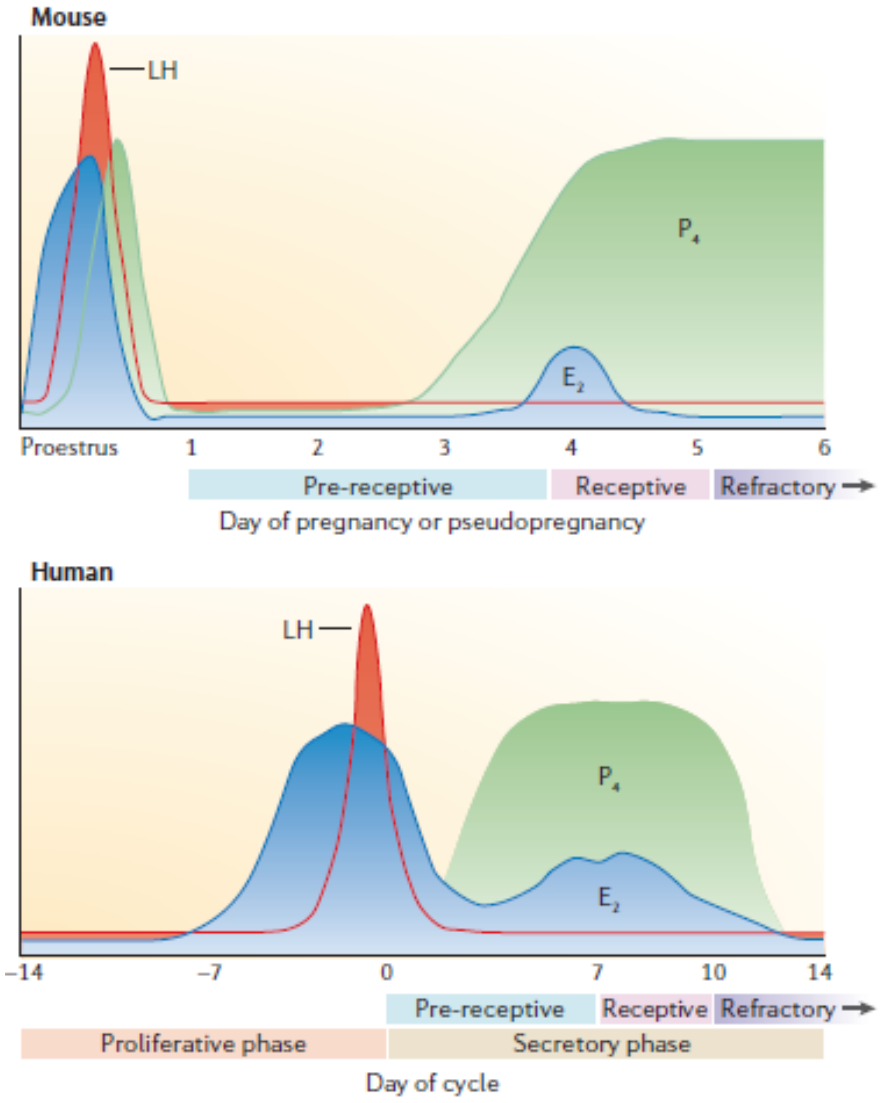

Figure 11 Réceptivité utérine chez la souris et l'humain. Adapté de (Wang and Dey 2006)

1.5.1.3 Développement embryonnaire précoce

À chaque cycle, les souris sont capables de libérer 8 à 10 ovules. Après l'ovulation, les œufs rentrent dans l'oviducte. Par l'accouplement, les spermatozoïdes voyagent à travers les deux cornes utérines pour atteindre le lieu de la fécondation.

La présence d'un bouchon vaginal le matin suivant la copulation avec un mâle indique un accouplement réussi. L'éjaculat du mâle va former un bouchon de courte durée, blanchâtre dans le tractus vaginal de la femelle. La présence d'un bouchon vaginal indique uniquement un accouplement réussi, mais ne signifie pas toujours que la gestation se produira à partir de cet accouplement.

Après un accouplement « réussi », la fécondation de l'ovule se produit dans l'ampoule (fin de l'ovaire). Les spermatozoïdes pénètrent dans les cellules du cumulus pour fertiliser les œufs. Habituellement, plus d'un spermatozoïde pénètre dans l'espace périvitellin. Toutefois, un seul spermatozoïde pénètre et féconde l'ovule. Dès lors, le zygote se divise par mitose pour finalement atteindre le stade de blastocyste. La stimulation de l'accouplement provoque la libération de prolactine par l'hypophyse, ce qui conduit à la formation d'un corps jaune fonctionnel dans l'ovaire et bloque la poursuite du cycle œstral.

La transformation du zygote en blastocyste se poursuit en quatre jours. Du stade zygote au stade morula, l'embryon migre le long de l'oviducte pour se retrouver au quatrième jour dans l'utérus et débuter sa nidation. L'attachement du blastocyste à l'épithélium endométrial reste superficiel entre le $4^{ème}$ et le $5^{ème}$ jour de gestation. À ce moment l'attachement est réversible, les embryons peuvent être collectés par lavage de l'utérus. L'attachement devient irréversible après le jour 5, lorsque les cellules trophoblastiques envahissent l'endomètre. Autour du blastocyste, un œdème apparaît et enrobe celui-ci. L'épithélium subit localement une apoptose qui

permet l'accès de l'embryon au stroma. La première vague d'invasion déclenche la réaction déciduale qui va s'étendre dans le stroma. Celle-ci est accompagnée d'une angiogenèse locale permettant l'infiltration des cellules immunitaires et de soutenir la croissance de l'embryon.

1.5 Enoncé de recherche

1.5.1 Objectifs

Au laboratoire, des travaux antérieurs ont mis en évidence que l'expression de l'IL1R1 et de l'IL1R2 est dépendante du cycle menstruel (Bigonnesse, Labelle et al. 2001; Boucher, Kharfi et al. 2001). Aussi, il a été démontré que l'IL1 pouvait exercer une régulation sur ses propres récepteurs dans un modèle *in vitro* de cellules endométriales (Bellehumeur, Blanchet et al. 2009). De plus, des travaux effectués avec un modèle de culture primaire de cellules épithéliales d'endomètre (considéré comme la première interface en contact avec l'embryon) ont démontré que la hCG pouvait déséquilibrer l'effet de l'IL1 en régulant négativement l'expression du récepteur IL1R2 (Herrmann-Lavoie, Rao et al. 2007). Par la suite la question était de savoir si des modifications dans le système IL1, présent à la surface des cellules de l'endomètre, pouvaient être affectées en fenêtre d'implantation par la présence de faibles doses d'hCG.

Comme, d'après la littérature, les cellules épithéliales, constituant une barrière, subissent une apoptose locale au lieu de l'adhésion, nous avons choisi d'étudier le compartiment stromal de l'endomètre. Devant la complexité des phénomènes autour de l'implantation embryonnaire, ainsi que les limites éthiques quant à l'exploration de la réceptivité utérine humaine, nous avons d'abord choisi d'étudier, en terme moléculaire, la relation entre la hCG et l'IL1, à faibles doses, sur des

cultures primaires d'endomètre, issues de volontaires saines et fertiles en phase mi-sécrétoire. Le but est « d'approcher au mieux » le comportement des cellules stromales (ESC), au cours de la fenêtre d'implantation. Ensuite, avec un modèle de lignée cellulaire endothéliale, nous avons évalué l'effet du couple hCG/IL1 sur le compartiment vasculaire. Le but est d'observer si la hCG et l'IL1 peuvent cibler différents types cellulaires. Enfin nous avons utilisé un modèle murin pour suivre l'expression du système IL1 dans les étapes précoces de la gestation. Le but est de vérifier si nos différentes observations *in vitro* corrèlent avec un modèle *in vivo*.

1.5.2 Projet 1

Hypothèse : la hCG cible les récepteurs de l'IL1.

Dans un premier temps, nous étudierons l'effet d'une faible dose d'hCG sur l'expression des récepteurs de l'IL1. Les principaux résultats montreront un déséquilibre dans le système IL1, issu d'une diminution des antagonistes naturels et d'une augmentation du récepteur fonctionnel.

1.5.3 Projet 2

Hypothèse : le déséquilibre dans les récepteurs de l'IL1 a un impact physiologique.

Consécutivement aux premiers résultats nous voulions savoir comment était modifiée la réponse des cibles de l'IL1β dans l'endomètre, suite au déséquilibre dans le système IL1. Sur les mêmes cultures qui ont servi à récolter les premiers résultats, nous avons mené une étude transcriptomique globale par « micro-array » en collaboration avec le service génomique du CHUL. Les résultats éloquents montrent que bien plus qu'un déséquilibre fonctionnel du système et de la famille IL1, la hCG agirait au travers de ce médiateur primordial.

1.5.4 Projet 3

Hypothèse : d'autres types cellulaires peuvent être touchés par le déséquilibre dans l'expression des récepteurs à l'IL1 causé par une exposition à la hCG.

Puis, notre curiosité nous à conduit à explorer les relations complexes de la hCG et de l'IL1 sur un autre type cellulaire présent dans l'endomètre et dont la participation est essentielle au processus implantatoire : les cellules endothéliales. Les travaux ont été menés sur une lignée endothéliale humaine (HMVEC). Comme avec les cellules stromales, nous avons mis en évidence un déséquilibre du système IL1 en présence de faibles concentrations d'hCG, pouvant favoriser l'angiogenèse.

1.5.5 Projet 4

Hypothèse : les changements d'expression des récepteurs de l'IL1 ont-ils lieux in vivo ?

Enfin nous voulions voir si l'étude de l'endomètre, en phase d'implantation, d'un modèle animal de souris gestante nous permettrait de corroborer les observations obtenues *in vitro*. Les résulats montreront un profile d'expression des récepteurs membres du système IL1 en concordance avec les différents phénomènes englobant l'implantation embryonnaire (réaction immunitaire au sperm, ouverture de lafenêtre d'implantation, et l'implantation des embryons), ainsi qu'une correlation positive entre l'expression de l'il1r1 et le mcp1 aux sites d'implamtations.

Nous espérons que nos travaux sur la réceptivité utérine, plus particulièrement sur le dialogue embryo-maternel, permettront d'améliorer la compréhension globale du phénomène unique de l'implantation embryonnaire.

Chapitre 2 : Human chorionic gonadotropin triggers angiogenesis via the modulation of endometrial stromal cell responsiveness to interleukin 1: a new possible mechanism underlying embryo implantation

Amélie Bourdiec[2], Rong Shao[3], C.V. Rao[4], and Ali Akoum[1,2]

1-2 Endocrinologie de la reproduction, Centre de recherche-Hôpital Saint-François d'Assise, Centre Hospitalier Universitaire de Québec, Faculté de médecine, Université Laval, Quebec, Canada

3Pioneer Valley Life Sciences Institute, University of Massachusetts Amherst, Springfield, Massachusetts

4Herbert Wertheim College of Medicine, Florida International University, Miami, Florida

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2.1 Résumé

Lors de l'implantation embryonnaire, de profonds changements fonctionnels, orchestrés par des signaux embryonnaires et maternels, se produisent dans l'endomètre. La gonadotrophine chorionique humaine (hCG), un signal embryonnaire majeur, joue un rôle essentiel dans l'initiation et le maintien de la grossesse. L'interleukine 1 (IL1), l'un des premiers signaux embryonnaires, semble exercer un impact direct sur l'endomètre réceptif et induire des changements moléculaires majeurs qui sont essentiel pour l'implantation de l'embryon. Ici nous examinons si la hCG peut moduler la réceptivité des cellules stromales de l'endomètre (ESC) à l'IL1 pendant la fenêtre d'implantation, et d'évaluer quel en sera l'impact sur la promotion *in vitro* de l'angiogenèse. Les cultures primaires d'ESC de femmes fertiles saines, prélevées lors de la fenêtre d'implantation, ont été traitées pendant 24h avec différentes concentrations d'hCG (0-100 ng/ml) puis stimulées pendant 24h avec l'IL1B (0-0.1 ng/ml). Les récepteurs de l'IL1 (IL1Rs), et la protéine chimiotactique des monocytes (MCP) 1, ont été analysés par PCR quantitative en temps réel, ELISA et Western blot. L'activité angiogenique a été étudiée *in vitro* avec la lignée endothéliale microvasculaire humaine (HMVEC), en utilisant le test de la cicatrice et en quantifiant la proliferation cellulaire *via* l'incorporation de BrdU dans l'ADN. Dans les cellules stromales, la hCG induit un déséquilibre dose-dépendant affectant la réceptivité à l'IL1 par une augmentation considérable du récepteur fonctionnel IL1R1, et régule à la baisse, de façon concomitante, les récepteurs leurres (IL1R2) et inhibiteurs (IL1RN) ulterieurement à la stimulation de l'IL1B. Une exposition préalable à la hCG amplifie la sécrétion de MCP1 par les ESC en réponse à l'IL1B. Le MCP1 semble jouer un rôle important dans la réponse pro angiogenique qui en résulte. En utilisant la transfection cellulaire, la surexpression de l'IL1R2 inhibe la sécrétion de MCP1 médiée par l'IL1B et hCG/IL1B.

Ces résultats suggèrent que l'interaction coordonnée de la hCG embryonnaire avec l'endomètre maternel décrit une nouvelle voie possible par laquelle ces facteurs peuvent promouvoir la croissance embryonnaire.

2.2 Abstract

Deep functional changes occurring within the endometrium during implantation are orchestrated by embryonic and maternal signals. Human chorionic gonadotropin (hCG), a major embryonic signal, plays a critical role in the initiation and maintenance of pregnancy. Interleukin (IL) 1, one of the earliest embryonic signals, appears to exert a direct impact on the receptive endometrium and to induce major molecular changes that are essential for embryo implantation. Herein we investigate whether hCG can modulate endometrial stromal cell (ESC) receptivity to IL1 during the implantation window and assess the impact on angiogenesis in vitro. Primary cultures of ESCs from normal fertile women during the implantation window were treated for 24 h with different concentrations of hCG (0–100 ng/ml) and stimulated for 24 h with IL1B (0–0.1 ng/ml). IL1 receptors (IL1Rs), IL1R antagonist (IL1RN), and monocyte chemotactic protein (MCP) 1 were analyzed by real-time PCR, ELISA, and Western blotting. The angiogenic activity in vitro was studied using human microvascular endothelial cell line, scratch wound assay, and cell proliferation via BrdU incorporation into DNA. Human CG induced a dose-dependent imbalance in ESC receptivity to IL1 by significantly upregulating the functional signaling IL1R1 and concomitantly downregulating the decoy inhibitory IL1R2 and IL1RN upon subsequent exposure to IL1B. Prior exposure to hCG amplified MCP1 secretion by ESCs in response to IL1B and triggered the release of angiogenic activity in vitro in which MCP1 appeared to play a significant role. Overexpression of IL1R2 using cell transfection inhibited IL1 and hCG/IL1B-mediated MCP1 secretion. These findings suggest that hCG coordinates embryonic signal interaction with the maternal

endometrium, and point to a new possible pathway by which it may promote embryonic growth.

2.3 Introduction

Successful blastocyst implantation requires precise synchronization between the embryo and the uterine environment. The endometrium is a specialized, hormonally regulated organ that does not adhere to embryos throughout most of the menstrual cycle in human as well as other mammals [1].

The endometrium acquires a receptive phenotype to the embryo during a special, limited period of the menstrual cycle called the “implantation window,” through specific structural and functional changes allowing blastocyst adhesion. Estradiol induces endometrial tissue proliferation, which is followed by progesterone-induced differentiation and the establishment of an implantation window [2]. This appears to occur between Days 19 and 24 of the menstrual cycle in humans. The dynamics of this transition from a nonreceptive to a receptive endometrium are poorly understood, but the correct spatiotemporal synthesis and balance of various factors regulated by steroid hormones is thought to play an important role in uterine preparation for implantation [3–7].

During this crucial period, numerous cytokines and growth factors are produced by both the preimplanted embryo and the uterine microenvironment [8]. In this intricate network of interactions involved in embryo implantation, cytokines and other hormonal factors act as mediators for maternal-fetal cross-talk. In this study, we addressed the interaction between two early embryonic signals: human chorionic gonadotropin (hCG) and interleukin (IL) 1.

Human CG is a major embryonic signal that plays a critical role in the initiation and maintenance of pregnancy [9]. Human CG acts on the intrauterine environment via the luteinizing hormone (LH)/hCG receptor (hLHCGR). Human endometrium, the natural host site where the embryo implants and develops, has been shown to contain functional membrane-bound hLHCGRs (mb-hLHCGRs), which have been detected in glandular and luminal epithelial cells, as well as in stromal cells [10, 11]. At the time of implantation, hCG has been shown to be involved in a wide spectrum of cell targets and biological actions. Synthesized early by the trophoblast, hCG appeared to influence endometrial receptivity and implantation [9], promote the decidualization of human endometrial stromal cells (ESCs) [12], and to possess both direct and indirect angiogenic properties [13–15].

IL1 is one of the earliest embryonic signals. Human embryos cultured in vitro produce high concentrations of IL1A and IL1B, and the presence of these cytokines has been correlated with successful implantation after transfer to the uterine cavity [16]. The IL1 system is composed of two agonists (IL1A and IL1B), one inhibitor (i.e., the IL1 receptor [IL1R] antagonist [IL1RN]), and two receptors (IL1R1 and IL1R2). IL1B is the circulating form of IL1, and IL1A is the membrane-bound form. Both IL1B and IL1A activate IL1R1 and exert similar effects, but IL1B has more affinity for IL1R2 [17]. IL1R1 acts as functional receptor and can transduce a signal upon IL1 binding, with the recruitment of a second subunit, termed IL1 accessory protein (IL1 RACP or IL1R3) [18]. IL1R2 rather acts as a negative regulator (or antagonist) of IL1 action and has been termed a “decoy receptor” [19]. Expression of functional IL1R1 has been detected throughout endometrial tissue, but is only moderately upregulated during the luteal phase [20]. Expression of the decoy IL1R2 follows a regulated cycle phase-dependent pattern. Thus, IL1R2 first increases in the midproliferative phase, decreases at the time of implantation, and then increases again in the late secretory phase [21]. Secretion of embryonic IL1B in response to the receptive endometrium

might induce molecular changes that are essential for embryonic implantation. Our first studies had revealed that hCG downregulates the expression of the decoy antagonist IL1R2 in endometrial epithelial cells without affecting the expression of the functional activating receptor type I (IL1R1) [22]. This points to a possible mechanism by which hCG could modulate endometrial receptivity to IL1B at the time of implantation.

The objective of the present study was to investigate whether hCG can target human ESCs during the implantation window to modulate cell responsiveness to IL1, and assess the possible impact on angiogenesis in vitro. Herein we show that hCG creates a significant imbalance in IL1Rs expression, amplifies cell responsiveness to ILB, and leads to a significant increase in monocyte chemotactic protein (MCP)-1 secretion and the release of angiogenic activity in vitro in which MCP1 appears to play a significant role. In view of the role of IL1 as a major and early embryonic signal, the present data point to a potentially novel mechanism by which hCG sustains human pregnancy and promotes embryonic growth.

2.4 Materials and methods

2.4.1 Subjects and Tissue Handling

Endometrial tissue specimens were obtained during the implantation window (Days 19–24) from normal fertile women with a regular menstrual cycle, who were undergoing laparoscopy for tubal ligation and had not received hormonal or anti-inflammatory therapy for at least 3 mo prior to surgery (mean age ± SD, 35.6 ± 4.9 yr; n = 7). Menstrual cycle day was determined according to the histological criteria of Noyes et al. [23]. Written informed consent was obtained from participants under a study protocol approved by the Ethics Committee on Human Research at Laval

University, Quebec, Canada. Endometrial tissue was immediately placed in cold, sterile Hanks balanced salt solution (HBSS) (Invitrogen Life Technologies, Burlington, ON, Canada) containing 1% antibiotics, then directly transported to the laboratory. A part of the biopsy was taken for cell culture.

2.4.2 Cell Culture and Treatment

Biopsies used in this study were devoid of any visible blood contamination. Tissues were immediately washed with cold HBSS and ESCs were isolated and cultured at 37°C, 5% CO2, in Dulbecco modified Eagle medium (DMEM):F12 (1:1) containing 10% charcoal-treated fetal bovine serum (FBS), insulin, transferrin, and a mix of antibiotics-antimycotics according to our previously described procedure [24]. ESCs prepared using this protocol were vimentin positive and found to be free of cytokeratin-positive epithelial cells, von Willbrand factor-positive endothelial cells, or CD45-positive leukocytes [24], and used between passages 1 and 3. At preconfluence, cells were incubated overnight with phenol and FBS-free medium and treated with hCG (0–1000 ng/ml; Sigma-Aldrich Co., St. Louis, MO) for 24 h. Cells were then stimulated with IL1B (0–1 ng/ml; R&D Systems, Minneapolis, MN) for 24 h and culture supernatants were recovered, centrifuged to eliminate cell debris, and divided into small aliquots; cells were trypsinized and collected and both supernatants, and cells were stored at −80°C until further use.

2.4.3 Real-Time PCR

Total RNA was extracted with TRIzol (Invitrogen), reverse transcribed, and analyzed by quantitative real-time RT-PCR (qRT-PCR), according to a previously reported procedure [25]. Following a 95°C denaturation for 2 min, the reactions were cycled 40 times with denaturation at 95°C for 15 sec and annealing at 60°C for 60 sec. IL1R1 primers (forward, 5’-AGAGGAAAACAAACCCACAAGG-3’; reverse, 5’-

CTGGCCGGTGACATTACAGAT-3′; amplimer size, 106 bp), IL1R2 primers (forward, 5′-TGGCACCTACGTCTGCACTACT-3′; reverse, 5′-TTGCGGGTATGAGATGAACG-3′; amplimer size, 112 bp), MCP1 primers (forward, 5′-CTCTGCCGCCCTTCTGT-3′, reverse, 5′-CTTCTTTGGGACACTTGCTG-3′, amplimer size, 109 bp), and GAPDH (forward, 5′-CAGGGCTGCTTTTAACTCTGG-3′; reverse, 5′-TGGGTGGAATCATATTGGAACA; amplimer size, 102 bp) were designed with Primer Express version 2.0 (Applied Biosystems), spanned intron-exon boundaries to avoid amplification of genomic DNA, and were selected for melting temperature (Tm) value compatibility (57–61°C). For each experimental sample, IL1R1, IL1R2, and MCP1 mRNA levels were normalized to GAPDH mRNA levels. Quantification of IL1R1, IL1R2, and IL1RN mRNA was performed using a relative quantification method. After each run, melting curve analysis (55–95°C) was performed to verify the specificity of the PCR. All samples were tested in duplicate, each run including a no-reverse transcription control.

2.4.4 Western Blot Analysis

Proteins were extracted from cultured cells in a buffer containing 0.5% Triton-X100, 10 mM Hepes (pH 7.4), 150 mM NaCl, 2 mM ethylene glycol tetraacetic acid, 2 mM ethylene diamine tetraacetic acid, 0.05% NaN3, and a mixture of antiproteases, composed of 5 µM aprotinin, 63 µM leupeptin, and 3 mM phenylmethylsulfonylfluoride. Cell homogenate was then incubated at 4°C for 45 min under gentle shaking and centrifuged at 11000 × g for 30 min to recover the soluble extract. Total protein concentration was determined using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Mississauga, ON, Canada). Protein extracts (10 µg) were then separated by SDS-PAGE, transferred onto 0.45-µm nitrocellulose membranes, and analyzed by Western blotting, as described previously [26, 27]. Briefly, IL1R1 and IL1R2 were detected using specific goat polyclonal antibodies

(1:100 dilution in PBS containing 5% skim milk and 0.1% Tween 20 [blocking solution]; R&D systems), while IL1RN was detected using a specific rabbit polyclonal antibody (Genzyme). Fc-specific peroxidase-labeled rabbit anti-goat antibody (1:10000 in blocking solution; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) was then used for IL1R1 and IL1R2, whereas an Fc-specific peroxidase-labeled goat anti-rabbit antibody (1:150000 in blocking solution; Jackson ImmunoResearch Laboratories) was used for IL1RN. ECL reagent (GE Healthcare, Chalfont St. Giles, UK) and exposure to Super-RX films (Fuji, Tokyo, Japan) for 5–30 min for optimal detection (all bands visible, but not overexposed). Controls included incubation with equivalent concentrations of normal goat (IL1R1 and IL1R2) and rabbit (IL1RN) IgGs. Membranes were stripped and reblotted with a monoclonal antibody specific to α-actin (1:50000 dilution in PBS-0.01% Tween-20; Sigma-Aldrich Co.) used as internal control for protein loading and transfer.

2.4.5 Cell Transfection

Cells were seeded into 24-well plates and grown until 70% confluence. Cells were then transfected with the eukaryotic expression vector pcDNA3 either alone or containing a cDNA coding for IL1R2 [28]. Transfection was performed using Lipofectamine Plus reagent according to manufacturer's instructions (Life Technologies Inc.) and our previously described procedure [26]. Cells were exposed for 24 h to the minimal culture medium (MM) alone or containing hCG (100 ng/ml), IL1B (0.1 ng/ml) or hCG/IL1B (100/0.1 ng/ml). The culture supernatants were then collected and kept in small aliquots at −80°C until use by ELISA.

2.4.6 Enzyme-Linked Immunosorbent Assay

MCP1, soluble (s) IL1R2, and IL1RN concentrations in the culture medium were measured using previously reported sandwich ELISAs [27, 29].

2.4.7 Scratch Wound Assay

In this study, we used a human microvascular endothelial cell (HMVEC) line. This line of immortalizing primary HMVECs was generated by engineering the human telomerase catalytic protein. Cells were shown to maintain inherent features of primary endothelial cells and to produce angiogenic response [30]. Cells were seeded into 24-well plates and grown to confluence in MCDB 131 medium. Cell monolayers were carefully wounded with a 200-µl pipette tip to generate a cut of ~1 mm in width. After two washing steps, cells were incubated for 24 h under various positive control conditions (VEGF and FGF [R&D Systems], or MCP1 [Prospec, East Brunswick, NJ]) or ESC-conditioned medium (1:3 dilution in FBS-free DMEM-F12). MCP1 was neutralized in culture using a neutralizing MCP1 antibody (5 µg/ml; MAB 279; R&D Systems). Cells were then fixed with 10% formalin for 20 min, stained with 4',6-diamidino-2-phenylindole, and wound healing was determined by measuring scratch width at seven random locations along the wound to examine the extent of closure using fluorescence microscopy. Measures were performed in duplicate and repeated three times.

2.4.8 DNA Synthesis Assay by BrdU Incorporation

HMVECs were seeded in 96-well microtiter plates (1 × 104 cells/well), cultured overnight, starved for 48 h in DMEM-F12 containing 0.2% FBS, and incubated for 48 h with ESC-conditioned media from cultures treated with hCG (100 ng/ml), IL1 (0.1 ng/ml), and hCG (100 ng/ml)/IL1 (0.1 ng/ml), equivalent concentrations of hCG, IL1, and hCG/IL1 similarly diluted in MM, MCP1 (250 and 350 ng/ml, which correspond to the mean levels of MCP1 found in the culture medium following IL1 or hCG/IL1 stimulation), VEGF (25 and 50 ng/ml), and FGF (25 and 50 ng/ml) (R&D Systems). For inhibitory assays, MCP1 was neutralized in culture with MCP1 antibody (5 µg/ml).

After 24 h, cells were labeled with 10 µM BrdU for 24 h at 37°C, and cell proliferation was assessed by ELISA according to the manufacturer's instructions (GE Healthcare, Mississauga, ON, Canada). Briefly, cells were fixed and genomic DNA was denatured by adding 200 µl/well of blocking reagent for 30 min at room temperature. Peroxidase-labeled anti-BrdU antibody was then added (100 µl/well) and incubated for 90 min at room temperature. Wells were washed three times, 3,3',5,5'-tetramethylbenzidine substrate was added (100 µl/well) and incubated for 15 min at room temperature, and the optical density was measured at 450 nm.

2.4.9 Statistical Analysis

Three experiments were carried out for each result shown, and all measurements were performed in duplicate. Data followed a parametric distribution, and were expressed as means ± SEM. The significance of statistical differences was determined using one-way ANOVA followed by the Boferroni test post hoc for multiple analyses, and the Student t-test for comparing two groups. All analyses were performed with GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA).

2.5 Results

2.5.1 Human CG Regulates Expression of IL1Rs in ESCs

Cell cultures were exposed to different concentrations of hCG (0–100 ng/ml) for 24 h before being stimulated for 24 h with IL1B (0-0.1 ng/ml). As shown in Figure 1, alone, each stimulus induced small and statistically nonsignificant variations of IL1R1 mRNA expression (Fig. 1A). However, a pretreatment with hCG followed by IL1B (0.1 ng/ml) resulted in an induction of IL1R1 mRNA expression, which was statistically significant for 10 ($P < 0.05$) and 100 ($P < 0.001$) ng/ml hCG. Furthermore, as shown in Figure 1B, IL1B (0.1 ng/ml) significantly upregulated IL1R2 mRNA expression in ESCs

($P < 0.001$), but pretreatment of cells with hCG (10 and 100 ng/ml) resulted in a significant downregulation of the IL1B-induced IL1R2 mRNA expression ($P < 0.05$ and $P < 0.01$, respectively). Furthermore, IL1B (0.1 ng/ml) significantly upregulated IL1RN mRNA expression in ESCs ($P < 0.05$). However, cell pretreatment with hCG (10 and 100 ng/ml) significantly downregulated the IL1B-induced IL1RN mRNA expression ($P < 0.05$; Fig. 1C).

Western blot analysis of IL1R1 showed that treatment of ESCs with IL1B (1 ng/ml) increased the intensity of membrane (mb) and soluble (s) IL1R2 bands (68 and 45 kDa) and that of IL1RN (17k Da). However, cell pretreatment with hCG (100 ng/ml) decreased the IL1B-indued IL1R2 and IL1R2 expression, but appeared to increase the expression of mb and sIL1R1 (Fig. 2A). Naturally occurring sIL1Rs are released following natural proteolytic cleavage and shedding from the extracellular domain of mbIL1R's [31–33]. Soluble IL1R1 was not detectable in ESC culture supernatants. Conversely, sIL1R2 was measurable in the culture medium and found to be increased by IL1B (0.1 ng/ml) ($P < 0.001$), but decreased in cells pretreated with hCG (100 ng/ml) prior to ILB exposure ($P < 0.001$) (Fig. 2B). Measurement of IL1RN showed comparable effects of IL1B which increased the antagonist concentration in the culture medium ($P < 0.05$), while prior treatment with hCG significantly decreased this IL1B-mediated IL1RN secretion ($P < 0.05$) (Fig. 2C).

2.5.2 ESCs Secrete High Concentrations of MCP1 in Response to Embryonic Stimuli

The data presented above show that hCG regulates the expression of IL1Rs 1 and 2 in an opposite, but functionally complementary, manner, and suggest that hCG amplifies the cellular response to this cytokine. In order to investigate this, we first assessed its impact on the synthesis and secretion levels of MCP1, one of the known

IL1 signaling targets [34]. This chemokine is responsible for monocyte trafficking and activation, and has been recognized as a potent tissue remodeling and angiogenic factor [35]. Quantitative real-time PCR analysis showed a dose-dependent upregulation of MCP1 mRNA steady-state levels in response to IL1B (Fig. 3A). Human CG had no significant effect on MCP1 mRNA. However, ESCs pretreated with 10 and 100 ng/ml hCG showed a significant stimulation of MCP1 mRNA expression in response to 0.01 ($P < 0.01$ and $P < 0.001$, respectively) and 0.10 ($P < 0.001$ and $P < 0.001$, respectively) ng/ml IL1B as compared with cells incubated with the culture medium alone, or with cells exposed to 0.01 ($P < 0.05$ and $P < 0.01$, respectively) and 0.1 ($P < 0.05$) ng/ml ILB without any prior hCG treatment. This suggests an hCG-mediated enhancement of ESC activation by IL1B (Fig. 3A). Furthermore, analysis of MCP1 secretion in the culture medium showed a significant dose-dependent increase in response to IL1B (Fig. 3B). Human CG alone had no significant effect on MCP1 secretion by ESCs. However, treatment with hCG followed by incubation with IL1B enhanced MCP1 secretion by ESCs. In fact, cell pretreatment with 10 and 100 ng/ml hCG significantly increased MCP1 concentration in the conditioned medium in response to 0.01 ($P < 0.01$) and 0.10 ($P < 0.001$ and $P < 0.01$, respectively) ng/ml IL1B as compared with the culture medium, or to 0.01 ($P < 0.01$) and 0.1 ($P < 0.01$) ng/ml IL1B without any prior hCG treatment (Fig. 3B).

Determination of the ratio of IL1R2:IL1R1 mRNA expression in these cells showed that it increased in cells exposed to 0.01 and 0.1 ng/ml IL1B as compared with untreated cells ($P < 0.05$ and $P < 0.01$, respectively), but decreased in cells pretreated for 24 h with 10 ($P < 0.05$ and $P < 0.01$, respectively) and 100 ($P < 0.05$ and $P < 0.001$, respectively) ng/ml of hCG. Interestingly, the diminution of the IL1R2:IL1R1 ratio occurred in parallel with the increase in MCP1, either in cells exposed to hCG alone or in cells exposed to hCG prior to IL1B stimulation (Fig. 3C). However, coincubation of ESCs with hCG and IL1B for 24 h without prior

pretreatment with hCG significantly reduced the IL1R2:IL1R1 ratio and increased MCP1 mRNA, but did not result in any significant increase in MCP1 protein secretion, suggesting that the hCG-mediated imbalance in IL1Rs occurs prior to the augmentation of MCP1 secretion (data not shown). ESCs were then assessed for MCP1 secretion in response to hCG, ILB, or hCG/ILB following transfection with the pcDNA3 expression vector alone (control) or containing IL1R2 cDNA. Cells transfected with the control expression vector showed a significant increase in MCP1 secretion in response to IL1B (0.1 ng/ml) and hCG (100 ng/ml)/ILB (0.1 ng/ml) ($P < 0.05$ and $P < 0.001$, respectively) as compared with the control medium. Furthermore, MCP1 secretion was significantly increased in cells stimulated with hCG/ILB compared with cells incubated with an equivalent concentration of IL1B ($P < 0.01$). However, cell transfection with IL1R2 cDNA dampened these effects and resulted in a significant decrease of hCG/IL1B-induced MCP1 production ($P < 0.001$; Fig. 4A). It is noteworthy that sIL1R2 was noticeably upregulated in cells that were transfected with IL1R2 cDNA compared with pcDNA3 vector-transfected cells (Fig. 4B).

2.5.3 MCP1 and Angiogenic Effects In Vitro of ESC-Conditioned Media

In view of the significant increase in the IL1-induced MCP1 secretion caused by priming ESCs with hCG, we next addressed the hypothesis that medium conditioned by ESCs upon such exposure to these two embryonic signals might possess angiogenic activity. We thus examined the potential of conditioned ESC medium to induce angiogenic activity in vitro using the scratch wound assay. As shown in Figure 5A, conditioned media from hCG-treated ESC cultures significantly induced HMVEC scratch closure ($P < 0.05$). Moreover, when HMVECs were exposed to medium conditioned by ESCs sequentially stimulated with hCG (100 ng/ml), then with 0.01 or 0.1 ng/ml IL1B, a significant dose-dependent closure of the scratch was observed

compared with the culture medium ($P < 0.01$), hCG ($P < 0.05$), or IL1B ($P < 0.01$ and $P < 0.05$, respectively). Meanwhile, no significant effect of controls (hCG, IL1B, or hCG/ILB used at equivalent concentrations) on HMVEC scratch closure was noted (Fig. 5B).

To evaluate whether MCP1 detected in media conditioned by ESCs exhibit angiogenic activities in vitro, we used a blocking monoclonal antibody (MAB-279) (5 µg/ml) against human MCP1. Medium conditioned by ESCs sequentially stimulated with hCG and IL1B was incubated with the MCP1 antibody for 1 h at room temperature before being added to endothelial cell cultures for 24 h. HMVECs treated with recombinant human (rh) MCP1 (250 ng/ml), FGF (250 ng/ml), or VEGF (250 ng/ml) as positive controls showed a significant closure of the scratch ($P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively) (Fig. 6, A and B). Further analysis showed a significant decrease in wound closure when rhMCP1 ($P < 0.05$) or hCG/IL1B-treated cell conditioned media were pre-incubated with anti-MCP1 antibody ($P < 0.001$ and $P < 0.01$). Importantly, the IgG control had no significant effect on scratch closure (Fig. 6, C and D). These data demonstrate that HMVECs migrate in response to rhMCP1, as well as to MCP1 secreted by ESCs in response to hCG and IL1B.

One essential component of angiogenesis is endothelial cell proliferation. We thus performed BrdU incorporation assays under conditions similar to the scratch tests reported above. Like positive controls (i.e., direct addition of rhMCP1 [250 and 350 ng/ml], FGF [25 and 50 ng/ml], or VEGF [25 and 50 ng/ml]), conditioned media from ESCs treated with 100 ng/ml hCG, 0.1 ng/ml IL1B, or a combination of these two factors induced a significant increase in BrdU incorporation into endothelial cell DNA (Fig. 7A), whereas hCG, IL1B, and hCG/ILB used at equivalent concentrations had no significant effect (Fig. 7B). Furthermore, MAB-279 inhibited the conditioned medium-induced BrdU incorporation into HMVEC DNA (Fig. 7C). The latter two tests

provide support for the hypothesis that hCG and IL1B may exert a combined stimulatory effect on angiogenesis, as observed in vitro using endothelial cell migration and proliferation and schematized in Figure 8.

2.6 Discussion

Similarly to other key embryonic signals, such as hCG, IL1 triggers a cascade of intricate events at the fetomaternal interface, which facilitate embryonic implantation and growth within endometrial host tissue [9, 22]. Accordingly, it is not surprising to see elaborate mechanisms operating locally at the level of the cytokine's targets, which tightly regulate and counter-regulate the biological effects of IL1 in order to ensure normal reproductive functions.

In the present study, we showed that hCG is able to modulate IL1B receptivity in primary ESCs during the implantation window. Curiously, hCG alone had no significant effect on IL1R1 or IL1R2 expression. However, it appeared to counterbalance the IL1-induced increase in IL1R2 and IL1RN and further enhance the IL1-induced increase in IL1R1, thereby decreasing the IL1R2:IL1R1 ratio. IL1R1 is a signaling functional receptor, whereas IL1R2, which has no signaling properties, rather acts as a decoy receptor that captures IL1 and prevents its effects on target cells [19]. Therefore, these opposite, but functionally complementary, effects of hCG on the expression of IL1Rs create an imbalance in ESC receptivity to IL1, which may favor cell responsiveness and amplify IL1-induced cell activation.

It is noteworthy that hCG per se had a significant downregulatory effect on ILR2 expression in endometrial epithelial cells [22]. The reasons for such an endometrial cell type-dependent difference in hCG action remains unknown, but it is of interest that, according to our previous studies [21], endometrial epithelial cells display a

higher expression level of ILR2 than stromal cells which may explain the detectable effect of hCG on these cells.

In the present study, we assessed the possible impact of hCG-mediated changes in the expression of IL1Rs in ESCs on the release of MCP1. Our data showed a significant hCG-mediated amplification of ESC responsiveness to IL1B and an upregulation of MCP1 transcripts. In order to determine whether this may also occur at the protein level, analysis of ESC supernatant showed that prior treatment with hCG leads to a significant augmentation of the IL1B-induced MCP1 protein secretion as well. These observations are clearly consistent with the changes in ESC receptivity to IL1B that follow hCG treatment [36]. In addition, the observation of a substantial increase in MCP1 secretion (2000% to 4500% of control) under regulation by hCG and IL1B may be particularly interesting in view of previous studies finding MCP1 in the category of genes that become upregulated in ESCs upon exposure to embryonic stimuli [37, 38] and the biological properties of this factor. MCP1 was named for its capability to chemoattract monocytes, but is also known for its potent growth-promoting and angiogenic properties [36, 39]. MCP1 has been implicated in angiogenesis as a direct as well as indirect proangiogenic factor. Indirectly, MCP1 acts on various cell types in inducing the secretion of proangiogenic molecules [40]. MCP1 can also act directly on endothelial cells to induce angiogenesis [36]. However, the mechanism by which MCP1 mediates these effects on angiogenesis is poorly understood. The recently identified MCP1-induced protein enhances endothelial cell proliferation, migration, and expression of angiogenesis-related genes, resulting in capillary-like tube formation. The role of MCP1 in monocyte/macrophage recruitment and activation is of relevance as well for embryonic implantation and growth. Actually, macrophages were shown to contribute to decidualization and implantation, and to remain present at high levels at the implantation site throughout pregnancy [41, 42]. Instead of impairing the

growth of the semiallogeneic embryo, uterine macrophages rather appeared to maintain immune tolerance toward trophoblastic antigens, favor embryonic cell proliferation, mediate the extent of trophoblast invasion, and play a protective role against possible infections. Interestingly, the available literature suggests that trophoblastic cells regulate monocyte migration and differentiation to create an adequate environment to promote trophoblast growth and survival and modulate monocyte response to bacterial stimuli. This strengthens the relevance of our findings and broadens the spectrum of the impact of hCG on early embryonic growth and development.

The available literature supports a role for IL1 in embryo implantation. Simon et al. [43] demonstrated that IL1R1 mRNA is upregulated by its ligand IL1B in ESCs isolated during the luteal phase, and suggested a role for the IL1 system in human implantation. Using a mouse model, these authors further showed that blastocysts did not attach or implant in IL1RN-treated animals, suggesting that blockade of IL1R1 interferes with the attachment of mouse blastocysts to maternal endometrium [44]. Furthermore, IL1RN appeared to act on the endometrial epithelium by inhibiting epithelial plasma membrane transformation at the time of implantation, presumably via transcriptional downregulation of α4 and posttranscriptional decrease of αv and β3 integrin subunits [45].

To characterize the angiogenic activity in vitro of conditioned media, we used HMVECs as a model [30]. The line was immortalized by engineering the human telomerase catalytic protein and found to generate a prominent angiogenic response in vitro [46]. Using the in vitro scratch wound assay, our data showed a significant stimulatory effect of hCG and IL1B on the capability of ESC-conditioned media to favor HMVEC scratch wound healing and a significant involvement of MCP1 secreted by ESCs. Assessment of HMVEC proliferation via the incorporation of BRdU

into cell DNA revealed a similar action. This strongly suggests that the modulation of endometrial cell receptivity to ILB in response to hCG may translate into increased cell responsiveness to IL1B and potentiate the secretion of angiogenic factors. Nevertheless, factors other than MCP1 may contribute to the detected angiogenic activity, as the reduction of BrdU incorporation by MCP1 antibody was partial and the closure was significantly induced by supernatant from stromal cells solely treated by hCG, although MCP1 was not induced by hCG alone. Investigations are underway in order to identify the other possible angiogenic factors that mediate hCG, IL1B, and/or hCG/IL1B effects.

Because IL1R1 signal transduction requires IL1 accessory protein (IL1R3), future studies will also evaluate if IL1R3 is regulated by hCG in ESCs at time of implantation. Furthermore, it will be interesting to address the identification of other potential targets of hCG and IL1 involved in embryonic growth and development, and ultimately study the impact of possible related defects on infertility. Some infertility cases may result from diminished uterine receptivity or delayed implantation [47]. On the other hand, the incidence of pregnancy loss after implantation is high, estimated at 25%–40% [48]. Although many losses involve genetic abnormalities, there is often no known cause [49]. Hormonal factors, leukemia-inhibiting factor, and prostanoid pathways play an important role in successful implantation. However, given the complexity of early development, it is likely that many other mechanisms are also involved, and a better understanding of the mechanisms responsible for implantation is crucial.

In conclusion, this study reports, for the first time, a close interaction between IL1B and hCG in signaling to uterine stromal cells in vitro. Human CG appeared to amplify ESC receptivity/responsiveness to IL1B and to enhance (alter expression of IL1Rs and induce) the expression and release of MCP1, which is shown to have effects on the

motility and proliferation of HMVECs. This is relevant to physiology of embryo implantation, as IL1B and hCG are both produced by embryos, and angiogenesis is an early uterine response to embryo implantation.

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2.8 Footnotes

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2.9 References

1. Psychoyos A. Uterine receptivity for nidation. Ann N Y Acad Sci 1986;476:36–42.

2. Wilcox AJ, Baird DD, Weinberg CR. Time of implantation of theconceptus and loss of pregnancy. N Engl J Med 1999; 340:1796–1799.

3. Navot D, Scott RT, Droesch K, Veeck LL, Liu HC, Rosenwaks Z. Thewindow of embryo transfer and the efficiency of human conception invitro. Fertil Steril 1991; 55:114–118.

4. Develioglu OH, Hsiu JG, Nikas G, Toner JP, Oehninger S, Jones HW Jr.Endometrial estrogen and progesterone receptor and pinopode expressionin stimulated cycles of oocyte donors. Fertil Steril 1999; 71:1040–1047.

5. Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA,Yoshinaga K. Embryo implantation. Dev Biol 2000; 223:217–237.

6. Lim H, Song H, Paria BC, Reese J, Das SK, Dey SK. Molecules inblastocyst implantation: uterine and embryonic perspectives. Vitam Horm2002; 64:43–76.

7. Giudice LC. Elucidating endometrial function in the post-genomic era.Hum Reprod Update 2003; 9:223–235.

8. Castro-Rendon WA, Castro-Alvarez JF, Guzman-Martinez C, Bueno-Sanchez JC. Blastocyst-endometrium interaction: intertwining a cytokinenetwork. Braz J Med Biol Res 2006; 39:1373–1385.

9. Filicori M, Fazleabas AT, Huhtaniemi I, Licht P, Rao Ch V, Tesarik J,Zygmunt M. Novel concepts of human chorionic gonadotropin:reproductive system interactions and potential in the management ofinfertility. Fertil Steril 2005; 84:275–284.

10. Bernardini L, Moretti-Rojas I, Brush M, Rojas FJ, Balmaceda JP. Status ofhCG/LH receptor and G proteins in human endometrium during artificialcycles of hormone replacement therapy. J Soc Gynecol Investig 1995; 2:630–635.

11. Reshef E, Lei ZM, Rao CV, Pridham DD, Chegini N, Luborsky JL. Thepresence of gonadotropin receptors in nonpregnant human uterus, humanplacenta, fetal membranes, and decidua. J Clin Endocrinol Metab 1990;70:421–430.

12. Tang B, Gurpide E. Direct effect of gonadotropins on decidualization ofhuman endometrial stroma cells. J Steroid Biochem Mol Biol 1993; 47:115–121.

13. Zygmunt M, Herr F, Keller-Schoenwetter S, Kunzi-Rapp K, Munstedt K,Rao CV, Lang U, Preissner KT. Characterization of human chorionicgonadotropin as a novel angiogenic factor. J Clin Endocrinol Metab 2002;87:5290–5296.

14. Zygmunt M, Herr F, Munstedt K, Lang U, Liang OD. Angiogenesis and vasculogenesis in pregnancy. Eur J Obstet Gynecol Reprod Biol 2003; 110(Suppl 1):S10–S18.

15. Islami D, Bischof P, Chardonnens D. Modulation of placental vascular endothelial growth factor by leptin and hCG. Mol Hum Reprod 2003; 9: 395–398.

16. Sheth KV, Roca GL, al-Sedairy ST, Parhar RS, Hamilton CJ, al-Abdul Jabbar F. Prediction of successful embryo implantation by measuring interleukin-1-alpha and immunosuppressive factor(s) in preimplantation embryo culture fluid. Fertil Steril 1991; 55:952–957.

17. Dinarello CA. Interleukin-1 and its biologically related cytokines. Adv Immunol 1989; 44:153–205.

18. Greenfeder SA, Nunes P, Kwee L, Labow M, Chizzonite RA, Ju G. Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. J Biol Chem 1995; 270:13757–13765.

19. Colotta F, Re F, Muzio M, Bertini R, Polentarutti N, Sironi M, Giri JG, Dower SK, Sims JE, Mantovani A. Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. Science 1993; 261:472–475.

20. Bigonnesse F, Labelle Y, Akoum A. Triphasic expression of interleukin-1 receptor type I in human endometrium throughout the menstrual cycle of fertile women and women with unexplained infertility. Fertil Steril 2001; 75:79–87.

21. Boucher A, Kharfi A, Al-Akoum M, Bossu P, Akoum A. Cycle-dependent expression of interleukin-1 receptor type II in the human endometrium. Biol Reprod 2001; 65:890–898.

22. Herrmann-Lavoie C, Rao CV, Akoum A. Chorionic gonadotropin downregulates the expression of the decoy inhibitory interleukin 1 receptor type II in human endometrial epithelial cells. Endocrinology 2007; 148: 5377–5384.

23. Noyes RW, Hertig AT, Rock J. Dating the endometrial biopsy. Am J Obstet Gynecol 1975; 122:262–263.

24. Akoum A, Lemay A, Brunet C, Hebert J. Secretion of monocyte chemotactic protein-1 by cytokine-stimulated endometrial cells of women with endometriosis. Le groupe d'investigation en gynecologie. Fertil Steril 1995; 63:322–328.

25. Carli C, Metz CN, Al-Abed Y, Naccache PH, Akoum A. Up-regulation of cyclooxygenase-2 expression and prostaglandin E2 production in human endometriotic cells by macrophage migration inhibitory factor: involvement of novel kinase signaling pathways. Endocrinology 2009; 150: 3128–3137.

26. Akoum A, Lawson C, Herrmann-Lavoie C, Maheux R. Imbalance in the expression of the activating type I and the inhibitory type II interleukin 1 receptors in endometriosis. Hum Reprod 2007; 22:1464–1473.

27. Bellehumeur C, Blanchet J, Fontaine JY, Bourcier N, Akoum A. Interleukin 1 regulates its own receptors in human endometrial cells via distinct mechanisms. Hum Reprod 2009; 24:2193–2204.

28. Bossu´ P, Visconti U, Ruggiero P, Macchia G, Muda M, Bertini R, Bizzarri C, Colagrande A, Sabbatini V, Maurizi G, Del Grosso E, Tagliabue A, et al. Transfected type II interleukin-1 receptor impairs responsiveness of human keratinocytes to interleukin-1. Am J Pathol 1995; 147:1852–1861.

29. Akoum A, Lemay A, McColl S, Turcot-Lemay L, Maheux R. Elevated concentration and biologic activity of monocyte chemotactic protein-1 in the peritoneal fluid of patients with endometriosis. Fertil Steril 1996; 66: 17–23.

30. Shao R, Guo X. Human microvascular endothelial cells immortalized with human telomerase catalytic protein: a model for the study of in vitro angiogenesis. Biochem Biophys Res Commun 2004; 321:788–794.

31. Cui X, Rouhani FN, Hawari F, Levine SJ. Shedding of the type II IL-1 decoy receptor requires a multifunctional aminopeptidase, aminopeptidase regulator of TNF receptor type 1 shedding. J Immunol 2003; 171: 6814–6819.

32. Bellehumeur C, Collette T, Maheux R, Mailloux J, Villeneuve M, Akoum A. Increased soluble interleukin-1 receptor type II proteolysis in the endometrium of women with endometriosis. Hum Reprod 2005; 20: 1177–1184.

33. Mantovani A, Garlanda C, Locati M, Rodriguez TV, Feo SG, Savino B, Vecchi A. Regulatory pathways in inflammation. Autoimmun Rev 2007; 7:8–11.

34. Akoum A, Jolicoeur C, Boucher A. Estradiol amplifies interleukin-1-induced monocyte chemotactic protein-1 expression by ectopic endometrial cells of women with endometriosis. J Clin Endocrinol Metab 2000; 85:896–904.

35. Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ, Murphy WJ. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. Blood 2000; 96:34–40.

36. Niu J, Azfer A, Zhelyabovska O, Fatma S, Kolattukudy PE. Monocyte chemotactic protein (MCP)-1 promotes angiogenesis via a novel transcription factor, MCP-1-induced protein (MCPIP). J Biol Chem 2008; 283:14542–14551.

37. Hess AP, Hamilton AE, Talbi S, Dosiou C, Nyegaard M, Nayak N, Genbecev-Krtolica O, Mavrogianis P, Ferrer K, Kruessel J, Fazleabas AT, Fisher SJ, et al. Decidual stromal cell response to paracrine signals from the trophoblast: amplification of immune and angiogenic modulators. Biol Reprod 2007; 76:102–117.

38. Rossi M, Sharkey AM, Vigano P, Fiore G, Furlong R, Florio P, Ambrosini G, Smith SK, Petraglia F. Identification of genes regulated by interleukin-1beta in human endometrial stromal cells. Reproduction 2005; 130: 721–729.

39. Kayisli UA, Mahutte NG, Arici A. Uterine chemokines in reproductive physiology and pathology. Am J Reprod Immunol 2002; 47:213–221.

40. Hong KH, Ryu J, Han KH. Monocyte chemoattractant protein-1-induced angiogenesis is mediated by vascular endothelial growth factor-A. Blood 2005; 105:1405–1407.

41. van Mourik MS, Macklon NS, Heijnen CJ. Embryonic implantation:cytokines, adhesion molecules, and immune cells in establishing an implantation environment. J Leukoc Biol 2009; 85:4–19.

42. Chen SJ, Liu YL, Sytwu HK. Immunologic regulation in pregnancy: from mechanism to therapeutic strategy for immunomodulation. Clin Dev Immunol 2012; 2012:258391.

43. Simon C, Piquette GN, Frances A, el-Danasouri I, Irwin JC, Polan ML. The effect of interleukin-1 beta (IL-1 beta) on the regulation of IL-1 receptor type I messenger ribonucleic acid and protein levels in cultured human endometrial stromal and glandular cells. J Clin Endocrinol Metab 1994; 78:675–682.

44. Simon C, Frances A, Piquette G, Hendrickson M, Milki A, Polan ML. Interleukin-1 system in the materno-trophoblast unit in human implantation: immunohistochemical evidence for autocrine/paracrine function. J Clin Endocrinol Metab 1994; 78:847–854.

45. Simon C, Valbuena D, Krussel J, Bernal A, Murphy CR, Shaw T, Pellicer A, Polan ML. Interleukin-1 receptor antagonist prevents embryonic implantation by a direct effect on the endometrial epithelium. Fertil Steril 1998; 70:896–906.

46. Hahn WC, Stewart SA, Brooks MW, York SG, Eaton E, Kurachi A, Beijersbergen RL, Knoll JH, Meyerson M, Weinberg RA. Inhibition of telomerase limits the growth of human cancer cells. Nat Med 1999; 5: 1164–1170.

47. Lim HJ, Wang H. Uterine disorders and pregnancy complications: insights from mouse models. J Clin Invest 2010; 120:1004–1015.

48. Wilcox AJ, Weinberg CR, O'Connor JF, Baird DD, Schlatterer JP, Canfield RE, Armstrong EG, Nisula BC. Incidence of early loss of pregnancy. N Engl J Med 1988; 319:189–194.

49. Simpson JL. Genes, chromosomes, and reproductive failure. Fertil Steril 1980; 33:107–116.

2.10 Figure Legends

FIG. 1. Human CG modulates the expression of IL1R mRNA in ESCs. Confluent ESC cultures were incubated with MM or hCG (10 and 100 ng/ml) for 24 h before being exposed, or not, to IL1B (0.01 and 0.1 ng/ml) for an additional 24 h. Total RNA was extracted and reverse transcribed. IL1R1, IL1R2, IL1RN, and GAPDH (internal control) mRNA levels were quantified by real-time PCR, as described in Materials and Methods. IL1R1 (A), IL1R2 (B), and IL1RN (C) mRNA ratio was then determined following normalization to GAPDH mRNA. Data were from ESC cultures issued from three different subjects and expressed as percent of control (ratio of IL1R1, IL1R2, or IL1RN mRNA levels found in cells incubated with IL1B, hCG, or hCG/ILB to those found in cells incubated with the control culture medium [MM] for an equivalent period of time). *$P < 0.05$, ***$P < 0.001$ relative to MM; †$P < 0.05$, ††$P < 0.01$ relative to cells stimulated with equivalent concentrations of IL1B.

FIG. 2. Human CG modulates the expression of the protein of IL1R in ESCs. Confluent ESC cultures were incubated with MM or 100 ng/ml hCG for 24 h before being exposed, or not, to IL1B (0.1 ng/ml) for an additional 24 h. Cells were recovered to

extract total proteins and analyze IL1R1, IL1R2, and IL1RN (A), and culture supernatants were collected to measure sIL1R2 (B) and IL1RN (C), as described in Materials and Methods. Data were from ESC cultures issued from three different subjects and expressed as percent of control (ratio of sIL1R2 or IL1RN levels found in cells incubated with IL1B, hCG, or hCG/ILB to those found in cells incubated with the control culture medium [MM] for an equivalent period of time). *P < 0.05, ***P < 0.001 relative to MM; †P < 0.05, †††P < 0.001 relative to cells stimulated with equivalent concentrations of IL1B.

FIG. 3. Human CG enhances MCP1 expression in ESCs in response to ILB. Confluent ESC cultures were incubated with MM or hCG (10 and 100 ng/ml) for 24 h before being exposed, or not, to IL1B (0.01 and 0.1 ng/ml) for an additional 24 h. Total cell RNA was extracted and reverse transcribed. MCP1, IL1R1, IL1R2, and GAPDH were quantified by real-time PCR, as described in Materials and Methods. MCP1 mRNA (A) and IL1R2:IL1R1 mRNA ratio (C) were then determined following normalization to GAPDH mRNA. The culture supernatants were recovered for determination of MCP1 secretion by ELISA (B). Data are from ESC cultures issued from three different subjects and expressed as percent of control (ratio of MCP1 mRNA levels or protein secretion in cells incubated with IL1B, hCG, or IL1B/hCG to those in cells incubated with the control minimal culture medium [MM] for an equivalent period of time). *P < 0.05, **P < 0.01, ***P < 0.001 relative to MM; †P < 0.05, ††P < 0.01, †††P < 0.001 relative to IL1B control dose.

FIG. 4. Overexpression of IL1R2 using cell transfection decreases of IL1B- and hCG/IL1B-induced MCP1 production by ESCs. Cells were transiently transfected with pcDNA3-IL1R2 or with the control vector pcDNA3 and incubated for 24 h with MM, hCG (100 ng/ml), IL1B (0.1 ng/ml), or hCG/IL1B (100/0.1 ng/ml), and MCP1 (A) and sIL1R2 (B) production was measured in the culture medium by ELISA. *P < 0.05, **P

< 0.01, ***P < 0.001 relative to MM; †P < 0.05, †††P < 0.001 relative to IL1B control dose; +++P < 0.001 relative to cells transfected with the empty pcDNA3 expression vector stimulated with an equal concentration of IL1B or hCG/IL1B.

FIG. 5. Human CG and IL1B trigger ESCs to stimulate HMVEC wound healing. At confluence, HMVEC monolayers were wounded by scraping and incubated for 24 h with ESC-conditioned media (culture supernatant [S]) following ESC treatment with hCG (100 ng/ml) and IL1B (0.01 and 0.1 ng/ml), as described in Figures 1 and 2. ESC-conditioned media were used at 1:3 dilution in MM (A), and hCG (100 ng/ml), IL1 (0.01 and 0.1 ng/ml), and hCG/IL1 combinations were similarly diluted (1:3 in MM) and included as controls (B). Cell migration to the wound surface was quantified by microscopy at 4× magnification. Data are from ESC cultures issued from three different subjects and expressed as percent of closure. *P < 0.05, **P < 0.01 relative to MM; †P < 0.05 relative to hCG control dose; +P < 0.05, ++P < 0.01 relative to IL1B control dose.

FIG. 6. Human CG and IL1B-induced HMVEC wound healing involves MCP1. At confluence, HMVEC monolayers were wounded by scraping and incubated for 24 h with MCP1, FGF, or VEGF as positive controls, and cell migration was quantified by microscopy at 4× magnification (A). Data are expressed as percent of closure (B). *P < 0.05, **P < 0.01 relative to MM. In parallel, ESC-conditioned media (culture supernatant [S]) of cultures treated with hCG/IL1B (1:3 dilution in MM) or MCP1 were incubated with either IgG control or with MCP1 antibody (MAB-279), and cell migration was quantified by microscopy at 4× magnification (C). Data are from ESC cultures issued from three different subjects and expressed as percent of closure (D). *P < 0.05, **P < 0.01, ***P < 0.001 relative to ESC conditioned media or MCP1 without neutralization with anti-MCP1 antibody.

FIG. 7. Human CG and IL1B trigger ESCs to stimulate HMVEC proliferation. HMVECs (104 cells/well in 96-well microtiter plates) were cultured overnight, starved in DMEM-F12 containing 0.2% FBS, and incubated with MCP1, VEGF, and FGF (positive controls), and ESC-conditioned media (1:3 dilution in MM) from cultures treated with hCG (100 ng/ml), IL1 (0.1 ng/ml), and hCG (100 ng/ml)/IL1 (0.1 ng/ml) (A) or with equivalent concentrations of hCG, IL1, and hCG/IL1 similarly diluted in MM (B). MCP1 was neutralized in ESC-conditioned media with anti-MCP1 antibody (MAB 279) (C). Cell proliferation was assessed by ELISA using BrdU incorporation into HMVEC DNA. Data are from ESC cultures issued from three different subjects and expressed as percent of control (S-MM in A and C; MM in B). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ as compared to control; †$P < 0.05$ as compared to S-hCG100/MM; +$P < 0.05$ as compared to S-MM/IL1B 0.1.

FIG. 8. Hypothesis of hCG and IL1 cross-talk in early pregnancy. Synthesized early by trophoblastic cells, hCG and IL1B act through the endometrial tissue on ESCs via their respective receptors (LHCGR, IL1R2, and IL1R1). Human CG amplifies IL1 signaling in ESCs, leading to increased cell responsiveness to IL1, the release of angiogenic factors, and the induction of angiogenesis. One of these identified factors is MCP1. EEC, endometrial epithelial cells.

2.11 Figures

Figure 1 Human CG modulates the expression of IL1R mRNA in ESCs.

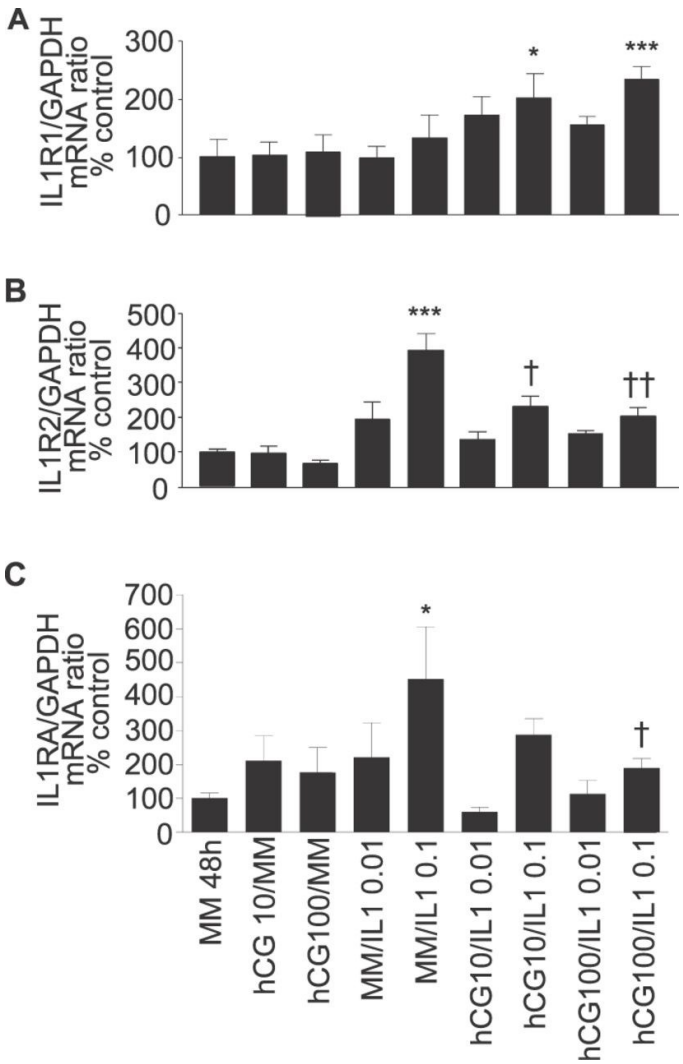


Figure 2 Human CG modulates the expression of the protein of IL1R in ESCs.

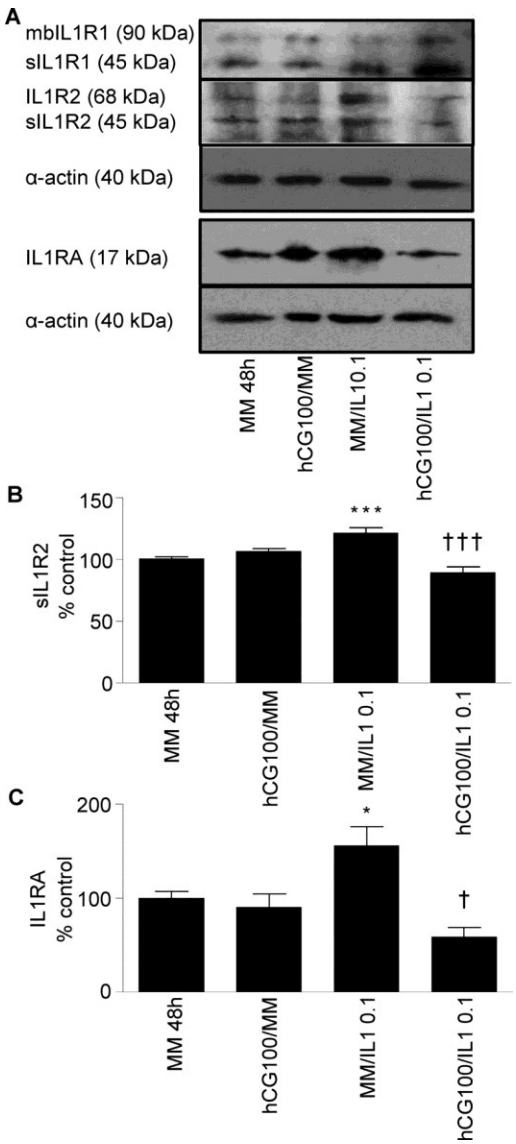

A
mbIL1R1 (90 kDa)
sIL1R1 (45 kDa)
IL1R2 (68 kDa)
sIL1R2 (45 kDa)
α-actin (40 kDa)
IL1RA (17 kDa)
α-actin (40 kDa)
MM 48h
hCG100/MM
MM/IL10.1
hCG100/IL1 0.1
B
sIL1R2
% control
150
100
50
0
***
†††
MM 48h
hCG100/MM
MM/IL1 0.1
hCG100/IL1 0.1
C
IL1RA
% control
200
100
0
*
†
MM 48h
hCG100/MM
MM/IL1 0.1
hCG100/IL1 0.1

Figure 3 Human CG enhances MCP1 expression in ESCs in response to ILB.

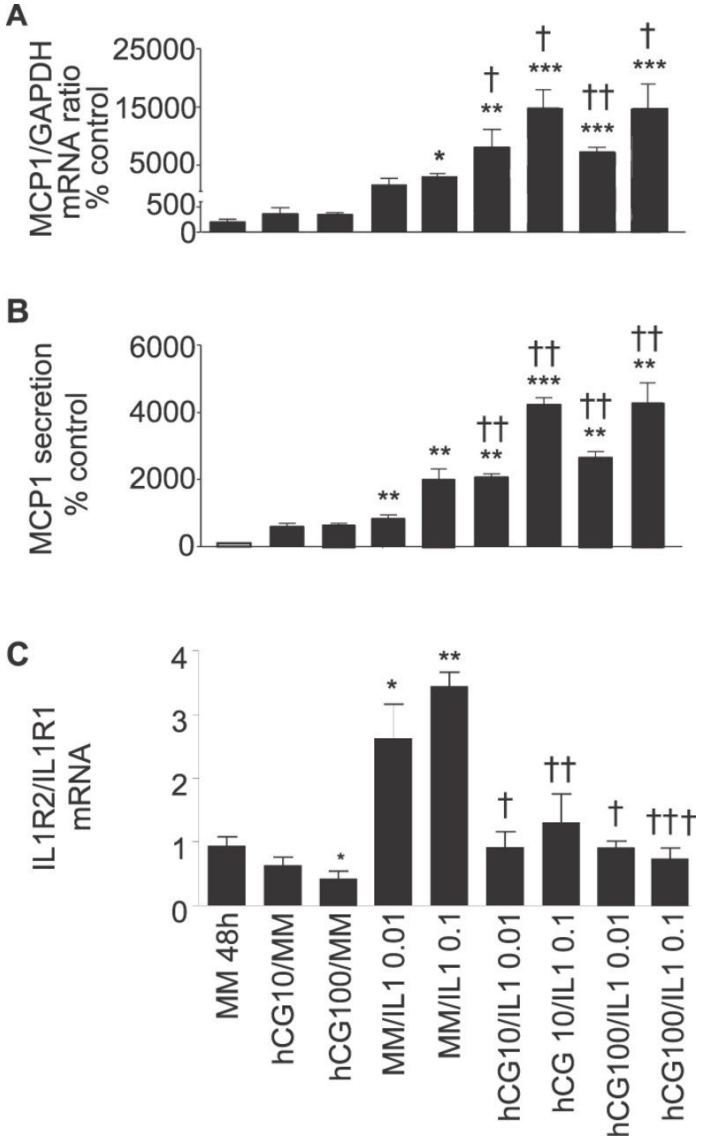


Figure 4 Overexpression of IL1R2 using cell transfection decreases of IL1B- and hCG/IL1B-induced MCP1 production by ESCs.

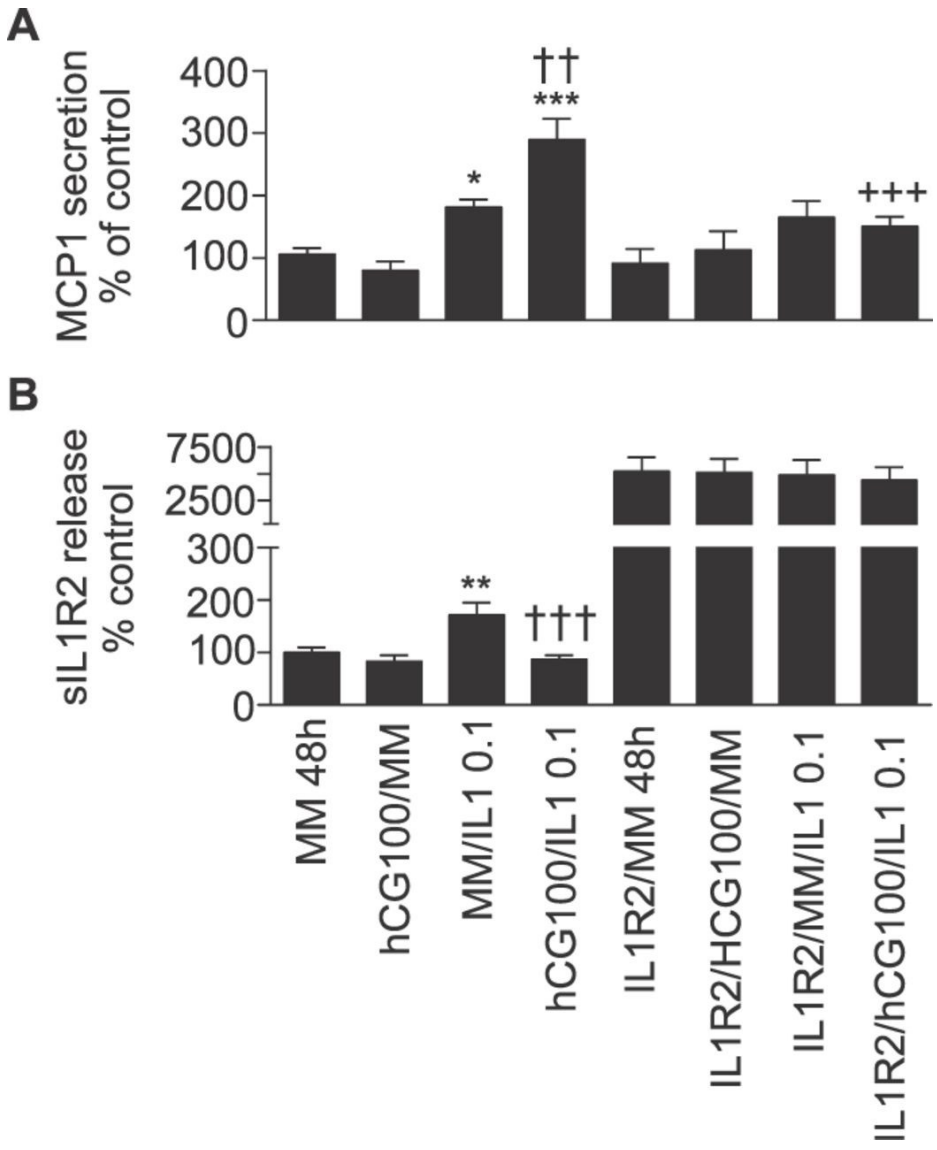


Figure 5 Human CG and IL1B trigger ESCs to stimulate HMVEC wound healing.

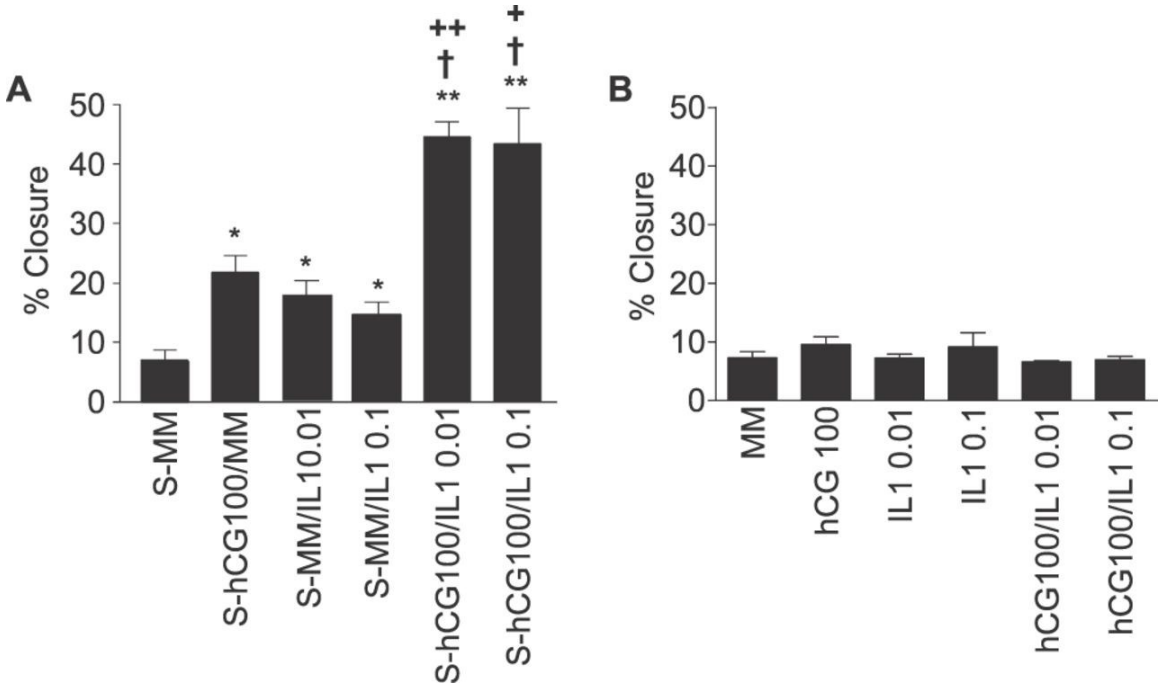


Figure 6 Human CG and IL1B-induced HMVEC wound healing involves MCP1.

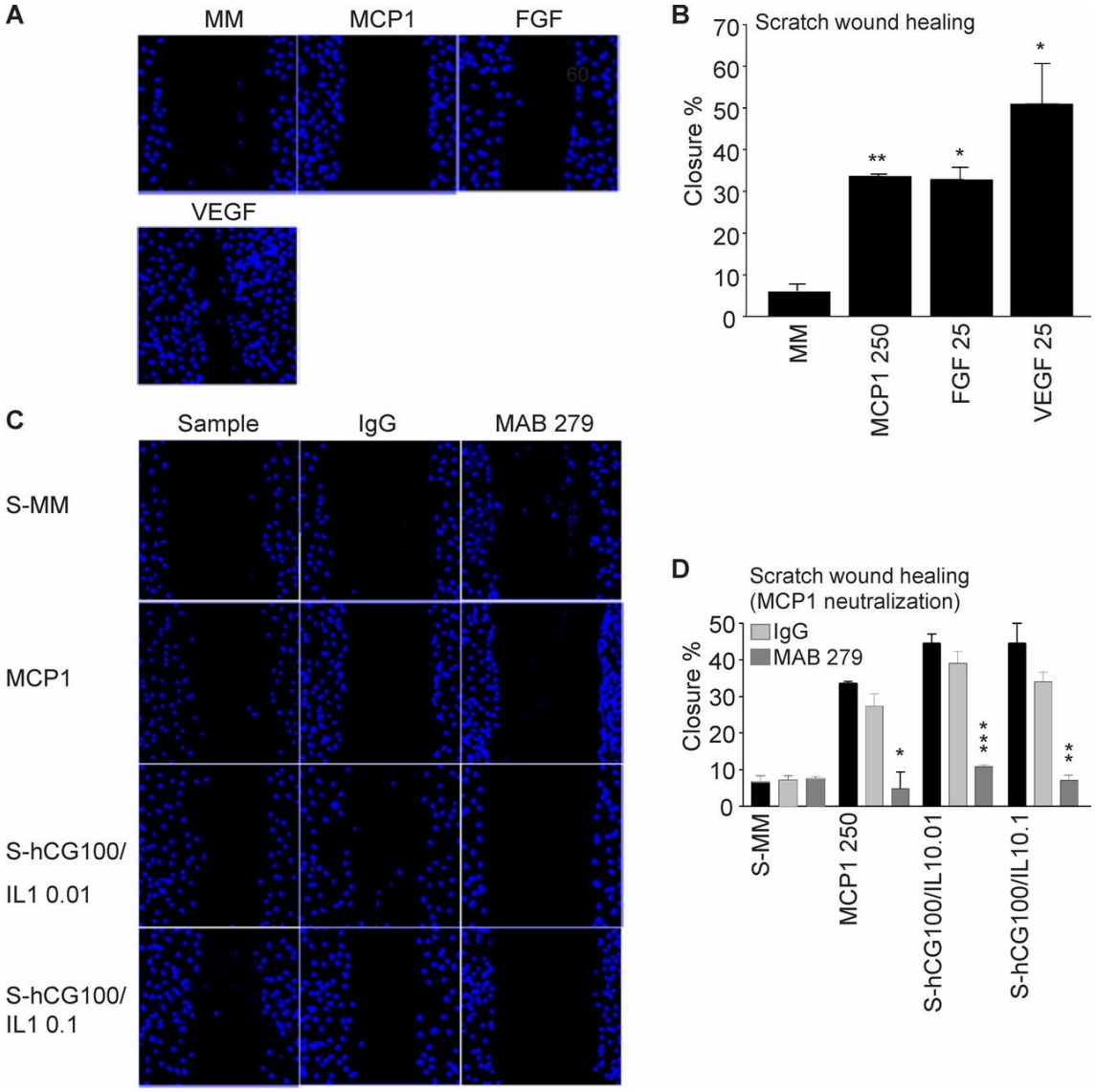


Figure 7 Human CG and IL1B trigger ESCs to stimulate HMVEC proliferation.

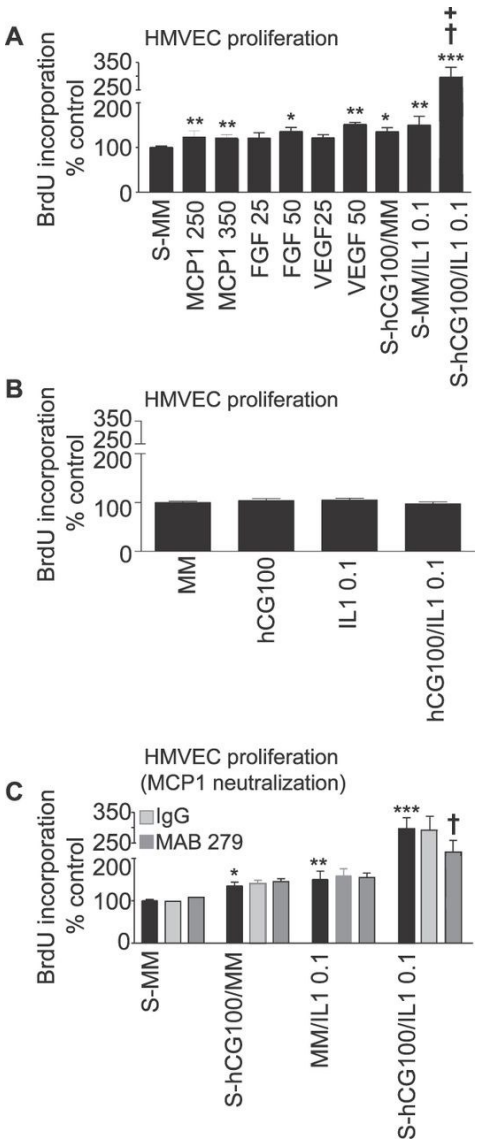
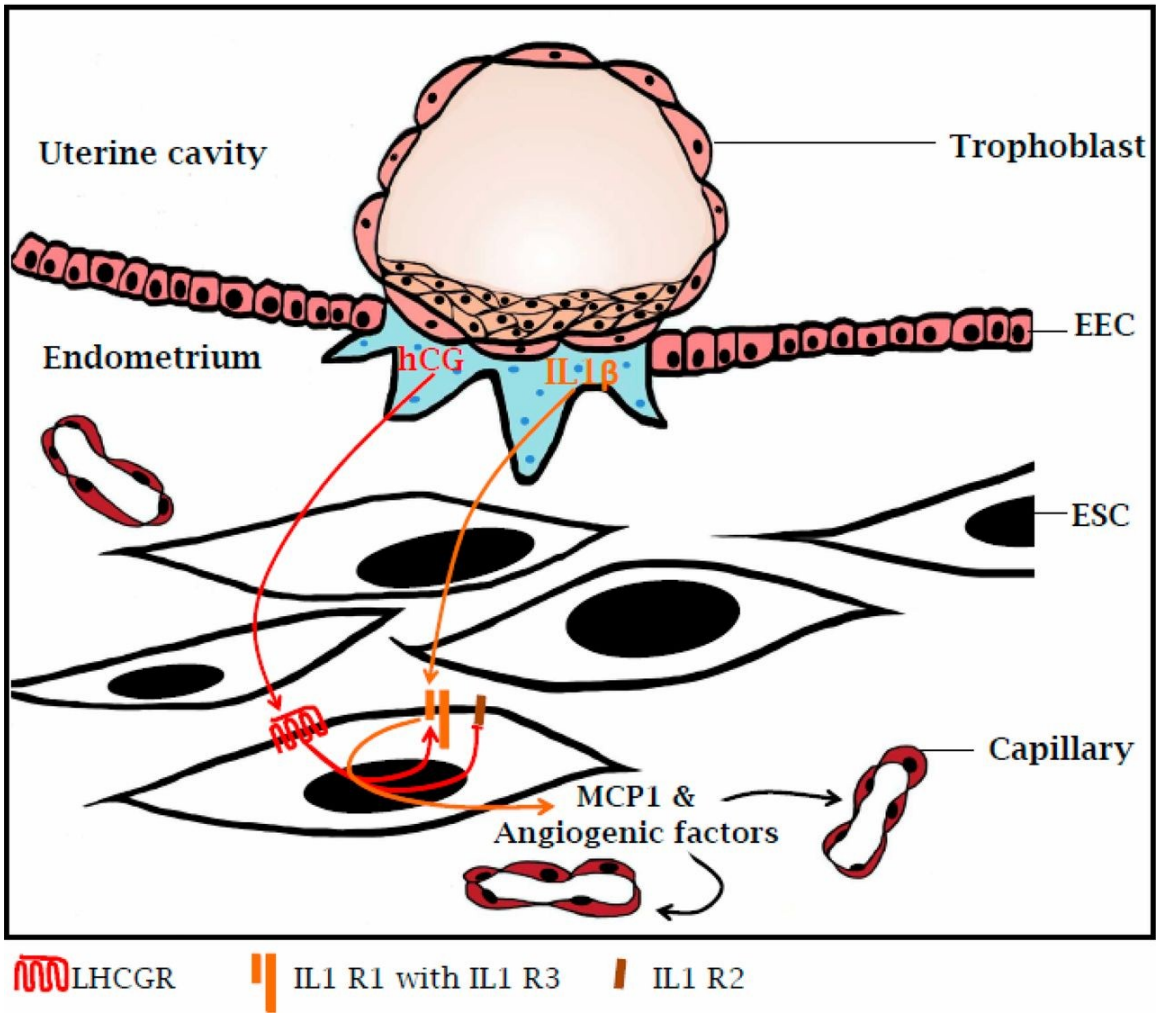


Figure 8 Hypothesis of hCG and IL1 cross-talk in early pregnancy.


Uterine cavity
Trophoblast
Endometrium
EEC
hCG
IL-1β
ESC
Capillary
MCP1 &
Angiogenic factors
LHCGR
IL1 R1 with IL1 R3
IL1 R2

Chapitre 3 : Transcriptome analysis reveals new insights into the modulation of endometrial stromal cell receptive phenotype by embryo-derived signals interleukin-1 and human chorionic gonadotropin: possible involvement in early embryo implantation.

Amélie Bourdiec[1], Ezequiel Calvo[2], C. V. Rao[3], Ali Akoum[1]

1 Endocrinologie de la reproduction, Département d'obstétrique, gynécologie et reproduction, Centre de recherche du CHU de Québec, Hôpital Saint-François d'Assise, Québec, Québec, Canada, 2 Département de médecine moléculaire, Centre de recherche du CHU de Québec, CHUL, Faculté de médecine, Université Laval, Québec, Québec, Canada, 3 Department of Cellular Biology and Pharmacology, Herbert Wertheim College of Medicine, Florida International University, Miami, Florida, United States of America

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3.1 Résumé

L'implantation du conceptus dans la cavité utérine nécessite un réseau complexe d'intéractions entre l'embryon et l'endomètre réceptif. Nous croyons que les signaux qui dérivent de l'embryon jouent un rôle important dans le remodelage et la prolongation de la réceptivité de l'endomètre. Nos études antérieures ont fourni des preuves d'une participation originale de la gonadotrophine chorionique humaine (hCG) dans la modulation et la potentialisation de la sensibilité des cellules épithéliales et stromales de l'endomètre à l'interleukine 1 (IL1), l'un des premiers signaux embryonnaires, ce qui peut présenter une nouvelle voie par laquelle l'embryon favorise sa propre implantation et sa croissance au sein de l'endomètre maternel. La présente étude à été conçue pour acquérir une meilleure compréhension de l'impact de la hCG sur la modulation de la réceptivité des cellules stromales de l'endomètre, et en particulier sur la réactivité cellulaire à l'IL1 dans la promotion de la croissance et dans l'acquisition d'un phénotype capable de recevoir, de soutenir et de promouvoir le développement de l'embryon. Nos résultats ont montré d'importants changements dans l'expression des gènes impliqués dans la prolifération cellulaire, la modulation immunitaire, le remodelage tissulaire, l'apoptose et le processus angiogénique. Cela met en évidence un impact significatif de ces signaux embryonnaires sur la réceptivité de l'endomètre maternel, son adaptation à l'implantation d'un embryon et la création d'un environnement favorable à la croissance de ce dernier au sein d'un tissu hôte, probablement hostile. Il est également interessant que nos résultats aient identifié une interaction complexe entre l'IL1 et la hCG, laquelle, malgré une action synergique sur plusieurs gènes cibles importants dans l'endomètre, peut exercer un contrôle étroit de l'IL1 qui s'étend à d'autres membres de la famille IL1.

3.2 Abstract

The presence of the conceptus in uterine cavity necessitates an elaborate network of interactions between the implanting embryo and a receptive endometrial tissue. We believe that embryo-derived signals play an important role in the remodeling and the extension of endometrial receptivity period. Our previous studies provided original evidence that human Chorionic Gonadotropin (hCG) modulates and potentiates endometrial epithelial as well as stromal cell responsiveness to interleukin 1 (IL1), one of the earliest embryonic signals, which may represent a novel pathway by which the embryo favors its own implantation and growth within the maternal endometrial host. The present study was designed to gain a broader understanding of hCG impact on the modulation of endometrial cell receptivity, and in particular, cell responsiveness to IL1 and the acquisition of growth-promoting phenotype capable of receiving, sustaining, and promoting early and crucial steps of embryonic development. Our results showed significant changes in the expression of genes involved in cell proliferation, immune modulation, tissue remodeling, apoptotic and angiogenic processes. This points to a relevant impact of these embryonic signals on the receptivity of the maternal endometrium, its adaptation to the implanting embryo and the creation of an environment that is favorable for the implantation and the growth of this latter within a new and likely hostile host tissue. Interestingly our data further identified a complex interaction between IL1 and hCG, which, despite a synergistic action on several significant endometrial target genes, may encompass a tight control of endogenous IL1 and extends to other IL1 family members.

3.3 Introduction

Embryonic implantation and establishment of successful pregnancy require a dynamic process of interactions between the embryo and a receptive maternal endometrium. This embryo/maternal cross-talk involves an elaborate and coordinated network of communication via timely released embryonic and maternal-derived signals and well-targeted actions. Optimal receptivity of the human endometrium to the implantation of a competent blastocyst occurs during a limited period of time within the menstrual cycle called “implantation window”, which is generally believed to span d6-10 following luteinizing hormone (LH) peak in the normal menstrual cycle [1,2]. Numerous studies showed major and specific changes arising within this specific time interval, which encompass adhesion, invasion, survival, growth, differentiation and immune-modulating factors that shape up endometrial receptivity. The dynamics of this transition from a non-receptive to a receptive endometrium are poorly understood, but the correct spatio-temporal synthesis and balance of various factors is thought to play an important role in human uterine preparation for implantation [3,4,5].

Indeed, under the influence of a developing embryo, endocrine factors, particularly ovarian hormones, play a critical role in the regulation of the molecular changes that occur. Embryonic human chorionic gonadotropin (hCG) maintains for instance the production of progesterone by the corpus luteum, which is critical to sustain early pregnancy. However, direct interactions at the fetal-maternal interface and appropriate coordination between embryonic and maternal signals at the implantation site are essential for providing the synergistic environment needed for the establishment of pregnancy [6,7].

hCG is a major embryonic signal playing a key role in the initiation and maintenance of pregnancy [8]. It is transcribed as early as the 2-cell embryo stage [9] and is produced abundantly by the trophectodermal cells of the pre-implantation blastocyst [10]. Following implantation, hCG is produced by syncytiotrophoblast of the developing conceptus [11]. Recent evidence suggests that hCG is also produced in glandular and luminal epithelium of human endometrium, primarily during the secretory phase [12,13]. hCG production by embryonic cells may directly regulate the expression of endometrial factors and extend the period during which the endometrium is receptive [2,14].

hCG acts on the intrauterine environment via the luteinizing hormone (LH)/hCG receptor (hLHCGR), which was detected in various cell types including human uterus and decidua, placenta and fetal membranes [15,16]. Synthesized early by the trophoblast, hCG may therefore have a wide spectrum of cell targets and biological actions that influence endometrial receptivity and embryo implantation. It promotes human endometrial stromal cell (ESC) decidualization [17] via functional differentiation resulting in an up-regulation of cyclooxygenase 2 (COX2) gene expression and increased production of prostaglandin (PG)E2 [18], possesses both direct and indirect angiogenic properties [19], induces tissue specific human uterine natural killer (uNK) cell proliferation [20] and regulates embryonic autocrine and maternal paracrine factors involved in embryo attachment, endometrial remodeling, antioxidant defense and immune mechanisms around the implanting blastocyst [2,21,22].

Several studies provide strong evidence that interleukin (IL1)B may play a pivotal role at the embryo-maternal interface and represents one of the earliest signals [23,24,25,26,27]. IL1 is synthesized by the human embryo during its initial stages, and the concentration of this cytokine has been positively correlated with successful

implantation after in vitro fertilization and transfer to the uterine cavity [28,29]. A key regulator of the inflammatory response, IL1 is currently recognized as a multifunctional cytokine with a wide spectrum of effects on numerous cell types (eg nervous system cells, immune cells, connective tissue cells, endometrial cells, hepatocyte, fibroblast and endothelial cells) [30,31]. IL1 acts on human endometrial cells to induce the secretion of leukemia inhibitory factor (LIF) and PGE2 [32,33], which play an important role in the implantation process [34], up-regulates the expression of integrin β3, a marker of uterine receptivity, in human endometrial epithelial cells [35] and stimulates the migration of human first-trimester villous cytotrophoblast cells via endometrium-derived factors [36]. Purified human cytotrophoblasts in culture release IL1B in the manner that parallels their invasive potential [37]. IL1B stimulates the release of human placental metalloproteinase (MMP)9 [37], proMMP3 expression in baboon stromal cells [38] and hCG by first trimester human trophoblastic cells [39].

The IL1 system is composed of two receptors (IL1R1 and IL1R2), one accessory protein (IL1 RAP) also called IL1R3, one receptor antagonist (IL1RN) and two agonists (IL1A and IL1B), which both trigger cell activation via the functional signaling IL1R1 [40]. IL1R2 rather acts as a negative regulator of IL1 action. Either the membrane-bound (mb) or the soluble (s) form of IL1R2, which is released by proteolysis from the cell surface, acts by capturing IL1, thereby inhibiting IL1-mediated cell activation [41].

Our previous studies pointed to new mechanisms by which the embryo may fine-tune the receptivity of the maternal endometrium, and revealed the ability of hCG to interact with different human endometrial cell types and modulate cell receptivity to IL1. Actually, hCG appeared to down-regulate the expression of the inhibitory IL1R2 in endometrial epithelial cells without affecting that of the

activating IL1R1 [42]. Comparable effects were observed in ESCs during the implantation window with, interestingly, a concomitant up-regulation of IL1R1, a down-regulation of IL1RN, an increased angiogenic activity and a higher secretion of monocyte chemotactic protein1 (MCP1) [23]. First identified as a specific factor for macrophage recruitment and activation, MCP1 was later found to be endowed with various immune modulating, proangiogenic and growth-promoting properties [43,44]. The aim of the present work was to gain a broader understanding of the global impact of hCG on the modulation of human endometrial cell responsiveness to IL1 and the acquisition of growth-promoting phenotype capable of sustaining active embryonic implantation and growth. Using micro-array analysis of hCG, IL1 and hCG/IL1-treated ESCs from the implantation window, our data identified several significantly regulated genes targeted by hCG/IL1 synergy and implicated in angiogenesis, proliferation, tissue remodeling, cell signaling and immune modulation, which is relevant to early embryo implantation process, and a wide spectrum of targets encompassing IL1 family members.

3.4 Materials et methods

3.4.1 Subjects and tissue handling

Endometrial tissue specimens were obtained during the implantation window (days 19 to 24) from normal fertile women with a regular menstrual cycle, who were undergoing laparoscopy for tubal ligation and had not received hormonal or anti-inflammatory therapy for at least 3 months prior to surgery (mean age ± SD, 35.6 ± 4.9 yr.; n = 7). Menstrual cycle day was determined according to the histological criteria of Noyes et al [45]. A written informed consent was obtained from participants under a study protocol approved by the Ethics Committee on Human Research of Laval University, Quebec, Canada. Collection of endometrial tissue biopsies was performed using a Pipelle (Unimar Inc., Prodimed, Neuilly-En-Thelle, France). Tissue samples were kept at 4°C in sterile Hank's balanced salt solution (HBSS) containing 100 U/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B (Invitrogen Life Technologies, Burlington, ON, Canada) and immediately transported to the laboratory.

3.4.2 Cell culture and treatment

ESCs were isolated and characterized according to our previously described procedure [46]. Concisely, tissue was minced into small pieces, dissociated with collagenase before ESCs were separated by differential sedimentation and adhesion. The purity of primary ESC cultures was tested morphologically by light microscopy and immunocytochemically on parallel cultures, as previously described. Cultures were free of CD45-positive leukocytes and contamination by factor VIII-positive endothelial cells was generally less than 1%. ESCs were cultured at 37 °C in DMEM:F12 (1:1) supplemented with 10% fetal bovine serum (FBS), insulin,

transferrin, and a mix of antibiotics–antimycotics. Preconfluent cells were washed with HBSS, incubated overnight with charcoal-treated FBS-supplemented medium, washed with phenol red-free DMEM:F12 (1:1) and cultured with phenol red- and FBS-free medium containing hCG (100 ng/mL, recombinant protein expressed in a mouse cell line, 10,000 IU/mg; Sigma-Aldrich Co., St. Louis, MO) for 24h. Cells were then incubated with a fresh phenol red- and FBS-free medium containing IL1B (0.1 ng/mL, R&D Systems, Minneapolis, MN) for additional 24h. hCG and IL1B concentrations were determined based on our previous studies with human ESCs where different doses were used (Bourdiec A et al, Biol Reprod, 2012). hCG and IL1B concentrations are within the range of the molecules' physiological concentrations [28,47].

3.4.3 RNA preparation and micro-array analysis

Total RNA of ESC cultures issued from 3 different women was extracted with Trizol according to the manufacturer's directions (Invitrogen). Then they were washed using the micro RNeasy Kit (Quiagen). Total RNA quantity was measured with Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., DE,USA) and RNA integrity was assessed by capillary electrophoresis using the Bioanalyzer 2100 (Agilent Technologies, Mississauga, Ontario, Canada). DNA micro-array analyses were carried out with Affymetrix Human Gene 1.0 ST at the Gene Expression Platform of the Research Centre of Laval University Hospital Centre, Quebec, Canada. The array interrogates 28,869 well-annotated genes with 764,885 distinct probes. The design of the Human Gene 1.0 ST Array was based on the March 2006 human genome sequence assembly (UCSC Hg18, NCBI build 36) with comprehensive coverage of RefSeq, Ensembl and putative complete CDS GenBank transcripts. Chips were processed according to the Affymetrix standard protocol. Briefly, total RNA (150 ng per sample) was labeled using the Ambion WT Expression kit and Affymetrix

GeneChip® WT Terminal Labeling kit, and hybridized to the arrays as described by the manufacturer (Affymetrix, Santa Clara, CA). The cDNA hybridization cocktail was incubated overnight at 45°C while rotating in a hybridization oven. After 17±1h of hybridization, the cocktail was removed and the arrays were washed and stained in an Affymetrix GeneChip fluidics station 450, according to Affymetrix protocol (http://media.affymetrix.com/support/downloads/manuals/wt_sensetarget_label_manual.pdf). The arrays were scanned using the Affymetrix GCS 3000 7G and the Affymetrix Expression Console Software (Affymetrix, Santa Clara, CA), to produce the intensity files.

Data analysis, background subtraction, and intensity normalization were performed using Robust Multiarray Analysis [48]. Differentially expressed genes and false discovery rate were estimated from t test ($P < 0.05$) and corrected using Bayes approach [49]. Data analysis, hierarchical clustering, and ontology was performed using the OneChanelGUI to extend affylmGUI graphical interface capabilities [50] and Partek Genomics Suite, version 6.5 (Partek Inc., St. Louis, MO) with analysis of variance analysis.

3.4.4 Quantitative real time PCR.

RNA was extracted using the standard Trizol® protocol (Invitrogen) as previously reported [51]. Quantitative real time (qRT)-PCR was performed on the same 3 cultures used for microarray analysis and 4 more different cultures. An ABI 7000 Thermal Cycler (Applied Biosystems, Foster City, CA) was used. Each standard PCR reaction contained 2 µL reverse transcriptase (RT) product, 0.5 µL of primer (final concentration, 0.1 mM), 12.5 µL of SYBR Green PCR Master Mix (Invitrogen) consisting of Taq DNA polymerase reaction buffer, Taq DNA polymerase, SYBR green I, deoxynucleotide triphosphate mix and MgCl2. The reaction melting temperature

(Tm) and the list of primers are reported in the Table 1. Primers were designed with Primer Premier 5 software to cross intron-exon boundaries. All samples were tested in duplicate and for each reaction negative controls without RNA and without reverse transcriptase were included.

3.4.5 Enzyme-Linked Immunosorbent Assay (ELISA)

CCL2 and CCL5 concentrations in the culture medium were measured using previously reported sandwich ELISAs [52,53]. VEGFC, TIMP3, MMP9 and prolactin were measured using DuoSet kit (DuoSet, R&D Systems, Minneapolis, MN), according to the manufacturer's instructions.

3.4.6 Statistical analysis

qRT-PCR and ELISA data followed a parametric distribution and were expressed as means ± SEM. Statistical analyses were performed with GraphPad Software Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA). The significance of statistical differences was determined using one way analysis of variance (ANOVA) followed by the Bonferroni test post hoc, for multiple comparisons, and the Student's t-test for the comparison of two groups.

3.5 Results

3.5.1 Gene expression profile for all experiments.

Total RNA was isolated from primary human ESCs treated with hCG (100 ng/mL) for 24h before being stimulated or not with IL1B (0.1 ng/mL) for additional 24h. RNA from all treated groups was compared by micro-array with the corresponding non-treated control using Affymetrix GeneChip Human Genome. Using Significance Analysis of Micro-arrays (SAM), all genes significantly regulated between treated

versus the non-treated group were selected [fold change (FC) 1.5 and a false discovery rate (FDR) < 5%]. The unsupervised hierarchical clustering analysis of the array data showed specific molecular signatures of the global gene expression for each group and a noticeable discrimination between IL1B-treated and IL1B-untreated cells with and without hCG pre-treatment (Figure 1A). Three dimensional Principal Component Analysis (PCA) further showed different patterns of gene expression and a clear segregation between the four groups included in this study [MM/MM (control minimal medium), hCG/MM, MM/IL1B and hCG/IL1B treatments). Also, samples from the same group were very tightly clustered together, which corroborates the robustness of the Affymetrix micro-arrays (Figure 1B).

3.5.2 Gene expression profile in ESCs is under embryonic stimuli

The different gene lists identified using SAM analysis (FC 1.5 and an FDR < 5%) of treatment versus control groups were then intersected to determine their overlap. The results showed that 9 significantly regulated genes were common to all treatments, and 97, 211 and 497 genes were independently regulated by hCG, IL1B and hCG/IL1, respectively (Figure 1C), thereby suggesting highly specific expression profiles.

3.5.3 Genes ontology (GO) of biological processes

Gene ontology (GO) annotations were then used to explore the specific functional properties of the molecular signatures. A functional enrichment analysis was performed using Partek software (Figure 2). Only significant biological functions were reported. The molecular signature of hCG/MM-treated group was enriched in genes associated with the regulation of cell response to stimulus, cellular and

metabolic processes and cellular component organization. Analysis of the IL1B-treated group identified several enriched GO categories that were linked to multi-organism process, immune system process, death, biological adhesion, locomotion, biological regulation, developmental process and response to stimulus, with a marked increase in the latter process. In hCG/IL1 treated group, most enriched GO categories were similar to those found in the IL1B-treated group, but the enrichment scores appeared to be different. The most perceptible increase over hCG and IL1B/hCG was related to cellular, biological regulation and immune system processes, whereas other processes such as biological adhesion and response to stimulus seemed to be lessened to some extent. As observed in the forest plot shown in Figure 2B, IL1B increased the percentage of differentially up- and down-regulated gene populations with a fold change above 2 in each of these biological processes, while co-exposure to hCG led globally to a more moderated regulation. These observations are noteworthy considering the possible involvement of these critical biological processes in the embryo-maternal crosstalk and the establishment of pregnancy.

3.5.4 Identification of differentially expressed genes implicated in early embryo implantation

Tables 2-5 summarize significantly up- and down-regulated genes in ESCs in response to hCG, IL1 and hCG/IL1 compared to untreated control cells. Genes were ranked according to the average of the fold change. To validate changes in the level of RNA transcripts, we have selected some genes found to be significantly regulated upon hCG, IL1B or hCG/IL1B treatment by micro-array and known for being involved in proliferation, immune modulation, tissue remodeling, cell signaling, apoptosis and angiogenesis, which are crucial for early embryo implantation. qRT-PCR was performed for the MCPs [chemokine C-C motif ligand (CCL)2 or MCP1, CCL8 or MCP2

and CCL7 or MCP3], vascular cell adhesion molecule 1(VCAM1), IL6, CCL5 or regulated and normal T cell expressed and secreted (RANTES), PG synthase (PTGS)2 or COX2, vascular endothelial growth factor C (VEGFC), MMP9, tissue inhibitor of metalloproteinase 3 (TIMP3) and keratin 19 (KRT19), and for IL1 family members IL1R-like 1 (IL1RL1) or IL33R, IL18R1, their respective ligands (IL33 and IL18) and both IL1 isoforms A and B.

Our previous studies showed that hCG acts on ESCs, in synergy with IL1B, to stimulate the expression of CCL2 (MCP1), a monocyte/macrophage chemotactic factor with potent angiogenic properties, via the creation of an imbalance between IL1R1 and IL1R2 expression [23]. Micro-array analysis and qRT-PCR validation indicated that not only CCL2, but CCL8 (MCP2) and CCL7 (MCP3) were significantly upregulated by hCG/IL1 as well ($P < 0.001$, $P < 0.05$ and $P < 0.001$, respectively). Furthermore, a significant increase of CCL2, CCL8 and CCL7 mRNA transcripts in cells exposed to hCG/IL1B compared to hCG was noted ($P < 0.001$, $P < 0.05$ and $P < 0.001$, respectively), whereas only CCL2 and CCL7 mRNA transcripts were significantly increased in cells treated with hCG/IL1B compared to IL1B ($P < 0.05$) (Figure 3 A, B and C).

Many other cytokines and growth factors known for being involved in the regulation of immune responses, adhesion, cell proliferation and angiogenesis were also found to be targeted by hCG and IL1B synergistic action. Vascular cell adhesion protein 1, which mediates leukocyte adhesion to vascular endothelium, was significantly up-regulated by hCG/IL1B compared to the control minimal medium (MM) ($P < 0.01$), but neither hCG nor IL1B had a statistically significant stimulatory effect (Figure 4A). IL6 and CCL5 mRNA levels were significantly increased by hCG/IL1B compared to MM ($P < 0.05$ and $P < 0.01$, respectively) or to hCG ($P < 0.05$ and $P < 0.01$,

respectively). CCL5 levels were also significantly increased in cells treated with hCG/IL1B compared to IL1B ($P < 0.05$) (Figure 4B, C).

PTGS2, a major rate-limiting enzyme involved in PG synthesis, and VEGFC, an isoform of a potent angiogenic factor, also showed an increased mRNA expression in cells treated with hCG/IL1B compared to cells incubated with MM ($P < 0.001$ and $P < 0.01$, respectively) or with hCG alone ($P < 0.001$ and $P < 0.01$, respectively). IL1B stimulated PTGS2 and VEGFC mRNA synthesis as well ($P < 0.05$), but cell exposure to hCG significantly stimulated in the IL1B-induced PTGS2 expression ($P < 0.05$) (Figure 4D, E).

Also found to be regulated were some molecules shown to participate in endometrial tissue remodeling such as MMP9, TIMP3 and KRT19 (Figure4F, G, H). MMP9 was significantly up-regulated by IL1B and hCG/IL1B ($P < 0.01$ and $P < 0.05$, respectively). By itself, hCG had no statistically significant effect on MMP9 expression, but it moderated the IL1B-induced effect. TIMP3, a natural tissue inhibitor of MMPs, was down-regulated by IL1B either in the presence or the absence of hCG ($P < 0.05$). Cytokeratin 19 (KRT19), an intermediate filament protein associated with embryonic placenta development [54], was found to be significantly inhibited by IL1B ($P < 0.05$) and more by IL1B combined with hCG ($P < 0.01$).

3.5.5 hCG modulates IL1B effects on the expression of IL1 family members in ESCs

Our previous studies showed that hCG down-regulates the IL1B-induced increase of IL1R2 and IL1RN in ESCs and amplifies the IL1B-induced increase of IL1R1 [23]. Our current micro-array analysis further revealed that hCG/IL1B interaction may affect ESC responsiveness to other components of IL1 family including IL1 isoforms A and B as well as IL1RL1 and IL18R1 (Tables 2 and 4). To validate these findings, qRT-PCR

analysis was performed. Our results showed that IL1B induced both ILA and IL1B in ESCs ($P < 0.01$ and $P < 0.001$, respectively), but hCG moderated that endogenous IL1B-mediated expression (Figure5 A, D). However, both IL1 isoforms remained significantly up-regulated compared to the control medium ($P < 0.05$) despite the down-regulatory effect of hCG. Furthermore, the micro-array data predicted a synergistic interaction between hCG and IL1B, resulting in the induction of IL18R1 and IL1R1L expression (Figure 5 B, E). This was confirmed by qRT-PCR analysis, which showed a significant up-regulation of these two receptors as compared to control ($P < 0.05$ and $P < 0.001$, respectively) and to hCG ($P < 0.05$ and $P < 0,001$, respectively). However, despite a noticeable increase of IL1RL1 and IL1R18 in hCG/IL1B-treated compared to IL1B-treated cells, this increase was statistically significant only for IL18R1 ($P < 0.01$), but did not reach statistical significance for IL1RL1 with these small groups.

Because IL18R1 and IL1RL1 were targeted by hCG/IL1B, we have then investigated their ligands (IL18, IL33) by qRT-PCR. Our data showed that like IL1A and IL1B, both IL33 and IL18 were up-regulated in cells treated with IL1B ($P < 0.01$ and $P < 0.05$, respectively), but they were down-regulated in cells treated with hCG and IL1B compared to IL1B alone ($P < 0.01$) (Figure 5 C, F).

3.5.6 Validation of selected soluble proteins

To confirm the gene expression changes at the protein level, we have selected some genes found to be significantly regulated upon hCG, IL1B or hCG/IL1B treatment and known for being involved in immune modulation, tissue remodeling and angiogenesis. Results indicated that CCL2 was significantly upregulated by hCG/IL1B ($P < 0.001$), which corroborates the microarray data (Figure 6A). CCL5 secretion was upregulated either by IL1B or hCG/IL1B ($P < 0.05$), but hCG/IL1B synergism was not

perceptible at the protein level (Figure 6 B). hCG/IL1B treatment further appeared to upregulate VEGFC ($P < 0.001$) and downregulate TIMP3 secretion ($P < 0.001$) (Figure 6 C, D). MMP9 was significantly up-regulated by IL1B ($P < 0.05$), but hCG/IL1 did not show a statistically significant stimulatory effect (Figure 6E).

3.6 Discussion

Cumulating evidences point to an important role for embryo-endometrial dialogue in mediating the adhesion, invasion and growth of the embryo during implantation and early development. Well-known for rescuing the corpus luteum and maintaining the production of progesterone, embryo-derived signals such as hCG seem to orchestrate endometrial adaptation to the implantation of the newly formed embryo as well [22], but little is known about the involved pathways and the underlying mechanisms. Human CG is quite known, for instance, for stimulating cytokine/chemokine production by endometrial epithelial and stromal cells and playing direct and indirect roles in human ESC decidualization [17] and angiogenesis in human endothelial cells [19,23,55]. It is unclear if hCG is involved in blastocyst attachment in humans. However, it may have an indirect role, as suggested by our and other studies, and act via the modulation of human epithelial cell receptivity/responsiveness to other major embryonic signals such as IL1 [56]. A recent study demonstrated that the expression of β3-integrin-subunit, a cell adhesion mediator and marker of uterine receptivity [57], on the surface of human endometrial epithelial cells could be up-regulated by coculture with a human preimplantation embryo and blocked by anti-IL1 antibody [35]. Another study showed that the expression of trophinin, which mediates cell adhesion by homophilic binding, and the ability for apical cell adhesion with trophinin-expressing

human trophoblastic cells are increased in presence of hCG associated with IL1β [58].

The presence of LHCG receptor in various endometrial cell types [15,16] makes plausible that hCG has a broad spectrum of endometrial cell targets. After the intrusion of the embryo through the luminal endometrial epithelium, trophoblastic cells are in close contact with different maternal stromal cell types [59]. To achieve a successful pregnancy, an appropriate cross-talk between embryonic and maternal cells must therefore take place, where numerous embryo- as well as maternal-derived factors including steroid hormones, matrix degrading enzymes, integrins, cytokines, chemokines and growth factors could be involved [60,61].

Our previous studies revealed a new mechanism by which hCG can target different human endometrial cell types, including epithelial and stromal cells, to modulate their receptivity to IL1, an early potent embryonic signal, and amplify thereby the release of immune and angiogenic factors [23,42]. In the present study, we further showed that hCG acts on ESCs, either alone or via the modulation of the IL1-mediated cell responsiveness, to regulate numerous relevant genes involved in cell signaling, proliferation, apoptosis, immune modulation, tissue remodeling and angiogenesis, which are highly relevant mechanisms underlying the implantation process and the modulation of the immune response around the implanting embryo. Some of the genes were known for playing important roles in the various embryo implantation stages, but many genes were not known for being possibly involved and regulated by hCG or hCG/IL1B synergism.

MCPs were among the most significantly induced immune factors in ESCs in response to hCG and IL1B. hCG amplified the IL1B-induced expression of MCP1, 2 and 3. These chemokines are involved in the recruitment of

monocytes/macrophages, T cells and NK cells into inflammatory sites [62,63]. Moreover, MCPs stimulate angiogenesis, either directly via MCP1-induced protein (MCPIP) or indirectly via their activation of immune cells such as NK cells and macrophages, which are known for releasing growth and angiogenic factors [64]. Interestingly, the current micro-array data are in keeping with our previous findings of an increased IL1B-mediated secretion of MCP1 in human ESCs from the implantation window following hCG treatment [23] and consistent with a possible role for MCPs in embryo implantation. Actually, macrophages contribute to decidualization and implantation and remain abundant at the implantation site throughout pregnancy [65,66], and trophoblastic cells were shown to regulate human monocyte migration and differentiation [67]. However, uterine macrophages do not appear to impair the growth of the semi-allogeneic embryo. They rather seem to play a protective role against possible infections, maintain immune tolerance toward trophoblastic antigens, mediate trophoblast invasion and support embryonic growth [68]. This strengthens the relevance of our findings and broadens the spectrum of hCG's impact on early embryonic growth and development.

VCAM1, an adhesion molecule of endothelial cells playing an important role in immune cell trafficking [69], appeared to be up-regulated by hCG or IL1B, but significantly by hCG and IL1B in ESCs. During pregnancy, the few available reports suggest a possible role for VCAM1. The expression of this adhesion molecule is strongly induced in the endothelium of early pregnant sheep endometrium [70] and decreases in fetal membranes with advancing gestational age [71]. However, its role in human pregnancy and during embryo implantation remains to be elucidated.

Many other cytokines including IL6, CCL5 (RANTES) and VEGFC appeared to be targeted by hCG and IL1B synergistic action. These pluripotent factors are quite known for being involved in the regulation of immune response, cell proliferation,

tissue remodeling and angiogenesis. First identified as a promoter of B-cell differentiation and antibody production, IL6 is nowadays known as a pleiotropic cytokine that regulates cell growth, angiogenesis, inflammation and hematopoiesis [72]. IL6 expression was described in human granulosa and theca cells, endometrium and pre-implantation embryo [73]. Also, habitual abortion in women is associated with a decrease in expression of IL1B and IL6 [74], suggesting a role for these cytokines in the maintenance of pregnancy. A growing body of evidence implicates CCL5 in the induction of tolerance at immune-privileged sites. This cytokine seems to suppress maternal allogeneic responses, which is necessary for successful implantation [75,76,77,78]. VEGFC is primarily a potent angiogenic growth factor and may play an important role in embryonic cell growth. However, it was described recently as an immune modulator that induces immune tolerance in murine tumor cells [79]. Therefore, these hCG/IL1B-induced biological properties in endometrial cells may represent a relevant mechanism involved in the immune tolerance of the implanting embryo within the uterine maternal host. This is in keeping with a previous study reporting that in vivo infusion of IL1B and hCG induces endometrial changes that mimic early pregnancy events in the baboon and lead to the development of an immunotolerant environment [80].

Invasion of the trophoblast into the endometrium requires a delicate balance between tissue degradation and maintenance. Up-regulation of MMP9 expression in endometrial cells by IL1B in human endometrial cells [81] and secretion by cultured first trimester human trophoblastic cells and fibroblasts has been demonstrated [82]. In addition, the expression of MMPs correlated with the invasive potential of human trophoblast cells [82]. Our micro-array data and qRT-PCR validation revealed the regulation of several tissue remodeling mediators such as MMP9, TIMP3 and KRT19. MMP9 was significantly up-regulated by IL1B in ESCs and hCG seemed to

moderate this action, which, however, remained significant compared to non-stimulated cells. TIMP3, a natural tissue inhibitor of MMPs [83] was down-regulated by IL1B either in the presence or the absence of hCG. The reduction of TIMP3 expression levels, combined with the increased expression of MMP9, may create an imbalance that favors tissue matrix proteolysis and embryo implantation. However, the recent literature reporting that TIMP3 induces apoptosis, inhibits angiogenesis and impedes cell migration [84] makes highly relevant our present findings, considering the crucial importance of embryonic cell survival, proliferation and migration for the establishment of early pregnancy. Interestingly, KRT19, a molecule associated with embryonic placenta development [54] was found to be significantly inhibited by IL1B and more by IL1B combined with hCG. KRT19 is an intermediate filament protein and an epigenetically regulated tumor suppressor gene down-regulated in several cancerous tumors [85]. Also, down-regulation of KRT19 in human oral squamous cell carcinoma lines increases the invasive potential [86], but no other previous studies showed any eventual relationship with the invasive capacity of embryonic cells.

Micro-array and qRT-PCR validation data further revealed a synergistic interaction between hCG and IL1B to induce PTGS2, which is a rate limiting enzyme for PG synthesis. This is quite relevant considering the well-documented role of PGs as key regulators of female reproductive tract functions, including ovulation, menstruation and myometrial contractility, and vascular permeability and angiogenesis at the implantation site [87,88,89].

Interestingly, validation of the expression of some major selected genes at the protein level corroborates the combined role of hCG and IL1B in the modulation of angiogenic, immune and tissue remodeling functions of ESCs. Actually, assessment

of protein secretion showed that hCG and IL1B synergistically induced CCL2 and VEGFC, inhibited TIMP3 and moderate the IL1B-induced MMP9.

Our previous studies showed that hCG down-regulates the IL1B-induced increase in IL1R2 and IL1RN in human ESCs and further enhances the IL1B-induced increase in IL1R1, thereby amplifying in vitro the release of angiogenic activity [23]. Surprisingly, the results of the current micro-array analysis showed a broader spectrum of regulation encompassing other IL1 family members, and suggest a possible modulation of endometrial cell responsiveness to IL1 family. In fact, hCG appeared to potentiate the IL1B-induced expression of IL1R1L (IL33R) and IL18R and to moderate, on the other hand, the expression of endogenous IL33, IL18, IL1A and IL1B in ESCs. Nonetheless, the expression of IL1 isoforms was still up-regulated despite the down-regulatory effect of hCG. These results indicate a possible mechanism by which hCG may induce immunotropism and prevent undue local expression of proinflammatory cytokines, as excessive production levels may be associated with repeated miscarriage and fetal growth retardation [90].

It is noteworthy that according to the recent literature, hCG has been shown to be produced by human endometrial epithelial cells in the luteal phase [12]. Indeed, endogenous endometrial hCG may, though produced at low quantities, have a role in in embryo implantation and the hCG-mediated growth promoting effects, but this is still to be demonstrated.

In conclusion, our study showed that hCG induces major changes in human ESC phenotype and deeply modulates their responsiveness to a proinflammatory, but a growth mediator and a potent embryonic signal such as IL1B. Generally via synergistic stimulatory or inhibitory mechanisms, hCG induces significant alterations in the expression of genes known for being involved or having the potential to play

an important role in embryonic implantation and growth and the modulation of the immune response around the implanting blastocyst. Furthermore, our study revealed that the modulation of endometrial cell receptivity via hCG is not limited to IL1 receptors' agonists and antagonists, but also extends to other IL1 family members, which share numerous growth-promoting, immune-modulating and signaling pathways. This, together with our previous data showing that hCG can similarly target different endometrial cell types, strengthens the relevance of such a modulatory mechanism for implantation and early embryonic growth within the host maternal endometrial tissue and further suggests that hCG plays an important role in the establishment of a receptive endometrial phenotype.

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3.8 References

1. Wilcox AJ, Baird DD, Weinberg CR (1999) Time of implantation of the conceptus and loss of pregnancy. The New England journal of medicine 340: 1796-1799.

2. Evans J, Catalano RD, Brown P, Sherwin R, Critchley HO, et al. (2009) Prokineticin 1 mediates fetal-maternal dialogue regulating endometrial leukemia inhibitory factor. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 23: 2165-2175.

3. Navot D, Scott RT, Droesch K, Veeck LL, Liu HC, et al. (1991) The window of embryo transfer and the efficiency of human conception in vitro. Fertil Steril 55: 114-118.

4. Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, et al. (2000) Embryo implantation. Dev Biol 223: 217-237.

5. Giudice LC (2003) Elucidating endometrial function in the post-genomic era. Hum Reprod Update 9: 223-235.

6. Bazer FW, Wu G, Spencer TE, Johnson GA, Burghardt RC, et al. (2010) Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. Molecular human reproduction 16: 135-152.

7. Geisert R, Fazleabas A, Lucy M, Mathew D (2012) Interaction of the conceptus and endometrium to establish pregnancy in mammals: role of interleukin 1beta. Cell and tissue research.

8. Filicori M, Fazleabas AT, Huhtaniemi I, Licht P, Rao Ch V, et al. (2005) Novel concepts of human chorionic gonadotropin: reproductive system interactions and potential in the management of infertility. Fertil Steril 84: 275-284.

9. Jurisicova A, Antenos M, Kapasi K, Meriano J, Casper RF (1999) Variability in the expression of trophectodermal markers beta-human chorionic gonadotrophin, human leukocyte antigen-G and pregnancy specific beta-1 glycoprotein by the human blastocyst. Human reproduction 14: 1852-1858.

10. Lopata A, Hay DL (1989) The potential of early human embryos to form blastocysts, hatch from their zona and secrete HCG in culture. Human reproduction 4: 87-94.

11. Hoshina M, Boothby M, Hussa R, Pattillo R, Camel HM, et al. (1985) Linkage of human chorionic gonadotrophin and placental lactogen biosynthesis to trophoblast differentiation and tumorigenesis. Placenta 6: 163-172.

12. Zimmermann G, Ackermann W, Alexander H (2009) Epithelial human chorionic gonadotropin is expressed and produced in human secretory endometrium during the normal menstrual cycle. Biology of reproduction 80: 1053-1065.

13. Zimmermann G, Ackermann W, Alexander H (2012) Expression and production of human chorionic gonadotropin (hCG) in the normal secretory endometrium: evidence of CGB7 and/or CGB6 beta hCG subunit gene expression. Biology of reproduction 86: 87.

14. Filicori M, Fazleabas AT, Huhtaniemi I, Licht P, Rao Ch V, et al. (2005) Novel concepts of human chorionic gonadotropin: reproductive system interactions and potential in the management of infertility. Fertility and sterility 84: 275-284.

15. Bernardini L, Moretti-Rojas I, Brush M, Rojas FJ, Balmaceda JP (1995) Status of hCG/LH receptor and G proteins in human endometrium during artificial cycles of hormone replacement therapy. J Soc Gynecol Investig 2: 630-635.

16. Reshef E, Lei ZM, Rao CV, Pridham DD, Chegini N, et al. (1990) The presence of gonadotropin receptors in nonpregnant human uterus, human placenta, fetal membranes, and decidua. J Clin Endocrinol Metab 70: 421-430.

17. Tang B, Gurpide E (1993) Direct effect of gonadotropins on decidualization of human endometrial stroma cells. J Steroid Biochem Mol Biol 47: 115-121.

18. Han SW, Lei ZM, Rao CV (1999) Treatment of human endometrial stromal cells with chorionic gonadotropin promotes their morphological and functional differentiation into decidua. Mol Cell Endocrinol 147: 7-16.

19. Zygmunt M, Herr F, Keller-Schoenwetter S, Kunzi-Rapp K, Munstedt K, et al. (2002) Characterization of human chorionic gonadotropin as a novel angiogenic factor. J Clin Endocrinol Metab 87: 5290-5296.

20. Kane N, Kelly R, Saunders PT, Critchley HO (2009) Proliferation of uterine natural killer cells is induced by human chorionic gonadotropin and mediated via the mannose receptor. Endocrinology 150: 2882-2888.

21. Rao CV, Lei ZM (2007) The past, present and future of nongonadal LH/hCG actions in reproductive biology and medicine. Mol Cell Endocrinol 269: 2-8.

22. Sherwin JR, Sharkey AM, Cameo P, Mavrogianis PM, Catalano RD, et al. (2007) Identification of novel genes regulated by chorionic gonadotropin in baboon endometrium during the window of implantation. Endocrinology 148: 618-626.

23. Bourdiec A, Shao R, Rao CV, Akoum A (2012) Human Chorionic Gonadotropin Triggers Angiogenesis via the Modulation of Endometrial Stromal Cell Responsiveness to Interleukin 1: A New Possible Mechanism Underlying Embryo Implantation. Biology of reproduction.

24. Paulesu L, Jantra S, Ietta F, Brizzi R, Bigliardi E (2008) Interleukin-1 in reproductive strategies. Evolution & development 10: 778-788.

25. Simon C, Frances A, Piquette G, Hendrickson M, Milki A, et al. (1994) Interleukin-1 system in the materno-trophoblast unit in human implantation: immunohistochemical evidence for autocrine/paracrine function. The Journal of clinical endocrinology and metabolism 78: 847-854.

26. Simon C, Frances A, Piquette GN, el Danasouri I, Zurawski G, et al. (1994) Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist. Endocrinology 134: 521-528.

27. Krussel JS, Bielfeld P, Polan ML, Simon C (2003) Regulation of embryonic implantation. European journal of obstetrics, gynecology, and reproductive biology 110 Suppl 1: S2-9.

28. Baranao RI, Piazza A, Rumi LS, Polak de Fried E (1997) Determination of IL-1 and IL-6 levels in human embryo culture-conditioned media. American journal of reproductive immunology 37: 191-194.

29. Sheth KV, Roca GL, al-Sedairy ST, Parhar RS, Hamilton CJ, et al. (1991) Prediction of successful embryo implantation by measuring interleukin-1-alpha and immunosuppressive factor(s) in preimplantation embryo culture fluid. Fertil Steril 55: 952-957.

30. Bankers-Fulbright JL, Kalli KR, McKean DJ (1996) Interleukin-1 signal transduction. Life sciences 59: 61-83.

31. Rossi M, Sharkey AM, Vigano P, Fiore G, Furlong R, et al. (2005) Identification of genes regulated by interleukin-1beta in human endometrial stromal cells. Reproduction 130: 721-729.

32. Sawai K, Matsuzaki N, Okada T, Shimoya K, Koyama M, et al. (1997) Human decidual cell biosynthesis of leukemia inhibitory factor: regulation by decidual cytokines and steroid hormones. Biology of reproduction 56: 1274-1280.

33. Tabibzadeh S, Kaffka KL, Satyaswaroop PG, Kilian PL (1990) Interleukin-1 (IL-1) regulation of human endometrial function: presence of IL-1 receptor correlates with IL-1-stimulated prostaglandin E2 production. The Journal of clinical endocrinology and metabolism 70: 1000-1006.

34. Dey SK, Lim H, Das SK, Reese J, Paria BC, et al. (2004) Molecular cues to implantation. Endocrine reviews 25: 341-373.

35. Simon C, Gimeno MJ, Mercader A, O'Connor JE, Remohi J, et al. (1997) Embryonic regulation of integrins beta 3, alpha 4, and alpha 1 in human endometrial epithelial cells in vitro. The Journal of clinical endocrinology and metabolism 82: 2607-2616.

36. Hirota Y, Osuga Y, Hasegawa A, Kodama A, Tajima T, et al. (2009) Interleukin (IL)-1beta stimulates migration and survival of first-trimester villous cytotrophoblast cells through endometrial epithelial cell-derived IL-8. Endocrinology 150: 350-356.

37. Librach CL, Feigenbaum SL, Bass KE, Cui TY, Verastas N, et al. (1994) Interleukin-1 beta regulates human cytotrophoblast metalloproteinase activity and invasion in vitro. The Journal of biological chemistry 269: 17125-17131.

38. Strakova Z, Szmidt M, Srisuparp S, Fazleabas AT (2003) Inhibition of matrix metalloproteinases prevents the synthesis of insulin-like growth factor binding protein-1 during decidualization in the baboon. Endocrinology 144: 5339-5346.

39. Yagel S, Lala PK, Powell WA, Casper RF (1989) Interleukin-1 stimulates human chorionic gonadotropin secretion by first trimester human trophoblast. The Journal of clinical endocrinology and metabolism 68: 992-995.

40. Greenfeder SA, Nunes P, Kwee L, Labow M, Chizzonite RA, et al. (1995) Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. J Biol Chem 270: 13757-13765.

41. Colotta F, Re F, Muzio M, Bertini R, Polentarutti N, et al. (1993) Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. Science 261: 472-475.

42. Herrmann-Lavoie C, Rao CV, Akoum A (2007) Chorionic gonadotropin down-regulates the expression of the decoy inhibitory interleukin 1 receptor type II in human endometrial epithelial cells. Endocrinology 148: 5377-5384.

43. Niu J, Azfer A, Zhelyabovska O, Fatma S, Kolattukudy PE (2008) Monocyte chemotactic protein (MCP)-1 promotes angiogenesis via a novel transcription factor, MCP-1-induced protein (MCPIP). J Biol Chem 283: 14542-14551.

44. Hong KH, Ryu J, Han KH (2005) Monocyte chemoattractant protein-1-induced angiogenesis is mediated by vascular endothelial growth factor-A. Blood 105: 1405-1407.

45. Noyes RW, Hertig AT, Rock J (1975) Dating the endometrial biopsy. American journal of obstetrics and gynecology 122: 262-263.

46. Akoum A, Lemay A, Brunet C, Hebert J (1995) Cytokine-induced secretion of monocyte chemotactic protein-1 by human endometriotic cells in culture. The Groupe d'Investigation en Gynecologie. Am J Obstet Gynecol 172: 594-600.

47. Cole LA (2010) Biological functions of hCG and hCG-related molecules. Reproductive biology and endocrinology : RB&E 8: 102.

48. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, et al. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4: 249-264.

49. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I (2001) Controlling the false discovery rate in behavior genetics research. Behavioural brain research 125: 279-284.

50. Wettenhall JM, Simpson KM, Satterley K, Smyth GK (2006) affylmGUI: a graphical user interface for linear modeling of single channel microarray data. Bioinformatics 22: 897-899.

51. Carli C, Metz CN, Al-Abed Y, Naccache PH, Akoum A (2009) Up-regulation of cyclooxygenase-2 expression and prostaglandin E2 production in human endometriotic cells by macrophage migration inhibitory factor: involvement of novel kinase signaling pathways. Endocrinology 150: 3128-3137.

52. Bellehumeur C, Blanchet J, Fontaine JY, Bourcier N, Akoum A (2009) Interleukin 1 regulates its own receptors in human endometrial cells via distinct mechanisms. Human reproduction 24: 2193-2204.

53. Akoum A, Lemay A, Maheux R (2002) Estradiol and interleukin-1beta exert a synergistic stimulatory effect on the expression of the chemokine regulated upon activation, normal T cell expressed, and secreted in endometriotic cells. The Journal of clinical endocrinology and metabolism 87: 5785-5792.

54. Maurer J, Nelson B, Cecena G, Bajpai R, Mercola M, et al. (2008) Contrasting expression of keratins in mouse and human embryonic stem cells. PloS one 3: e3451.

55. Islami D, Bischof P, Chardonnens D (2003) Modulation of placental vascular endothelial growth factor by leptin and hCG. Mol Hum Reprod 9: 395-398.

56. Herrmann-Lavoie C, Rao CV, Akoum A (2007) Chorionic Gonadotropin Downregulates the Expression of the Decoy Inhibitory Interleukin 1 Receptor Type II in Human Endometrial Epithelial Cells. Endocrinology.

57. Lessey BA, Castelbaum AJ, Sawin SW, Buck CA, Schinnar R, et al. (1994) Aberrant integrin expression in the endometrium of women with endometriosis. J Clin Endocrinol Metab 79: 643-649.

58. Sugihara K, Kabir-Salmani M, Byrne J, Wolf DP, Lessey B, et al. (2008) Induction of trophinin in human endometrial surface epithelia by CGbeta and IL-1beta. FEBS letters 582: 197-202.

59. Norwitz ER, Schust DJ, Fisher SJ (2001) Implantation and the survival of early pregnancy. The New England journal of medicine 345: 1400-1408.

60. Dominguez F, Pellicer A, Simon C (2003) The chemokine connection: hormonal and embryonic regulation at the human maternal-embryonic interface--a review. Placenta 24 Suppl B: S48-55.

61. Castro-Rendon WA, Castro-Alvarez JF, Guzman-Martinez C, Bueno-Sanchez JC (2006) Blastocyst-endometrium interaction: intertwining a cytokine network. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica [et al] 39: 1373-1385.

62. Proost P, Wuyts A, Van Damme J (1996) Human monocyte chemotactic proteins-2 and -3: structural and functional comparison with MCP-1. Journal of leukocyte biology 59: 67-74.

63. Gu L, Rutledge B, Fiorillo J, Ernst C, Grewal I, et al. (1997) In vivo properties of monocyte chemoattractant protein-1. Journal of leukocyte biology 62: 577-580.

64. Niu J, Azfer A, Zhelyabovska O, Fatma S, Kolattukudy PE (2008) Monocyte chemotactic protein (MCP)-1 promotes angiogenesis via a novel transcription factor, MCP-1-induced protein (MCPIP). The Journal of biological chemistry 283: 14542-14551.

65. van Mourik MS, Macklon NS, Heijnen CJ (2009) Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment. Journal of leukocyte biology 85: 4-19.

66. Chen SJ, Liu YL, Sytwu HK (2012) Immunologic regulation in pregnancy: from mechanism to therapeutic strategy for immunomodulation. Clinical & developmental immunology 2012: 258391.

67. Fest S, Aldo PB, Abrahams VM, Visintin I, Alvero A, et al. (2007) Trophoblast-macrophage interactions: a regulatory network for the protection of pregnancy. American journal of reproductive immunology 57: 55-66.

68. Houser BL (2012) Decidual macrophages and their roles at the maternal-fetal interface. The Yale journal of biology and medicine 85: 105-118.

69. Lee S, Yoon IH, Yoon A, Cook-Mills JM, Park CG, et al. (2012) An Antibody to the Sixth Ig-like Domain of VCAM-1 Inhibits Leukocyte Transendothelial Migration without Affecting Adhesion. Journal of immunology.

70. Rahman AN, Snibson KJ, Lee CS, Meeusen EN (2004) Effects of implantation and early pregnancy on the expression of cytokines and vascular surface molecules in the sheep endometrium. Journal of reproductive immunology 64: 45-58.

71. Vega-Sanchez R, de Jesus-Torres E, Arenas-Hernandez M, Beltran-Montoya J, Maida-Claros R, et al. (2010) [Expression of cell adhesion molecules in the maternal fetal period of human gestation]. Ginecologia y obstetricia de Mexico 78: 677-684.

72. Altun T, Jindal S, Greenseid K, Shu J, Pal L (2011) Low follicular fluid IL-6 levels in IVF patients are associated with increased likelihood of clinical pregnancy. Journal of assisted reproduction and genetics 28: 245-251.

73. Tabibzadeh S, Kong QF, Babaknia A, May LT (1995) Progressive rise in the expression of interleukin-6 in human endometrium during menstrual cycle is initiated during the implantation window. Human reproduction 10: 2793-2799.

74. von Wolff M, Thaler CJ, Strowitzki T, Broome J, Stolz W, et al. (2000) Regulated expression of cytokines in human endometrium throughout the menstrual cycle: dysregulation in habitual abortion. Molecular human reproduction 6: 627-634.

75. Hornung D, Bentzien F, Wallwiener D, Kiesel L, Taylor RN (2001) Chemokine bioactivity of RANTES in endometriotic and normal endometrial stromal cells and peritoneal fluid. Molecular human reproduction 7: 163-168.

76. Huang SJ, Schatz F, Masch R, Rahman M, Buchwalder L, et al. (2006) Regulation of chemokine production in response to pro-inflammatory cytokines in first trimester decidual cells. Journal of reproductive immunology 72: 60-73.

77. Ramhorst R, Patel R, Corigliano A, Etchepareborda JJ, Fainboim L, et al. (2006) Induction of maternal tolerance to fetal alloantigens by RANTES production. American journal of reproductive immunology 56: 302-311.

78. Ramhorst R, Gutierrez G, Corigliano A, Junovich G, Fainboim L (2007) Implication of RANTES in the modulation of alloimmune response by progesterone during pregnancy. American journal of reproductive immunology 57: 147-152.

79. Lund AW, Duraes FV, Hirosue S, Raghavan VR, Nembrini C, et al. (2012) VEGF-C Promotes Immune Tolerance in B16 Melanomas and Cross-Presentation of Tumor Antigen by Lymph Node Lymphatics. Cell reports 1: 191-199.

80. Strakova Z, Mavrogianis P, Meng X, Hastings JM, Jackson KS, et al. (2005) In vivo infusion of interleukin-1beta and chorionic gonadotropin induces endometrial

changes that mimic early pregnancy events in the baboon. Endocrinology 146: 4097-4104.

81. Guay S, Akoum A (2007) Stable inhibition of interleukin, 1 receptor type II in Ishikawa cells augments secretion of matrix metalloproteinases: possible role in endometriosis pathophysiology. Reproduction (accepted with modifications).

82. Anacker J, Segerer SE, Hagemann C, Feix S, Kapp M, et al. (2011) Human decidua and invasive trophoblasts are rich sources of nearly all human matrix metalloproteinases. Molecular human reproduction 17: 637-652.

83. Butler GS, Apte SS, Willenbrock F, Murphy G (1999) Human tissue inhibitor of metalloproteinases 3 interacts with both the N- and C-terminal domains of gelatinases A and B. Regulation by polyanions. The Journal of biological chemistry 274: 10846-10851.

84. Black RA (2004) TIMP3 checks inflammation. Nature genetics 36: 934-935.

85. Caren H, Djos A, Nethander M, Sjoberg RM, Kogner P, et al. (2011) Identification of epigenetically regulated genes that predict patient outcome in neuroblastoma. BMC cancer 11: 66.

86. Crowe DL, Milo GE, Shuler CF (1999) Keratin 19 downregulation by oral squamous cell carcinoma lines increases invasive potential. Journal of dental research 78: 1256-1263.

87. Sales KJ, Grant V, Catalano RD, Jabbour HN (2011) Chorionic gonadotrophin regulates CXCR4 expression in human endometrium via E-series prostanoid receptor

2 signalling to PI3K-ERK1/2: implications for fetal-maternal crosstalk for embryo implantation. Molecular human reproduction 17: 22-32.

88. Jabbour HN, Kelly RW, Fraser HM, Critchley HO (2006) Endocrine regulation of menstruation. Endocrine reviews 27: 17-46.

89. Matsumoto H, Ma WG, Daikoku T, Zhao X, Paria BC, et al. (2002) Cyclooxygenase-2 differentially directs uterine angiogenesis during implantation in mice. The Journal of biological chemistry 277: 29260-29267.

90. Kauma S, Matt D, Strom S, Eierman D, Turner T (1990) Interleukin-1 beta, human leukocyte antigen HLA-DR alpha, and transforming growth factor-beta expression in endometrium, placenta, and placental membranes. American journal of obstetrics and gynecology 163: 1430-1437.

3.9 Figure Legends

Figure 1. Analysis of genes significantly modulated by each treatment. A) Headmap of probe sets corresponding to genes significantly modulated ($P < 0.05$) in each group. Increased signal intensities are displayed in red, whereas lower signal intensities are shown in blue. Cluster distances were evaluated by Spearman correlation on average linkage (Partek Genomics Suite). B) PCA scatter plot of all samples was generated to assess the variability of micro-array data. Each sphere represents a whole chip data. As shown in the legend, samples are colored by treatment and grouped by an ellipsoid that considers two standard deviations from the center of each group. C) Venn diagram of the respective gene lists showing the overlap of action between hCG/MM, MM/IL1B and hCG/IL1B. Data were obtained with ESC cultures issued from 3 different subjects.

Figure 2. Enrichment score of biological processes. A) GO analysis was used to identify the main biological processes targeted by gene lists and significantly modulated by each treatment. Each functional group was assigned with a GO enrichment score that was calculated using a chi2 test. B) A forest plot using the same gene list was also generated to show the percentage of differentially expressed genes that were up-regulated (red) or down-regulated (blue) for each biological process. Light color: gene populations with a fold change ranging from 1.5 to 2. Dark color: gene populations with a fold change above 2. Data were obtained with ESC cultures issued from 3 different subjects.

Figure 3. hCG modulates MCPs' mRNA expression in ESCs. Confluent ESC cultures were incubated with minimal medium (MM) (control) or hCG (100 ng/mL) for 24h

before being exposed or not to IL1B (0.1 ng/mL) for additional 24h. Total RNA was extracted and reverse transcribed. CCL2, CCL8, CCL7 and GAPDH (internal control) mRNA levels were quantified by real-time PCR. CCL2 (A), CCL8 (B) and CCL7 (C) mRNA ratio was then determined following normalization to GAPDH mRNA. Data were from ESC cultures issued from 7 different subjects and expressed as fold change (FC) over control (ratio of CCL2, CCL8 or CCL7 mRNA levels found in cells incubated with IL1B, hCG or hCG/ILB to those found in cells incubated with MM for an equivalent period of time). *P < 0.05, *** P < 0.001 relative to MM; †P < 0.05, †††P < 0.001 relative to cells stimulated with an equivalent concentration of hCG; +P < 0.05 relative to cells stimulated with an equivalent concentration of IL1B. Data were obtained with ESC cultures issued from 7 different subjects (the 3 cultures used for microarray analysis and 4 additional cultures).

Figure 4. hCG modulates the IL1B-mediated mRNA expression of immune modulating, adhesion, growth, angiogenic and tissue remodeling factors in ESCs. Confluent ESC cultures were incubated with minimal medium (MM) (control) or hCG (100 ng/mL) for 24h before being exposed or not to IL1B (0.1 ng/mL) for additional 24h. Total RNA was extracted and reverse transcribed, and mRNA levels were then quantified by qRT-PCR. VCAM1 (A), IL6 (B), CCL5 (C), PTGS2 (D), VEGFC (E), MMP9 (F), TIMP3 (G) and KRT19 (H) mRNA ratio was then determined following normalization to GAPDH mRNA (internal control). Data were from ESC cultures issued from 7 different subjects and expressed as fold change (FC) over control (ratio of VCAM1, IL6, CCL5, PTGS2, VEGFC, MMP9, TIMP3 or KRT19 mRNA levels found in cells incubated with IL1B, hCG or hCG/ILB to those

found in cells incubated with MM for an equivalent period of time). *P < 0.05, **P < 0.01, *** P < 0.001 relative to MM; †P < 0.05, ††P < 0.01, †††P < 0.001 relative to cells stimulated with an equivalent concentration of hCG; +P < 0.05 relative to cells stimulated with an equivalent concentration of IL1B. Data were obtained with ESC cultures issued from 7 different subjects (the 3 cultures used for microarray analysis and 4 additional cultures).

Figure 5. hCG modulates IL1B effects on the expression of IL1 family members in ESCs. Confluent ESC cultures were incubated with minimal medium (MM) or hCG (100 ng/mL) for 24h before being exposed or not to IL1B (0.1 ng/mL) for additional 24h. Total RNA was extracted and reverse transcribed, and mRNA levels were then quantified by qRT-PCR. IL1A (A), IL1B (D), IL1RL1 (B), IL18R1 (E), IL33 (C) and IL18 (F) mRNA ratio was then determined following normalization to GAPDH mRNA (internal control). Data were from ESC cultures issued from 7 different subjects and expressed as fold change (FC) over control (ratio of IL1A, IL1B, IL1RL1, IL18R1, IL33 or IL18 mRNA levels found in cells incubated with IL1B, hCG or hCG/ILB to those found in cells incubated with MM for an equivalent period of time). *P < 0.05, **P < 0.01, *** P < 0.001 relative to MM; †P < 0.05, †††P < 0.001 relative to cells stimulated with an equivalent concentration of hCG; ++P < 0.01 relative to cells stimulated with an equivalent concentration of IL1B. Data were obtained with ESC cultures issued from 7 different subjects (the 3 cultures used for microarray analysis and 4 additional cultures).

Figure 6. hCG modulates the expression of immune, angiogenic and tissue remodeling factors in ESCs at the protein level. Confluent ESC cultures were

incubated with minimal medium (MM) or hCG (100 ng/mL) for 24h before being exposed or not to IL1B (0.1 ng/mL) for additional 24h. Supernatant was collected and soluble proteins CCL2 (A) CCL5 (B) VEGFC (C) TIMP3 (D) MMP9 (E) were then quantified by ELISA. Data were from ESC cultures issued from 4 different subjects and expressed in pg/mL. *P < 0.05, *** P < 0.001 relative to MM; ††P < 0.01, †††P < 0.001 relative to cells stimulated with an equivalent concentration of hCG; +P < 0.05, +++P <0.001 relative to cells stimulated with an equivalent concentration of IL1B.

3.10 Figures

Figure 1 Analysis of genes significantly modulated by each treatment.

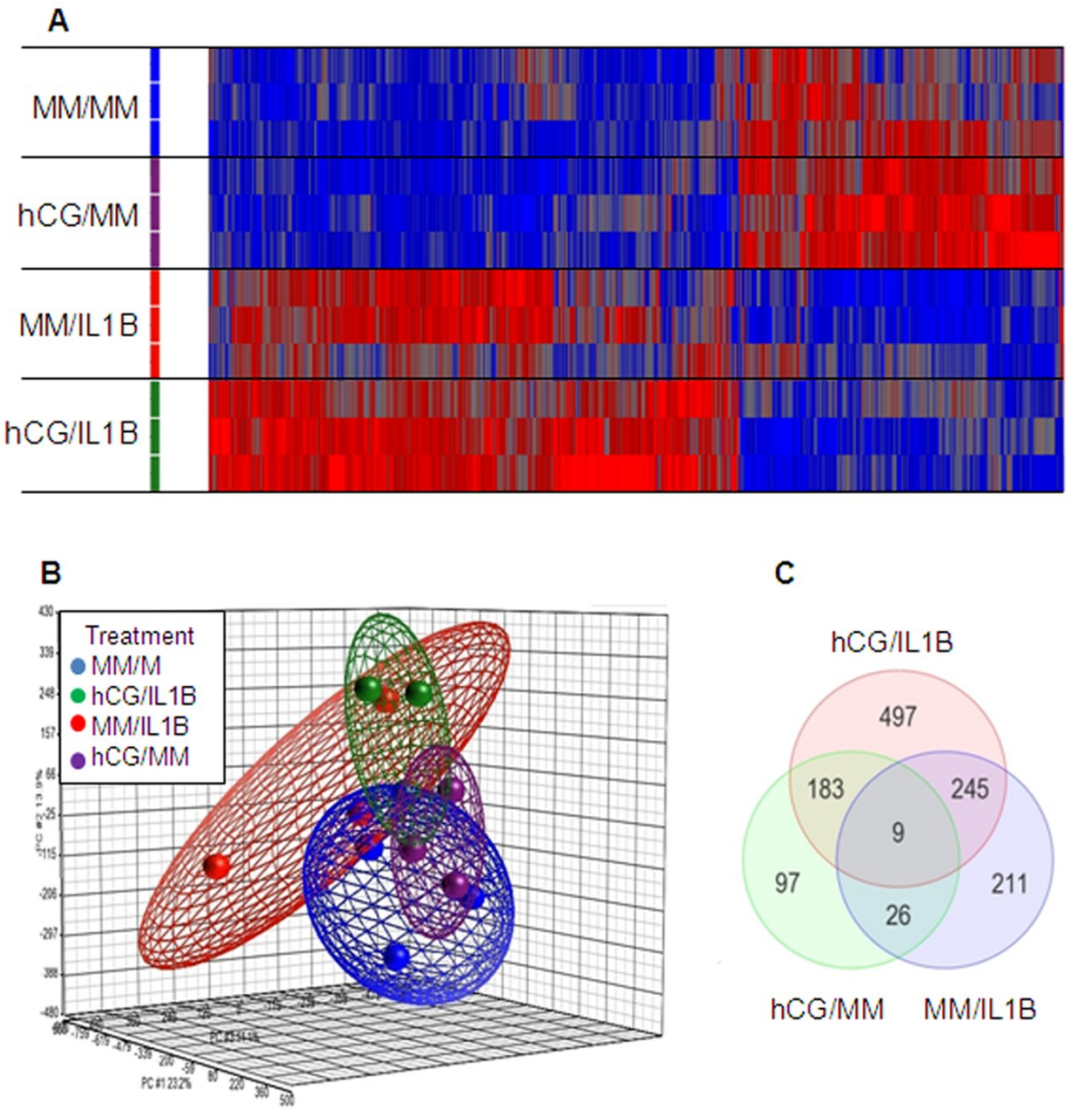

Figure 2 Enrichment score of biological processes.

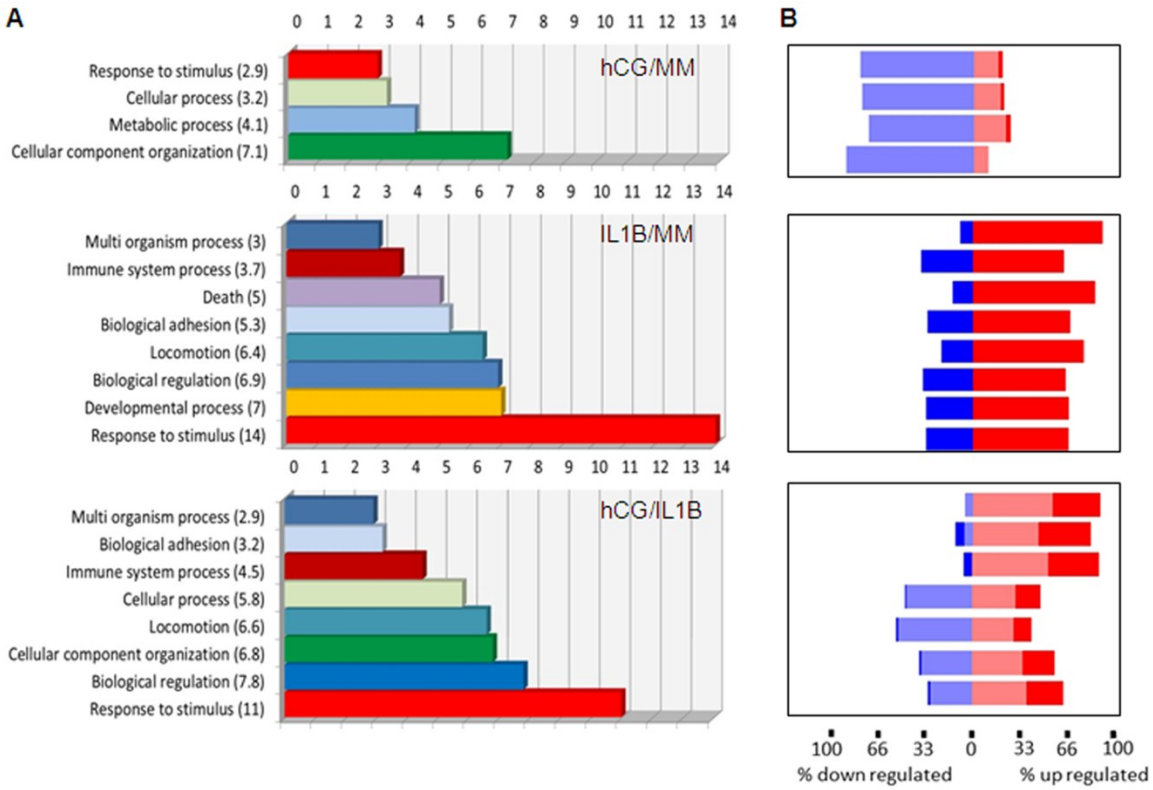

Figure 3 hCG modulates MCPs' mRNA expression in ESCs.

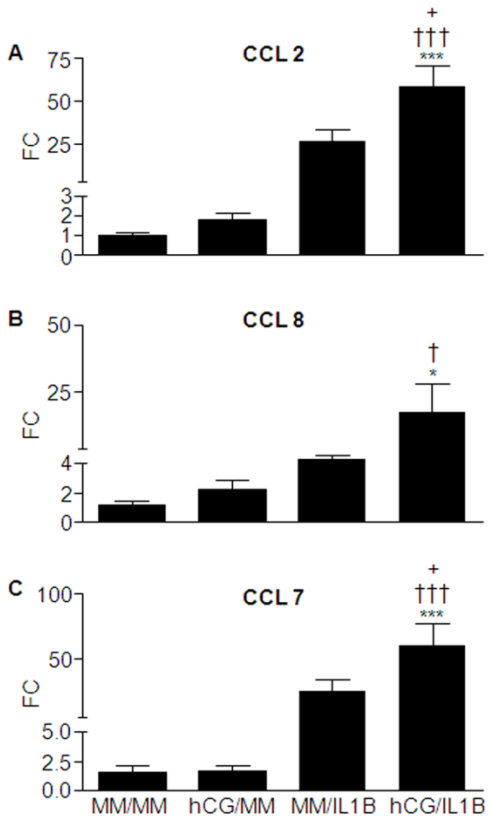

Figure 4 hCG modulates the IL1B-mediated mRNA expression of immune modulating, adhesion, growth, angiogenic and tissue remodeling factors in ESCs.

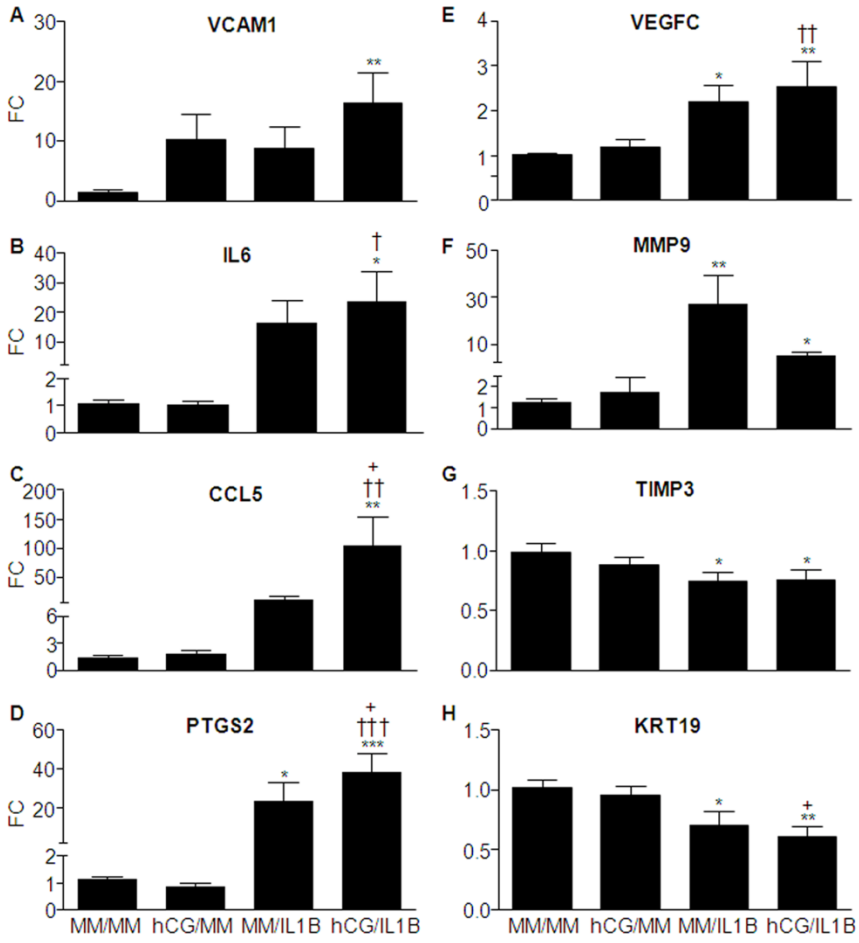

Figure 5 hCG modulates IL1B effects on the expression of IL1 family members in ESCs.

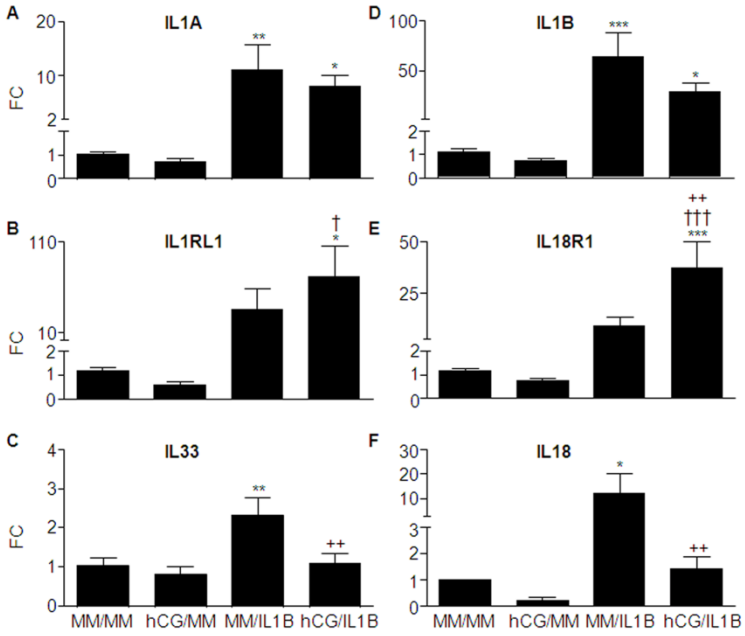

Figure 6 hCG modulates the expression of immune, angiogenic and tissue remodeling factors in ESCs at the protein level.

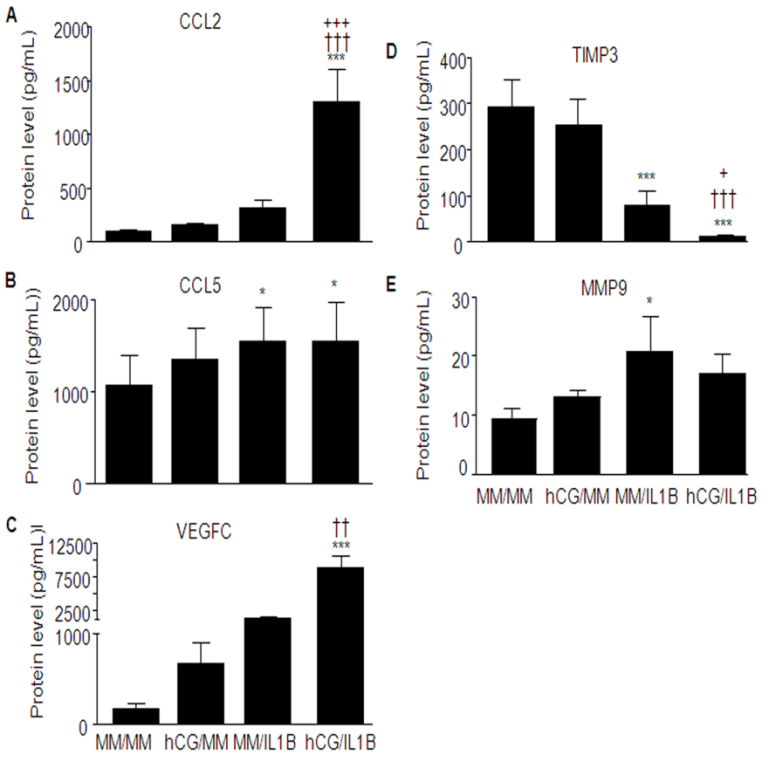

Table 1. List of PCR primers.

Gene	Primer	Tm°C
CCL2	F-CTCTGCCGCCCTTCTGT	60
	R-CTTCTTTGGGACACTTGCTG	
CCL5	F-CTCGCTGTCATCCTCA	56
	R-CACTTGCCACTGGTGTA	
CCL7	F-GCCTCTGCAGCACTTCTGTG	60
	R-CACTTCTGTGTGGGGTCAGC	
CCL8	F-CTTCAAGACCAAACGG	52
	R-GAATCCCTGACCCAT	
GAPDH	F- CAGGGCTGCTTTTAACTCTGG	60
	R-TGGGTGGAATCATATTGGAACA	
IL18R1	F-CTGGAGGAGCTGTTGT	60
	R-GATTAGTCTTCGGCTTT	
IL1A	F-AAGACAGTTCCTCCAT	52
	R-TTGCTACTACCACCAT	
IL1B	F-ACAGTGGCAATGAGGATG	58
	R-TGTAGTGGTGGTCGGAGA	
IL1RL1	F-CTGAGGACGCAGGTGA	54
	R-CTCCGATTACTGGAAACA	
IL18	F-GCCAGCCTAGAGGTATG	60
	R-GTTATCAGGAGGATTCATTT	
IL33	F-CAGGTGACGGTGTTG	56
	R-TGTAGGACTCAGGGTTA	
IL6	F-GGAGACTTGCCTGGTGAA	60
	R-GCATTTGTGGTTGGGTCA	
KRT19	F-CGACAATGCCCGTCTG	58
	R-GCCTGTTCCGTCTCAAA	
MMP10	F-CAAGAGGCATCCATAC	54
	R-AACCTTAGGCTCAACT	
MMP9	F-TTGACAGCGACAAGAAGTGG	54
	R-CCCTCAGTGAAGCGGTACAT	
PTGS2	F-TCCCTTGGGTGTCAAAGGTAA	60
	R-AAAACTGATGCGTGAAGTGCTG	
TIMP3	F-CTCCGACATCGTGATC	54
	R-TCCTTTACCAGCTTCTT	
VCAM1	F-TGAAGGATGCGGGAGT	58
	R-GCAGGTATTATTAAGGAGG	
VEGFC	F-GCCAGCAACACTACCA	58
	R-TTGAGTCATCTCCAGCAT	

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Table 2. Genes up-regulated after in vitro treatment of human endometrium stromal cells.

Gene	*p*-value hCG/IL1	FC hCG/IL1	*p*-value MM/IL1	FC MM/IL1	*p*-value hCG/MM	FC hCG/MM	IPA Pf.	IPA Im.	IPA Rm.	IPA CS.	IPA Ap.	IPA Ag.
CXCL6	0.00001	35.52	0.00002	21.51				X		X		
IL6[α]	0.00088	15.06	0.00147	12.16			X	X	X	X	X	X
CXCL1	0.00067	13.94	0.00090	12.35			X	X		X	X	
TNFAIP3	0.00005	13.72	0.00027	8.03						X	X	X
IL1B[α]	0.00076	13.13	0.00020	23.61			X	X	X	X	X	
CYP7B1	0.00000	12.96	0.00001	5.09	0.02288	1.60	X		X			
TNFAIP6	0.00013	11.79	0.00448	4.08						X		
PTGS2[α]	0.00203	10.48	0.00368	8.33			X	X	X	X	X	
CCL5[αβ]	0.01045	9.57	0.03962	5.30			X	X		X	X	
IL8	0.00318	9.27	0.00227	10.59			X	X		X	X	
C3	0.00337	8.42	0.01599	4.83			X	X		X	X	
VCAM1[α]	0.00099	8.08					X	X		X		
ITGB8	0.00002	8.07	0.00079	3.39			X			X		
CXCL2	0.00077	7.25	0.00343	4.69			X			X	X	
TNFAIP2	0.00004	7.10										
CCL2[αβ]	0.00249	6.74	0.01108	4.25			X	X		X	X	
CTSS	0.00014	6.68	0.00064	4.57			X	X			X	
IRAK3	0.00001	6.56	0.00023	3.58						X	X	X
CFB	0.00235	6.14					X			X		
EPSTI1	0.00639	5.55										
IL24	0.00217	5.26	0.00063	7.59			X	X		X	X	
IL13RA2	0.01786	5.17	0.03330	4.14			X					
MMP12	0.01798	4.49	0.01483	4.79								
NFKBIZ	0.00003	4.14	0.00005	3.84							X	
CX3CL1	0.02049	3.95						X	X	X	X	
IL7R	0.00167	3.92	0.00162	3.95			X	X		X	X	X
FGF7	0.00367	3.87			0.02245	2.57	X	X	X	X		
GNA15	0.00250	3.85								X		
CXCL3	0.00228	3.81					X	X		X		
CSF1	0.00613	3.60					X	X	X	X	X	
CCL8[α]	0.00031	3.47						X		X		
ZC3H12A	0.00003	3.23	0.00010	2.76								
ICAM1	0.00001	3.13	0.00002	2.94			X	X	X	X	X	
IL32	0.00011	3.07	0.00285	1.97			X	X		X	X	
ANK2	0.00002	3.05			0.00215	1.73						
CXCL5	0.01034	3.02	0.01015	3.04			X			X		
IL15RA	0.00093	2.94					X	X		X	X	
IL1A[α]	0.00012	2.88	0.00001	4.43			X	X	X	X	X	
PTGES	0.00004	2.84					X	X		X	X	
ITGA8	0.01123	2.76										
IRAK2	0.01232	2.67	0.00250	3.75						X		X
NFKBIA	0.00003	2.60	0.00007	2.37			X			X	X	X
WNT2	0.00299	2.54			0.00760	2.20				X	X	
LIF	0.00019	2.37	0.00078	2.01			X	X	X	X	X	
CYP1B1	0.01872	2.35					X			X	X	
BAMBI	0.04533	2.28								X		
IL1RL1[α]	0.01807	2.05					X	X		X	X	
FGF2	0.00006	1.93	0.00006	1.93			X			X	X	
HLA-DOB	0.00425	1.89										
C1R	0.00242	1.83										
CCL7[α]	0.03055	1.80						X		X		

Pf, proliferation; Im, immune functions; Rm, tissue remodeling; CS, cell signaling; Ap, apoptosis; Ag, angiogenesis
[α/β] Real-time PCR / ELISA validation performed

TABLE 2(ctd).

Gene	*p*-value hCG/IL1	FC hCG/IL1	*p*-value MM/IL1	FC MM/IL1	*p*-value hCG/MM	FC hCG/MM	IPA Pf.	IPA Im.	IPA Rm.	IPA CS.	IPA Ap.	IPA Ag.
C1S	0.00019	1.79			0.00136	1.54						
HLA-F	0.00278	1.71										
IL20	0.00004	1.69					X			X		
FOXP1	0.00162	1.66					X			X	X	
IL18R1$^{\alpha}$	0.00483	1.65						X		X		
IFNGR1	0.01156	1.65					X			X	X	
C2	0.02976	1.65										
SPAG1	0.01701	1.64										
IL7	0.00011	1.63					X	X	X	X	X	
TNFRSF1B	0.00134	1.62					X	X	X	X	X	
IL17RB	0.01191	1.62						X				
HLA-E	0.00189	1.61					X					
DUSP1	0.01584	1.57					X	X		X	X	
NFKB1	0.00005	1.56					X	X	X	X	X	
PDGFC	0.00670	1.52					X			X		
EGFR	0.00112	1.51					X			X	X	
CLDN12	0.00231	1.50								?		
INHBA			0.03972	2.82			X	X		X	X	
CLDN1			0.00226	2.42						X		
SERPINB3			0.01389	2.09						X	X	X
CYP19A1			0.04113	2.05							X	
VEGFC$^{\alpha\beta}$			0.03365	2.05			X			X	X	
MMP9$^{\alpha\beta}$			0.00034	1.93			X		X	X	X	
MMP3			0.02941	1.81			X				X	
IL4R			0.00190	1.77			X	X		X	X	X
SAT1			0.01189	1.68			X				X	
EPHA6			0.01086	1.67								
TRAF3IP2			0.00848	1.63						X	X	
NFKB2			0.00022	1.62			X	X		X	X	
MMP13			0.01643	1.62					X	X		
ADAM12			0.02572	1.60						X	X	
CASP3			0.00748	1.59			X	X		X	X	
ALDH1A1					0.00743	4.10				X	X	
CD34					0.00212	2.14				X		
FAT4					0.00889	2.14						
CXCL12					0.04752	2.06	X	X	X	X	X	
ANGPTL2					0.01814	1.96						
CLDN11					0.01104	1.96	X			X		
SEPP1					0.02775	1.93				X		
TMOD1					0.00298	1.92				X		
C1RL					0.02441	1.67						
CADM1					0.01684	1.66	X			X	X	
C4A					0.04847	1.66		X		X		
CRYAB					0.04510	1.62	X				X	
CNN1					0.03209	1.60	X					
SLC2A12					0.00483	1.58						
HSD17B6					0.02452	1.55						
SMAD9					0.02996	1.54						
ITPR1					0.00157	1.53	X			X	X	X

Pf, proliferation; Im, immune functions; Rm, tissue remodeling; CS, cell signaling; Ap, apoptosis; Ag, angiogenesis

$^{\alpha/\beta}$ Real-time PCR / ELISA validation performed

TABLE 3. Name of up-regulated genes

RefSeq	Gene symbol	Genes name
NM_002993	CXCL6	chemokine (C-X-C motif) ligand 6
NM_000600	IL6	interleukin 6 (interferon, beta 2)
NM_001511	CXCL1	chemokine (C-X-C motif) ligand 1
NM_006290	TNFAIP3	tumor necrosis factor, alpha-induced protein 3
NM_000576	IL1B	interleukin 1, beta
NM_004820	CYP7B1	cytochrome P450, family 7, subfamily B, polypeptide 1
NM_007115	TNFAIP6	tumor necrosis factor, alpha-induced protein 6
NM_000963	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
NM_002985	CCL5	chemokine (C-C motif) ligand 5
NM_000584	IL8	interleukin 8
NM_000064	C3	complement component 3
NM_001078	VCAM1	vascular cell adhesion molecule 1
NM_002214	ITGB8	integrin, beta 8
NM_002089	CXCL2	chemokine (C-X-C motif) ligand 2
NM_006291	TNFAIP2	tumor necrosis factor, alpha-induced protein 2
NM_002982	CCL2	chemokine (C-C motif) ligand 2
NM_004079	CTSS	cathepsin S
NM_007199	IRAK3	interleukin-1 receptor-associated kinase 3
NM_001710	CFB	complement factor B
NM_001002264	EPSTI1	epithelial stromal interaction 1 (breast)
NM_006850	IL24	interleukin 24
NM_000640	IL13RA2	interleukin 13 receptor, alpha 2
NM_002426	MMP12	matrix metallopeptidase 12 (macrophage elastase)
NM_031419	NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta
NM_002996	CX3CL1	chemokine (C-X3-C motif) ligand 1
NM_002185	IL7R	interleukin 7 receptor
NM_002009	FGF7	fibroblast growth factor 7
NM_002068	GNA15	guanine nucleotide binding protein (G protein), alpha 15
NM_002090	CXCL3	chemokine (C-X-C motif) ligand 3
NM_000757	CSF1	colony stimulating factor 1 (macrophage)
NM_005623	CCL8	chemokine (C-C motif) ligand 8
NM_025079	ZC3H12A	zinc finger CCCH-type containing 12A
NM_000201	ICAM1	intercellular adhesion molecule 1
NM_001012631	IL32	interleukin 32
NM_001148	ANK2	ankyrin 2, neuronal
NM_002994	CXCL5	chemokine (C-X-C motif) ligand 5
NM_002189	IL15RA	interleukin 15 receptor, alpha
NM_000575	IL1A	interleukin 1, alpha
NM_004878	PTGES	prostaglandin E synthase
NM_003638	ITGA8	integrin, alpha 8
NM_001570	IRAK2	interleukin-1 receptor-associated kinase 2
NM_020529	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NM_003391	WNT2	wingless-type MMTV integration site family member 2
NM_002309	LIF	leukemia inhibitory factor
NM_000104	CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1
NM_012342	BAMBI	BMP and activin membrane-bound inhibitor homolog
NM_016232	IL1RL1	interleukin 1 receptor-like 1
NM_002006	FGF2	fibroblast growth factor 2 (basic)
NM_002120	HLA-DOB	major histocompatibility complex, class II, DO beta
NM_001733	C1R	complement component 1, r subcomponent
NM_006273	CCL7	chemokine (C-C motif) ligand 7
NM_201442	C1S	complement component 1, s subcomponent
NM_001098479	HLA-F	major histocompatibility complex, class I, F
NM_018724	IL20	interleukin 20
NM_032682	FOXP1	forkhead box P1
NM_003855	IL18R1	interleukin 18 receptor 1
NM_000416	IFNGR1	interferon gamma receptor 1
NM_000063	C2	complement component 2
NM_003114	SPAG1	sperm associated antigen 1
NM_000880	IL7	interleukin 7
NM_001066	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B
NM_018725	IL17RB	interleukin 17 receptor B

TABLE 3 (ctd).

RefSeq	Gene symbol	Genes name
NM_005516	HLA-E	major histocompatibility complex, class I, E
NM_004417	DUSP1	dual specificity phosphatase 1
NM_003998	NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NM_016205	PDGFC	platelet derived growth factor C
NM_005228	EGFR	epidermal growth factor receptor
NM_001185072	CLDN12	claudin 12
NM_002192	INHBA	inhibin, beta A
NM_021101	CLDN1	claudin 1
NM_006919	SERPINB3	serpin peptidase inhibitor, clade B (ovalbumin), member 3
NM_031226	CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1
NM_005429	VEGFC	vascular endothelial growth factor C
NM_004994	MMP9	matrix metallopeptidase 9
NM_002422	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)
NM_000418	IL4R	interleukin 4 receptor
NR_027783	SAT1	spermidine/spermine N1-acetyltransferase 1
NM_001080448	EPHA6	EPH receptor A6
NR_028338	TRAF3IP2	TRAF3 interacting protein 2
NM_002502	NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2
NM_002427	MMP13	matrix metallopeptidase 13 (collagenase 3)
NM_003474	ADAM12	ADAM metallopeptidase domain 12
NM_004346	CASP3	caspase 3, apoptosis-related cysteine peptidase
NM_000689	ALDH1A1	aldehyde dehydrogenase 1 family, member A1
NM_001773	CD34	CD34 molecule
NM_024582	FAT4	FAT tumor suppressor homolog 4 (Drosophila)
NM_000609	CXCL12	chemokine (C-X-C motif) ligand 12
NM_012098	ANGPTL2	angiopoietin-like 2
NM_005602	CLDN11	claudin 11
NM_005410	SEPP1	selenoprotein P, plasma, 1
NM_003275	TMOD1	tropomodulin 1
NM_016546	C1RL	complement component 1, r subcomponent-like
NM_014333	CADM1	cell adhesion molecule 1
NM_007293	C4A	complement component 4A (Rodgers blood group)
NM_001885	CRYAB	crystallin, alpha B
NM_001299	CNN1	calponin 1, basic, smooth muscle
NM_145176	SLC2A12	solute carrier family 2 (facilitated glucose transporter)
NM_003725	HSD17B6	hydroxysteroid (17-beta) dehydrogenase 6 homolog (mouse)
NM_001127217	SMAD9	SMAD family member 9
NM_001168272	ITPR1	inositol 1,4,5-triphosphate receptor, type 1

TABLE 4. Genes down-regulated after in vitro treatment of human endometrium stromal cells

Gene	*p*-value hCG/IL1	FC hCG/IL1	*p*-value MM/IL1	FC MM/IL1	*p*-value hCG/MM	FC hCG/MM	IPA Pf.	IPA Im.	IPA Rm.	IPA CS.	IPA Ap.	IPA Ag.
ETV1	0.02017	-5.26										
HSD17B2	0.03976	-5.06										
LOXL4	0.00528	-4.74	0.00435	-5.01								
CDK1	0.03192	-4.25					X				X	
ITGA6	0.00651	-3.82	0.01105	-3.35			X			X	X	
DUSP6	0.00051	-3.56			0.01223	-2.08				X	X	
SLC20A1	0.00306	-3.08								X		
POLE2	0.00645	-2.86			0.01939	-2.31						
CCNA2	0.02166	-2.66					X			X	X	
MCM6	0.00275	-2.54										
KRT19[α]	0.00000	-2.50	0.00001	-2.26								
RFC3	0.00785	-2.49										
KRT34	0.00247	-2.45										
E2F7	0.04905	-2.38					X					
BRCA1	0.01466	-2.25			0.02641	-2.04	X				X	
PAK1	0.00399	-2.24	0.02263	-1.77			X		X	X	X	
HELLS	0.04234	-2.22									X	
ANGPTL4	0.01955	-2.09					X				X	
ITGA4	0.00435	-2.03	0.00371	-2.08					X	X	X	X
ACTA2	0.01071	-1.91	0.02904	-1.68						X		
CCND1	0.00041	-1.90					X			X	X	
HOXA11	0.00279	-1.88	0.00047	-2.31								
ITGA3	0.00146	-1.69					X	X		X		
FZD1	0.00697	-1.58	0.00048	-2.06						X		
POLA2	0.02091	-1.51			0.00795	-1.66						
ITGB5			0.03518	-1.53			X			X	X	X
ANXA4			0.02022	-1.53							X	
TIMP3[αβ]			0.01839	-1.57			X			X	X	X
BCL2L10			0.00814	-1.58							X	
SMAD9			0.01991	-1.61								
IGF2BP3			0.02094	-1.62			X					
C5			0.01257	-1.68			X	X	X	X	X	
TGFBR3			0.04598	-1.82			X	X		X	X	
ANGPTL2			0.02817	-1.84								
FAS			0.03925	-1.85			X	X		X	X	X
LAMB1			0.03736	-1.91			X			X		
TNFRSF19			0.02307	-2.26							X	
PDGFD			0.01484	-3.19			X			X		
MMP10[α]					0.01030	-3.91					X	
IL13RA2					0.04783	-3.64	X					
CCNE2					0.01264	-2.78	X			X		
PLAU					0.00713	-2.42	X			X	X	X
MELK					0.01473	-2.41						
ITGA2					0.01329	-2.37	X			X	X	X
CDC45					0.01108	-2.30	X				X	
SPAG5					0.03517	-2.16	?					
BRCA2					0.03864	-1.96	X			X	X	
E2F8					0.02061	-1.85	X					
MMP3					0.02542	-1.84	X				X	X
MCM5					0.01884	-1.79						
MCM3					0.02412	-1.62						
ORC1					0.00293	-1.60						
PCNA					0.02189	-1.59	X				X	
E2F1					0.00984	-1.55	X			X	X	X
IL1A[α]					0.02429	-1.52	X	X	X	X	X	X
FOSL1					0.01092	-1.50	X			X	X	

[α/β] Real-time PCR/ ELISA validation performed

Pf, proliferation; Im, immune functions; Rm, tissue remodeling; CS, cell signaling; Ap, apoptosis; Ag, angiogenesis

TABLE 5. Name of down-regulated genes

RefSeq	Gene symbol	Genes name
NM_004956	ETV1	ets variant 1
NM_002153	HSD17B2	hydroxysteroid (17-beta) dehydrogenase 2
NM_032211	LOXL4	lysyl oxidase-like 4
NM_001786	CDK1	cyclin-dependent kinase 1
NM_000210	ITGA6	integrin, alpha 6
NM_001946	DUSP6	dual specificity phosphatase 6
NM_005415	SLC20A1	solute carrier family 20 (phosphate transporter), member 20
NM_002692	POLE2	polymerase (DNA directed), epsilon 2 (p59 subunit)
NM_001237	CCNA2	cyclin A2
NM_005915	MCM6	minichromosome maintenance complex component 6
NM_002276	KRT19	keratin 19
NM_002915	RFC3	replication factor C (activator 1) 3, 38kDa
NM_021013	KRT34	keratin 34
NM_203394	E2F7	E2F transcription factor 7
NR_027676	BRCA1	breast cancer 1, early onset
NM_001128620	PAK1	p21 protein (Cdc42/Rac)-activated kinase 1
NM_018063	HELLS	helicase, lymphoid-specific
NM_139314	ANGPTL4	angiopoietin-like 4
NM_000885	ITGA4	integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor)
NM_001141945	ACTA2	actin, alpha 2, smooth muscle, aorta
NM_053056	CCND1	cyclin D1
NM_005523	HOXA11	homeobox A11
NM_002204	ITGA3	integrin, alpha 3 (antigen CD49C, alpha 3 subunit of VLA-3 receptor)
NM_003505	FZD1	frizzled homolog 1 (Drosophila)
NM_002689	POLA2	polymerase (DNA directed), alpha 2 (70kD subunit)
NM_002213	ITGB5	integrin, beta 5
NM_001153	ANXA4	annexin A4
NM_000362	TIMP3	TIMP metallopeptidase inhibitor 3
NM_020396	BCL2L10	BCL2-like 10 (apoptosis facilitator)
NM_001127217	SMAD9	SMAD family member 9
NM_006547	IGF2BP3	insulin-like growth factor 2 mRNA binding protein 3
NM_001735	C5	complement component 5
NM_003243	TGFBR3	transforming growth factor, beta receptor III
NM_012098	ANGPTL2	angiopoietin-like 2
NM_000043	FAS	Fas (TNF receptor superfamily, member 6)
NM_002291	LAMB1	laminin, beta 1
NM_148957	TNFRSF19	tumor necrosis factor receptor superfamily, member 19
NM_025208	PDGFD	platelet derived growth factor D
NM_002425	MMP10	matrix metallopeptidase 10 (stromelysin 2)
NM_000640	IL13RA2	interleukin 13 receptor, alpha 2
NM_057749	CCNE2	cyclin E2
NM_002658	PLAU	plasminogen activator, urokinase
NM_014791	MELK	maternal embryonic leucine zipper kinase
NM_002203	ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)
NM_001178010	CDC45	cell division cycle 45 homolog (S. cerevisiae)
NM_006461	SPAG5	sperm associated antigen 5
NM_000059	BRCA2	breast cancer 2, early onset
NM_024680	E2F8	E2F transcription factor 8
NM_002422	MMP3	matrix metallopeptidase 3 (stromelysin 1, progelatinase)
NM_006739	MCM5	minichromosome maintenance complex component 5
NM_002388	MCM3	minichromosome maintenance complex component 3
NM_004153	ORC1	origin recognition complex, subunit 1
NM_002592	PCNA	proliferating cell nuclear antigen
NM_005225	E2F1	E2F transcription factor 1
NM_000575	IL1A	interleukin 1, alpha
NM_005438	FOSL1	FOS-like antigen 1

Chapitre 4 : Human Chorionic Gonadotropin Regulates Endothelial Cell Responsiveness to Interleukin 1 and Amplifies the Cytokine-Mediated Effect on Cell Proliferation, Migration and the Release of Angiogenic Factors.

Amélie Bourdiec[1], David Bédard[1], C. V. Rao[2], Ali Akoum[1],

1 Endocrinologie de la reproduction, Centre de recherche-HSFA, CHUQ, Faculté de médecine, Université Laval, Québec, QC, Canada

2 Herbert Wertheim College of Medicine, Florida International University, Miami, FL, USA

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4.1 Résumé

Problème : La réussite de l'implantation embryonnaire nécessite un réseau de communication complexe approprié entre l'embryon et son proche environnement dans le site implantatoire. Ici nous avons examiné si la gonadotrophine chorionique humaine (hCG), le signal embryonnaire majeur, cible les cellules endothéliales et regule leur réactivité à l'IL1, l'un des premiers signaux émis par les cellules embryonnaires.

Méthode d'étude : la prolifération et la migration des cellules endothéliales microvasculaires humaines après exposition à différentes concentrations d'hCG et / ou d'IL1B pour des périodes différentes ont été analysées par incorporation de BrdU et des tests de cicatrice. L'expression des récepteurs solubles et membranaires de l'IL1, du récepteur de l'hormone luténéisante et de la gonadotrophine chorionique (LHCGR), ainsi que l'interleukine 8 (IL8) a été déterminée par PCR quantitative en temps réel, Western blot et ELISA.

Résultats : En réponse à l'IL1B, la prolifération et la migration cellulaire augmentent d'avantage en présence d'hCG. L'IL1B régule à la hausse ses propres récepteurs, tandis que la présence d'hCG diminue significativement l'expression du récepteur IL1R2. Cela se traduit par une augmentation de la sécrétion d'IL8, qui est inhibée par la surexpression artificielle d'IL1R2.

Conclusion : ces résultats révèlent un nouveau mécanisme par lequel la hCG peut cibler les cellules endothéliales et stimuler directement l'angiogenèse pour favoriser la croissance embryonnaire.

4.2 Abstract

Problem : Successful embryonic implantation requires an appropriate communication network between the embryo and its near environment within the implantation site. Herein, we examined whether human chorionic gonadotropin (hCG), the major embryonic signal, targets endothelial cells and regulate their responsiveness to interleukin 1 (IL1), one of the earliest signals released by embryonic cells.

Method of study : Human microvascular endothelial cell proliferation and migration following exposure to various concentrations of hCG and/or IL1B for different time periods were analyzed by BrdU incorporation and wound healing assays. The expression of soluble (s) and membrane-bound (mb) IL1 receptors (IL1Rs), IL1R antagonist (IL1RN), luteinizing hormone/choriogonadotropin receptor (LHCGR), and IL8 was determined by real-time PCR, Western blot, and ELISA.

Results : Cell proliferation and migration increased in response to IL1B and further in the presence of hCG. IL1B up-regulated both the signaling IL1R1 and the inhibitory IL1R2, while adding hCG further increased IL1R1 and significantly downregulated IL1R2. This translated into an increased secretion of IL8, which was inhibited in cells where IL1R2 was overexpressed.

Conclusions : These findings reveal a new mechanism by which hCG may target endothelial cells to directly stimulate angiogenesis and favor embryonic growth.

4.3 Introduction

Human chorionic gonadotropin (hCG), a glycoprotein hormone mainly produced by the syncytiotrophoblasts in the chorionic villi, plays a key role in the initiation and

maintenance of pregnancy. One of the major embryonic signals, hCG is detectable in maternal serum as early as 1 day after the initiation of embryo implantation. Its levels reach a peak value after sixty to ninety days of gestation and gradually drop within the second trimester.[1]

Stimulation of progesterone production by ovarian corpus luteal cells and maintenance of embryo implantation were first considered as the main biological activities of hCG.[2, 3] However, hCG was shown to be involved in multiple steps of placentation and fetal development. Functional luteinizing hormone/choriogonadotropin receptor (LHCGR) was detected in non-gonadal tissues such as the human endometrium and hCG were found to directly modulate endometrial functions.[4] Actually, hCG promotes human endometrial stromal cell decidualization,[5] induces the proliferation of tissue-specific uterine natural killer (uNK) cells,[6] and plays a key role in immunological modulation at the maternal–fetal interface.[4]

Implantation of human embryo involves a complex process of tissue remodeling and requires the development of an efficient new blood vessel network. Several studies showed that hCG promotes angiogenesis as well as vasculogenesis in the uterine vasculature during pregnancy.[7, 8] In vivo, hCG induces a neovascularization comparable to the activity of vascular endothelial growth factor (VEGF)[8] and promotes the production of angiogenic factors such as VEGF by the placenta[9] and decidual macrophages.[10] In vitro, it stimulates capillary formation[8] and directly up-regulates the synthesis and secretion of macrophage inhibitory factor (MIF), a multifunctional cytokine with potent immunomodulatory and angiogenic properties, in endometrial epithelial cells.[11]

Deep morphological, structural and functional changes occurring within the uterus are primarily orchestrated by embryonic factors/signals which coordinate embryonic

growth and development.[12] Interleukin 1 (IL1) is one of the earliest embryonic signals. Beside its major immunological effects, IL1 regulates cell growth and angiogenesis.[13, 14] IL1 was shown to increase tumor invasiveness and metastasis by enhancing the expression of adhesion molecules on endothelial and malignant cells.[15] IL1 also stimulates the proliferation of endothelial cells and production of cytokines.[14, 16] The addition of exogenous IL1B in IL1B-KO mice partially restored the angiogenic response, whereas the administration of IL1 receptor antagonist (IL1RN) to wild-type mice inhibited the growth of blood vessel networks.[17] IL1 has two known receptors: The type 1 (IL1R1) acts as signaling receptor and mediates cell activation by IL1 via the recruitment of IL1 accessory protein (IL1RAP),[18] while the type 2 (IL1R2) acts as a negative regulator of IL1 action.[19]

Our first studies revealed that hCG acts directly on endometrial epithelial cells to down-regulate the expression of the decoy IL1R2, without affecting IL1R1 expression.[20] These data point to a possible mechanism by which hCG could modulate IL1-mediated actions. The main objective of this study was to investigate whether the hCG-mediated modulation of cell receptivity to IL1 can operate in endothelial cells. Our data showed that hCG significantly amplifies the IL1B-induced endothelial cell proliferation and migration, IL8 production and IL1R1 expression, but it concurrently exerts an inhibitory effect on the IL1-induced IL1R2 expression. Such a dual complementary action may amplify endothelial cell responsiveness to IL1 and potentiate the release of angiogenic factors. This points to a possible new mechanism by which hCG stimulates angiogenesis and promotes consequently the development of new blood vessels required for the highly demanding embryonic growth.

4.4 Materials and methods

4.4.1 Cell Culture and Treatment

Human microvascular endothelial cell line (HMVEC) was used in this study (Gift from Dr. R. Shao, Pioneer Valley Life Sciences Institute, University of Massachusetts Amherst, Springfield, MA, USA). This cell line has been established by engineering the human telomerase catalytic protein (hTERT).[21] Cells were shown to maintain inherent features of primary endothelial cells and to produce angiogenic response.[21] HMVEC were cultured in endothelial cell basal medium MCDB 131 (Life Technologies Inc., Burlington, ON, Canada) supplemented with 10% fetal bovine serum (FBS), 10 µg/mL epithelial cell growth factor (EGF), 1 µg/mL hydrocortisone, 10 mM glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (complete MCDB 131 medium). Cells were seeded in 6-well plates until confluence, washed twice with MCDB 131 medium and cultured overnight with the same medium containing 10% charcoal-treated FBS. Cells were then washed twice with MCDB 131 and cultured for 24 and 48 h with fresh FBS-free MCDB 131 containing different concentrations of hCG (0–1000 ng/mL, recombinant expressed in mouse cell line, 10,000 IU/mg; Sigma Chemical Co., St. Louis, MO, USA) and IL1B (0–1 ng/mL) (R & D systems Inc., Minneapolis, MN, USA). Culture supernatants, centrifuged to remove culture debris, and cells, dissociated with 0.01% Trypsin and 0.01% EDTA in Hank's buffered salt solution (HBSS), were recovered and stored until further use at −80°C.

4.4.2 Immunocytofluorescence

Cells were seeded on coverslips in 24-well plates and grown in complete MCDB 131 medium. Pre-confluent cultures were incubated overnight in the same medium

containing 10% charcoal-treated FBS, then rinsed in serum-free MCDB 131 medium, and fixed in 10% Formalin. All immunostaining steps were carried out at room temperature. For IL1R1, IL1R2, and IL1RAP immunostaining, cells were washed in PBS/0.1% Tween 20 and incubated for 1 h with a monoclonal mouse anti-human IL1R1 (R & D Systems) [10 µg/mL in PBS containing 0.2% BSA and 0.01% Tween 20 (PBS/BSA/Tween 20)], a monoclonal mouse anti-human IL1R2 (R & D Systems) (10 µg/mL in PBS/BSA/Tween 20) or a polyclonal goat anti-human IL1RAP (R & D Systems) (10 µg/mL in PBS/BSA/Tween 20), respectively. Cells were then washed in PBS/0.1% Tween 20 and incubated for 1 h with a biotin-conjugated horse anti-mouse or rabbit anti-goat antibody (Vector Laboratories, Burlingame, CA, USA) (1:100 dilution in PBS/BSA/Tween 20). Cells were washed with PBS and incubated for 1 h with 1% streptavidin-fluorescein isothiocyanate in PBS/BSA/Tween 20. For LHCGR, cells were incubated with a polyclonal anti-hLHCGR rabbit antibody (1:300 dilution in PBS/0.1% Tween 20), rinsed in PBS/0.1% Tween 20, and incubated for 1 h with a biotin-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) (1:1000 dilution in PBS/0.2% BSA/0.1% Tween 20), then for 45 min at room temperature with Alexa 488-labeled streptavidin (1:100 dilution in PBS/0.2% BSA) (Molecular Probes Inc., Eugene, OR, USA). Cells were counterstained with 4',6-diaminido-2-phenyl-indole (1:2000 dilution in PBS/0.1% Tween 20) and covered with Mowiol containing an antifading agent (10% para-phenylenediamine) (Sigma). Cells incubated without the primary antibody were included as negative controls. Staining was observed under fluorescence microscopy (Olympus BX51; Olympus Corp., Tokyo, Japan) and photographs were captured using Image-Pro Express 5.1 software (Media Cybernetics Inc., Bethesda, MD, USA).

4.4.3 Scratch Wound Assay

Analysis of endothelial cell migration using the scratch wound assay has been carried out, as previously reported.[22, 23] Cells grown to pre-confluence in 24-well plates in complete MCDB 131 medium were kept overnight in MCDB 131 medium containing 10% charcoal-treated FBS and carefully wounded thereafter with a 200 µL pipette tip to create a cut with a mean width of 1 mm. Cells were then washed with HBSS and incubated for 24 h with hCG, ILB or hCG and IL1B in MCDB 131 before being fixed with 10% formalin for 20 min and stained with DAPI. Cell incubation period (24 h) was determined on the basis of preliminary assays showing that longer incubation periods with the indicated stimuli lead to spontaneous closure of the wound. Scratch width was measured using fluorescence microscopy to examine the extent of closure. Each condition was tested three times in duplicate, and a dozen of measurements were made all along the scratch. Measurements were expressed as % of closure of the scratch after 24 h of incubation relative to its initial width at time zero. Our preliminary validation tests to evaluate the sensitivity of the scratch assay showed a dose–dependent induction of endothelial cell migration for known angiogenic factors, such as VEGF, fibroblast growth factor (FGF), and monocyte chemotactic protein 1 (MCP1), with a detectable effect observed at 25 ng/mL. For hCG and IL1B, a detectable effect was observed at 0.05 and 100 ng/mL, respectively. This paralleled the dose–dependent stimulation of endothelial cell proliferation, as measured by BrdU incorporation into cell DNA, following exposure to these factors (data not shown).

4.4.4 Bromodeoxyuridine (BrdU) Incorporation into Cell DNA

To measure cell proliferation, cells were seeded at 1.104cell/well into 96-well microtitre plates and cultured until pre-confluence in complete MCDB 131 medium.

Cells were then incubated overnight in MCDB 131 medium containing 10% charcoal-treated FBS, starved for 48 h with 0.2% charcoal-treated FBS in MCDB 131 before being exposed to hCG, IL1B or hCG and IL1B for 48 h. After 24 h labeling at 37°C with 10 µm BrdU, cell proliferation was determined by ELISA as per the instructions of the manufacturer (GE Healthcare, Mississauga, ON, Canada). Concisely, cells were fixed in 10% formalin, DNA was denatured for 30 min at room temperature by 200 µL/well of blocking reagent (1:10) and peroxidase-labeled anti BrdU antibody (1:100) was added (100 µL/well). After 90 min incubation at room temperature followed by three washings, cells were incubated for 15 min at room temperature with TMB 3,3',5,5'-tetramethylbenzidine (TMB) (100 µL/well), then with 2M H_2SO_4 (100 µL/well) to stop the enzymatic reaction, and the optical density was measured at 450 nm.

4.4.5 Real-Time PCR

Total cell RNA was extracted using Trizol according to the manufacturer's instructions (Life Technologies Inc.). cDNA was synthesized using 100 ng of RNA and 2.5 µm random hexamers in 20 µL of a solution containing 50 mm KCl, 10 mm Tris–HCl, 5 mm $MgCl_2$, 1 mM of each deoxyribonucleotide triphosphate (dNTP), 20 U of RNase inhibitor, and 50 U of reverse mRNA expression using Gene Amp PCR Core Kit (Perkin-Elmer, Foster City, CA, USA). The reaction was then incubated for 15 min at 25°C, 30 min at 42°C, and 5 min at 99°C. Quantitative real-time PCR was performed using an ABI 7000 thermal cycler (Applied Biosystems, Foster city, CA, USA). Each standard PCR reaction contained 2.5 µL of RT product, 0.5 µL of each primer (final concentration, 5 pm/L), 12.5 µL SYBR Green PCR Master Mix consisting of Taq DNA polymerase reaction buffer, dNTP mix, SYBR Green I, $MgCl_2$, and Taq DNA polymerase. After 10 min denaturation at 95°C, the reactions were cycled 50 times with 15 s denaturation at 95°C and 60 s annealing at 60°C. IL1R1 primers (forward,

5’-AGAGGAAAACAAACCCACAAGG-3’; reverse, 5’-CTGGCCGGTGACATTACAGAT-3’; amplimer size 106 bp), IL1R2 primers (forward, 5’-TGGCACCTACGTCTGCACTACT-3’; reverse, 5’-TTGCGGGTATGAGATGAACG- 3’; amplimer size 112 bp), soluble (s) IL1RAP primers (forward, 5foGAAAGGTAATAGATGCGGTCAGTG-3T; reverse, 5 reverse, GATGCGGTCAGTG-3; amplimer size 122 bp), membrane-bound (mb) IL1RAP primers (forward, 5 forward, primers GTCAGTG-; reverse, 5 reverse, primers GTCAGTG-3TGAAC; amplimer size 122 bp), IL1RN primers (forward, 5’-AACAGAAAGCAGGACAAGCG-3’; reverse, 5’-CCTTCGTCAGGCATATTGGT-3’; amplimer size 149 bp), IL8 primers (forward, 5’-ATACTCCAAACCTTTCC-3’; reverse, 5’-CTTCTCCACAACCCTC-3’; amplimer size 157 bp) and GAPDH (forward, 5’-CAGGGCTGCTTTTAACTCTGG-3’; reverse, 5’-TGGGTGGAATCATATTGGAACA; amplimer size 102 bp), were designed with Primer express™, version 2.0 (Applied Biosystems) to span at least one intron-exon boundary to prevent the amplification of possible contaminating genomic DNA. Quantification of IL1R1, IL1R2, mbIL1RAP, sIL1RAP, IL1RN, and IL8 mRNA was performed using a relative quantification method. For each experimental sample, IL1R1, IL1R2, mbIL1RAP, sIL1RAP, IL1RN, and IL8 mRNA levels were normalized to GAPDH mRNA levels. All samples were tested in duplicate. Melting curve analysis (55–95°C) was performed after each run to verify the specificity of the PCR, and each run included no-template and no-reverse transcription controls.

4.4.6 Western Blot Analysis

Proteins were isolated with Trizol according to the manufacturer's instructions (Life Technologies Inc.). Total protein concentration was determined using Bio-Rad DC Protein Assay (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). Protein extracts (10 πg) and culture supernatants (40 πL) were then separated by SDS-polyacrylamide gel electrophoresis (PAGE), transferred onto 0.45 πm nitrocellulose

membranes and analyzed by Western blotting, as described previously.[24] Briefly, IL1R1, IL1R2, and IL1RAP were detected using specific goat polyclonal antibodies [2 µg/mL in PBS containing 5% skim milk and 0.1% Tween 20) (blocking solution)] (R & D systems), IL1RN was detected using a specific rabbit polyclonal antibody (2 µg/mL in blocking solution) (Genzyme), and hCG/LH receptor was detected using a rabbit polyclonal antibody raised against the synthetic 15–38 N-terminus amino acid sequence (1:1500 dilution in blocking solution).[20] Fc-specific peroxidase-labeled rabbit anti-goat antibody (Jackson ImmunoResearch Laboratories, Inc.) (1:10,000 in blocking solution) was then used for IL1R1, IL1R2, and IL1RAP, whereas an Fc-specific peroxidase-labeled goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, Inc.) (1:150,000 in blocking solution) was used for IL1RN and LHCGR. For a chemiluminescence-based detection, ECL reagent (GE healthcare, Chalfont St Giles, UK) was used. Membranes were exposed to Super RX films (Fuji, Tokyo, Japan) for 5–30 min so that bands are visible without being overexposed. To detect α-actin, used as internal control for protein loading and transfer, membranes were stripped and reblotted with a mouse monoclonal anti-α-actin antibody (Sigma Chemical Co.) (1:50,000 dilution in PBS-0.01% Tween 20).

4.4.7 Enzyme-Linked Immunosorbent Assay (ELISA)

IL8 concentrations in HMVEC supernatants were measured using an ELISA procedure developed in the laboratory. This assay uses a mouse monoclonal anti-human IL8 antibody and a rabbit polyclonal anti-human IL8 antibody (Biosource International, Life Technologies Inc. Burlington, ON, Canada). Briefly, 96-well plates were coated overnight at 4°C with the mouse monoclonal antibody. Plates were washed four times with PBS containing 0.1% Tween 20 (washing buffer), and recombinant human IL8 (R and D Systems), used at concentrations ranging from 100 pg/mL to 6.4 ng/mL, or samples diluted in serum-free RPMI were then added to the plates. After 60 min

incubation at 37°C, the plates were washed and incubated for 60 min at 37°C with the IL8 polyclonal antibody (1/8000 dilution in PBS containing 0.5% BSA). The plates were then washed and incubated for 60 min at 37°C with a goat anti-rabbit IgG peroxidase conjugate (Zymed Laboratories Inc., San Francisco, CA, USA) (1/2000 dilution in PBS/0.5% BSA). After a final wash, 100 µL of TMB-peroxidase substrate (Bio-Rad Laboratories Ltd) was added to each well, the enzymatic reaction was terminated by the addition of 50 µL of 2 N H2SO4, and the optical density was determined at 450 nm. sIL1R2 and IL1RN concentrations in the culture medium were measured using a previously reported sandwich ELISA.[25] IL8, sIL1R2, and IL1RN concentrations were calculated by interpolation from standard curves.

4.4.8 Cell Transfection

HMVEC were seeded into 24-well plates and grown in complete MCDB 131 medium before being incubated overnight in the same medium containing 10% charcoal-treated FBS and transfected at 70% confluence. Cells were transfected with the eukaryotic expression vector pcDNA3 alone, or containing a cDNA coding for IL1R2 to overexpress of IL1R2 (Gift from Dr Paola Bossù, Research Center Dompé SpA, L'Aquila, Italy) Cell transfection was carried out using Lipofectamine Plus reagent (Life Technologies Inc.), as described previously.[24] Before cell stimulation, the culture medium was replaced by a serum-free medium for 24 h. Cells were then exposed or not for 24 h to hCG, IL1B or hCG and IL1B. The culture supernatants were collected and centrifuged to remove cell debris, and cells were recovered by trypsinization for RNA extraction as described above. RNAs and culture supernatants were then aliquoted and kept at −80°C until analysis.

4.4.9 Statistical Analysis

All analyses were performed with GraphPad Software Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA). Data followed a parametric distribution and were expressed as means ± S.E.M. The significance of statistical differences was determined using one-way analysis of variance (anova) followed by the Bonferroni test post hoc, for multiple analyses, and the Student's t-test for the comparison of two groups.

4.5 Results

Immunocytofluorescence analysis first showed that HMVECs express hCG and IL1 receptors. A positive immunostaining for LHCGR, IL1R1, IL1R2, and IL1RAP was observed (Fig. 1a,c,e,g). No immunostaining in control cells incubated with rabbit (LHCGR), mouse (IL1R1 and IL1R2) or goat (IL1RAP) IgGs was noted, instead of the respective primary antibodies (Fig. 1b,d,f,h).

Further studies showed that hCG and IL1B can induce HMVEC proliferation and migration. HMVEC cultures were exposed to different concentrations of hCG (0–1000 ng/mL), IL1B (0–1 ng/mL) or a combination of hCG and IL1B. As shown in Fig. 2a,b, a dose–dependent increase in BrdU incorporation into DNA was observed in HMVECs in response to these stimuli, which was significant at 100 and 1000 ng/mL hCG ($P < 0.01$ and $P < 0.001$, respectively), and 0.1 and 1 ng/mL IL1B ($P < 0.05$ and $P < 0.001$, respectively). Interestingly, BrdU incorporation into HMVEC DNA was further increased in cells stimulated with a combination of hCG (100 ng/mL) and IL1B (0.1 ng/mL) ($P < 0.001$) as compared the control medium, as well as to each of these stimuli alone ($P < 0.01$), thus suggesting a synergistic stimulatory action on HMVEC proliferation (Fig. 2c). Assessment of cell migration using scratch wound assay showed a significant increase in response to hCG (100 ng/mL) and IL1B (0.1

ng/mL) ($P < 0.001$). However, in cells simultaneously incubated with equivalent concentrations of hCG and IL1B, HMVEC migration was significantly increased as compared with cells incubated with the control medium ($P < 0.001$), but also as compared with hCG or IL1B ($P < 0.05$), suggesting here too a synergistic stimulatory effect on HMVEC migration (Fig. 2d).

The above-described data suggest that HMVEC responsiveness is increased following exposure to hCG and IL1B. To determine whether this is related to any change in hCG and/or IL1 receptors and antagonists, we further investigated IL1's functional activating receptor and its accessory protein (IL1R1 and mbIL1RAP) together with the cytokine's-specific inhibitors (IL1R2, sIL1RAP, and IL1RN) and LHCGR. Cell cultures were exposed to hCG (100 ng/mL), IL1B (0.1 ng/mL) or hCG plus IL1B at similar concentrations for 24 and 48 h, as optimal cell proliferation and cell migration assays described earlier required 48 and 24 h, respectively.

Quantitative real-time PCR analysis showed that at 24 and 48 h, IL1R1 mRNA steady-state levels were significantly increased by IL1B ($P < 0.05$) and further by a combination of hCG and IL1B ($P < 0.05$ and $P < 0.01$, respectively), but hCG has no statistically significant effect (Fig. 3a,b). IL1R2 mRNA levels in HMVECs were also increased by IL1B at 24 and 48 h ($P < 0.05$). However, the IL1B-induced IL1R2 mRNA levels were significantly down-regulated by co-incubating HMVECs with hCG and IL1B ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 3c,d). Meanwhile, no significant effect of hCG and/or IL1B on mbIL1RAP, sIL1RAP, IL1RN or LHCGR mRNA expression was noted (Fig. 3e,g,i,k) at 24 h. However, a significant stimulation of mbIL1RAP in cells exposed for 48 h to IL1B and hCG/IL1B was observed ($P < 0.05$) (Fig. 3f), which in view of mbIL1RAP role in IL1 signaling suggests that 48-h stimulation is more suitable for HMVEC stimulation with IL1B or ILB/hCG.

Then, we assessed the effect of hCG and/or IL1B on IL1Rs and LHCGR protein expression. Western blot analysis showed that hCG reduced the IL1-induced expression of IL1R2 at the protein level as well, but had no noticeable effect on that of ILR1, mbIL1RAP, sIL1RAP or IL1RN (Fig. 4a). Measurement of soluble IL1Rs and IL1RN in the culture medium further showed that sIL1R2 levels were significantly up-regulated in cells stimulated with IL1B as compared with non-stimulated cells ($P < 0.001$), but co-incubation with hCG and IL1B led in contrast to a significant down-regulation of the IL1B-induced sIL1R2 secretion ($P < 0.01$) (Fig. 4b). No significant effect of hCG and/or IL1B on sIL1RAP or IL1RN was noted (Fig. 4c,d).

To investigate the impact of the hCG-mediated down-regulation of IL1R2 in endothelial cells, we assessed its effect on the synthesis and secretion levels of IL8. Quantitative real-time PCR and ELISA analyses showed a significant increase in IL8 mRNA and protein level in response to IL1B ($P < 0.05$) and further in response to IL1B and hCG together ($P < 0.01$), whereas hCG had no statistically significant effect. Furthermore, a significant increase of IL8 secretion in cultures simultaneously treated with hCG and IL1B compared with IL1B alone was seen ($P < 0.05$) (Fig. 5a,b). Overexpression of IL1R2 in HMVECs by cell transfection with the pcDNA3 expression vector containing IL1R2 cDNA, significantly inhibited IL8 secretion following exposure to IL1B alone or in combination with hCG ($P < 0.05$ and $P < 0.01$, respectively). It is noteworthy that sIL1R2 concentrations were increased in the culture medium of endothelial cells transfected with IL1R2 cDNA than in the culture media of pcDNA3 vector-transfected cells (Fig. 6a,b).

4.6 Discussion

Normal reproductive functions require highly coordinated interactions between the embryo and the maternal endometrium via an intricate network of signaling and

communication. Only <24 hr are required for the embryo to breakdown the uterine endometrial epithelial lining and implant within the maternal uterine endometrium. We believe that appropriate interactions with the different types of surrounding endometrial cells are crucial for the implanting embryo to modulate maternal endometrial cell receptivity and favor its own growth. Herein, we show that hCG, the major embryonic signal, acts directly on endothelial cells to up-regulate the receptivity of these cells to IL1, one of the earliest embryonic signals, which taking into account the immune-modulatory, growth-promoting, and tissue remodeling effects of IL1, may represent a possible mechanism underlying embryonic implantation and development.

Actually, our data first showed that hCG amplifies HMVEC proliferation and migration in response to IL1B. Analysis of IL1 receptors and antagonist revealed that the IL1B-induced expression of IL1R2, the decoy inhibitory receptor of IL1,[19] is significantly down-regulated in the presence of hCG, whereas the ILB-induced expression of the functional activating IL1R1[19] is rather up-regulated by this hormone. These data strongly suggest that hCG profoundly modulates endothelial cell receptivity to IL1 via intriguing selective bidirectional regulatory mechanisms, which may amplify endothelial cell responsiveness to IL1 and promote angiogenesis. The nature of such mechanisms and the possible involved signaling pathways remains to be elucidated. Nevertheless, our data showed that IL8 secretion is increased in response to IL1B and further in cells co-incubated with hCG. Overexpressing IL1R2 in HMVEC did not completely block IL8 secretion, but significantly reduced the levels of IL8 secreted in response to IL1B alone or in combination with hCG. This supports a role for IL1R2 in the regulation of the ILB- and hCG/IL1B-induced IL8 secretion in HMVEC but suggests the involvement of other mechanisms. However, our study did not point to a significant involvement of

IL1RN, which competes with IL1 for binding to IL1R1,[26] or sIL1RAP, which is known for increasing IL1R2 affinity for IL1B,[27] as hCG and/or IL1B had no statistically significant effect on these IL1 antagonists.

First reported as a specific factor for mononuclear cells chemoattraction and activation, IL8 is now known for promoting a wide variety of biological effects, including tissue remodeling, cell invasion, and angiogenesis. Thus, IL8 has been shown to promote angiogenic responses in endothelial cells, inducing the proliferation, survival, and migration of vascular endothelial cells[28] and to influence endometrial differentiation, survival, and tissue remodeling.[29, 30] Other studies showed that IL8 stimulates trophoblast secretion of progesterone, which plays an important role in maintaining successful pregnancy.[31] According to more recent data, IL1B stimulates migration and survival of first-trimester villous cytotrophoblast cells by inducing IL8 secretion in endometrial cells.[32] This is interesting as IL8 expression in the endometrium was found to be enhanced during early pregnancy[33] and IL8 receptor (IL8R) expression was detected in trophoblastic cells of the developing embryo.[34] Furthermore, NK cells, which are the most abundant leukocytes during early pregnancy, were found to produce IL8 and choriodecidual cells from early pregnancy tissues were also shown to produce considerable amounts of IL8.[35] Chemokines and chemoattractant cytokines mediate leukocyte migration and are known to play a role in endometrial function, embryo implantation, and recruitment of the pregnancy-associated leukocytes.[36] In view of the above-indicated findings, the IL1B-induced production of IL8 by endothelial cells may not only stimulate angiogenesis and modulate the immune environment at the implantation site, but may also directly promote embryo invasion and implantation.

The available literature supports a role for IL1 in embryo implantation. The expression of IL1B appeared to increase prior to the initiation of blastocyst implantation in the mouse[37] and might be an initiator of conceptus-uterine cross-talk during pregnancy in the human.[38] IL1B was shown to increase the expression of integrin β3 in human endometrial epithelial cells[39] and to up-regulate IL1R1[40] and prostaglandin-endoperoxide synthase 2 (PTGS2)[41] expression in human endometrial stromal cells. Previous studies also suggest a possible role for IL1B and hCG in the inhibition of apoptosis and the development of an immunotolerant environment.[42] According to more recent studies, the presence of serum IL1B can predict ongoing pregnancy, and positively affect the implantation rate.[43]

Investigations are underway to assess the impact of hCG on human uterine endothelial cells. Nevertheless, our current data using a HMVEC, together with our previous data using human primary endometrial epithelial[44] and stromal[23] cells, make plausible that the embryo can directly target, interact with and modulate the receptivity of different endometrial cell types. The IL1B/hCG synergy shown in the present study, and exerted at the level of endothelial cells, points to a novel mechanism that may underlie the capability of the embryo to modulate maternal endometrial cell receptivity and promote angiogenesis. This is relevant to physiology of embryo implantation, as IL1B and hCG are both produced by embryos, and broadens the spectrum of hCG's impact on early embryonic growth and development.

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4.8 References

1 Jaffe RB, Lee PA, Midgley AR Jr: Serum gonadotropins before, at the inception of, and following human pregnancy. J Clin Endocrinol Metab 1969; 29:1281–1283.

2 Strott CA, Yoshimi T, Ross GT, Lipsett MB: Ovarian physiology: relationship between plasma LH and steroidogenesis by the follicle and corpus luteum; effect of HCG. J Clin Endocrinol Metab 1969; 29:1157–1167.

3 Jaffe RB: Fetoplacental endocrine and metabolic physiology. Clin Perinatol 1983; 10:669–693.

4 Rao CV, Lei ZM: The past, present and future of nongonadal LH/hCG actions in reproductive biology and medicine. Mol Cell Endocrinol 2007; 269:2–8.

5 Han SW, Lei ZM, Rao CV: Treatment of human endometrial stromal cells with chorionic gonadotropin promotes their morphological and functional differentiation into decidua. Mol Cell Endocrinol 1999; 147:7–16.

6 Kane N, Kelly R, Saunders PT, Critchley HO: Proliferation of uterine natural killer cells is induced by human chorionic gonadotropin and mediated via the mannose receptor. Endocrinology 2009; 150:2882–2888.

7 Berndt S, Blacher S, Perrier d'Hauterive S, Thiry M, Tsampalas M, Cruz A, Pequeux C, Lorquet S, Munaut C, Noel A, Foidart JM: Chorionic gonadotropin stimulation of angiogenesis and pericyte recruitment. J Clin Endocrinol Metab 2009; 94:4567–4574.

8 Zygmunt M, Herr F, Keller-Schoenwetter S, Kunzi-Rapp K, Munstedt K, Rao CV, Lang U, Preissner KT: Characterization of human chorionic gonadotropin as a novel angiogenic factor. J Clin Endocrinol Metab 2002; 87:5290–5296.

9 Islami D, Bischof P, Chardonnens D: Modulation of placental vascular endothelial growth factor by leptin and hCG. Mol Hum Reprod 2003; 9:395–398.

10 Zygmunt M, Herr F, Munstedt K, Lang U, Liang OD: Angiogenesis and vasculogenesis in pregnancy. Eur J Obstet Gynecol Reprod Biol 2003; 110(Suppl 1):S10–S18.

11 Akoum A, Metz CN, Morin M: Marked increase in macrophage migration inhibitory factor synthesis and secretion in human endometrial cells in response to human chorionic gonadotropin hormone. J Clin Endocrinol Metab 2005; 90:2904–2910.

12 Robertson SA, Chin PY, Glynn DJ, Thompson JG: Peri-conceptual cytokines–setting the trajectory for embryo implantation, pregnancy and beyond. Am J Reprod Immunol 2011; 66(Suppl 1):2–10.

13 Sheth KV, Roca GL, al-Sedairy ST, Parhar RS, Hamilton CJ, al-Abdul Jabbar F: Prediction of successful embryo implantation by measuring interleukin-1-alpha and immunosuppressive factor(s) in preimplantation embryo culture fluid. Fertil Steril 1991; 55:952–957.

14 Apte RN, Voronov E: Is interleukin-1 a good or bad 'guy' in tumor immunobiology and immunotherapy? Immunol Rev 2008; 222:222–241.

15 Vidal-Vanaclocha F, Alvarez A, Asumendi A, Urcelay B, Tonino P, Dinarello CA: Interleukin 1 (IL-1)-dependent melanoma hepatic metastasis in vivo; increased endothelial adherence by IL-1-induced mannose receptors and growth factor production in vitro. J Natl Cancer Inst 1996; 88:198–205.

16 Meager A: Cytokine regulation of cellular adhesion molecule expression in inflammation. Cytokine Growth Factor Rev 1999; 10:27–39.

17 Bar D, Apte RN, Voronov E, Dinarello CA, Cohen S: A continuous delivery system of IL-1 receptor antagonist reduces angiogenesis and inhibits tumor development. FASEB J 2004; 18:161–163.

18 Greenfeder SA, Nunes P, Kwee L, Labow M, Chizzonite RA, Ju G: Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. J Biol Chem 1995; 270:13757–13765.

19 Colotta F, Re F, Muzio M, Bertini R, Polentarutti N, Sironi M, Giri JG, Dower SK, Sims JE, Mantovani A: Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. Science 1993; 261:472–475.

20 Herrmann-Lavoie C, Rao CV, Akoum A: Chorionic gonadotropin down-regulates the expression of the decoy inhibitory interleukin 1 receptor type II in human endometrial epithelial cells. Endocrinology 2007; 148:5377–5384.

21 Shao R, Guo X: Human microvascular endothelial cells immortalized with human telomerase catalytic protein: a model for the study of in vitro angiogenesis. Biochem Biophys Res Commun 2004; 321:788–794.

22 Staton CA, Reed MW, Brown NJ: A critical analysis of current in vitro and in vivo angiogenesis assays. Int J Exp Pathol 2009; 90:195–221.

23 Bourdiec A, Shao R, Rao CV, Akoum A: Human chorionic gonadotropin triggers angiogenesis via the modulation of endometrial stromal cell responsiveness to interleukin 1: a new possible mechanism underlying embryo implantation. Biol Reprod 2012; 87:1–10.

24 Akoum A, Lawson C, Herrmann-Lavoie C, Maheux R: Imbalance in the expression of the activating type I and the inhibitory type II interleukin 1 receptors in endometriosis. Hum Reprod 2007; 22:1464–1473.

25 Bellehumeur C, Blanchet J, Fontaine JY, Bourcier N, Akoum A: Interleukin 1 regulates its own receptors in human endometrial cells via distinct mechanisms. Hum Reprod 2009; 24:2193–2204.

26 Sims JE, Smith DE: The IL-1 family: regulators of immunity. Nat Rev Immunol 2010; 10:89–102.

27 Smith DE, Hanna R, Della F, Moore H, Chen H, Farese AM, MacVittie TJ, Virca GD, Sims JE: The soluble form of IL-1 receptor accessory protein enhances the ability of soluble type II IL-1 receptor to inhibit IL-1 action. Immunity 2003; 18:87–96.

28 Waugh DJ, Wilson C: The interleukin-8 pathway in cancer. Clin Cancer Res 2008; 14:6735–6741.

29 Gazvani R, Smith L, Fowler PA: Effect of interleukin-8 (IL-8), anti-IL-8, and IL-12 on endometrial cell survival in combined endometrial gland and stromal cell cultures derived from women with and without endometriosis. Fertil Steril 2002; 77:62–67.

30 Mulayim N, Savlu A, Guzeloglu-Kayisli O, Kayisli UA, Arici A: Regulation of endometrial stromal cell matrix metalloproteinase activity and invasiveness by interleukin-8. Fertil Steril 2004; 81(Suppl 1):904–911.

31 Tsui KH, Chen LY, Shieh ML, Chang SP, Yuan CC, Li HY: Interleukin-8 can stimulate progesterone secretion from a human trophoblast cell line, BeWo. In Vitro Cell Dev Biol Anim 2004; 40:331–336.

32 Hirota Y, Osuga Y, Hasegawa A, Kodama A, Tajima T, Hamasaki K, Koga K, Yoshino O, Hirata T, Harada M, Takemura Y, Yano T, Tsutsumi O, Taketani Y: Interleukin (IL)-1beta stimulates migration and survival of first-trimester villous cytotrophoblast cells through endometrial epithelial cell-derived IL-8. Endocrinology 2009; 150:350–356.

33 Milne SA, Critchley HO, Drudy TA, Kelly RW, Baird DT: Perivascular interleukin-8 messenger ribonucleic acid expression in human endometrium varies across the menstrual cycle and in early pregnancy decidua. J Clin Endocrinol Metab 1999; 84:2563–2567.

34 Dame JB, Juul SE: The distribution of receptors for the pro-inflammatory cytokines interleukin (IL)-6 and IL-8 in the developing human fetus. Early Hum Dev 2000; 58:25–39.

35 Fluhr H, Sauter G, Steinmuller F, Licht P, Zygmunt M: Nonapoptotic effects of tumor necrosis factor-related apoptosis-inducing ligand on interleukin-6, leukemia inhibitory factor, interleukin-8, and monocyte chemoattractant protein 1 vary between undifferentiated and decidualized human endometrial stromal cells. Fertil Steril 2009; 92:1420–1423.

36 Jones RL, Hannan NJ, Kaitu'u TJ, Zhang J, Salamonsen LA: Identification of chemokines important for leukocyte recruitment to the human endometrium at the times of embryo implantation and menstruation. J Clin Endocrinol Metab 2004; 89:6155–6167.

37 Kruessel JS, Huang HY, Wen Y, Kloodt AR, Bielfeld P, Polan ML: Different pattern of interleukin-1 beta-(IL-1 beta), interleukin-1 receptor antagonist- (IL-1ra) and interleukin-1 receptor type I- (IL-1R tI) mRNA-expression in single preimplantation

mouse embryos at various developmental stages. J Reprod Immunol 1997; 34:103–120.

38 Lindhard A, Bentin-Ley U, Ravn V, Islin H, Hviid T, Rex S, Bangsboll S, Sorensen S: Biochemical evaluation of endometrial function at the time of implantation. Fertil Steril 2002; 78:221–233.

39 Simon C, Gimeno MJ, Mercader A, O'Connor JE, Remohi J, Polan ML, Pellicer A: Embryonic regulation of integrins beta 3, alpha 4, and alpha 1 in human endometrial epithelial cells in vitro. J Clin Endocrinol Metab 1997; 82:2607–2616.

40 Simon C, Piquette GN, Frances A, el-Danasouri I, Irwin JC, Polan ML: The effect of interleukin-1 beta (IL-1 beta) on the regulation of IL-1 receptor type I messenger ribonucleic acid and protein levels in cultured human endometrial stromal and glandular cells. J Clin Endocrinol Metab 1994; 78:675–682.

41 Huang JC, Liu DY, Yadollahi S, Wu KK, Dawood MY: Interleukin-1 beta induces cyclooxygenase-2 gene expression in cultured endometrial stromal cells. J Clin Endocrinol Metab 1998; 83:538–541.

42 Strakova Z, Mavrogianis P, Meng X, Hastings JM, Jackson KS, Cameo P, Brudney A, Knight O, Fazleabas AT: In vivo infusion of interleukin-1beta and chorionic gonadotropin induces endometrial changes that mimic early pregnancy events in the baboon. Endocrinology 2005; 146:4097–4104.

43 Bonetti TC, Salomao R, Brunialti M, Braga DP, Borges E Jr, Silva ID: Cytokine and hormonal profile in serum samples of patients undergoing controlled ovarian stimulation: interleukin-1beta predicts ongoing pregnancy. Hum Reprod 2010; 25:2101–2106.

44 Boucher A, Kharfi A, Al-Akoum M, Bossu P, Akoum A: Cycle-dependent expression of interleukin-1 receptor type II in the human endometrium. Biol Reprod 2001; 65:890–898.

4.9 Figure Legends

Figure 1. Immunocytofluorescence analysis of LHCGR, IL1Rs, and IL1RN in HMVECs. The receptors were detected using antibodies specific to LHCGR (a), IL1R1 (c), IL1R2 (e), and IL1RAP (g). Streptavidin-fluorescein isothiocyanate (green) was used for detection of IL1Rs receptors and Alexa 488 (red) for LHCGR. Cells were counterstained with 4',6-diaminido-2-phenyl-indole (blue). Cells incubated without the primary antibody were included as negative controls (b, d, f, h). Data are representative of experiments from three different cultures.

Figure 2. hCG and IL1B stimulate HMVEC proliferation and migration. For cell proliferation (a, b, c), HMVEC (104 cells/well in 96-well microtiter plates) were cultured overnight, starved in the endothelial cell basal medium (MCDB 131) containing 0.2% FBS and incubated in quadricate for 48 h with this medium alone (control medium) or supplemented with hCG, IL1 and hCG/IL1 at the indicated concentrations. Cell proliferation was assessed by ELISA using BrdU incorporation into HMVEC DNA. Data are from four different cultures and expressed as % of control. For cell migration, (d), confluent HMVEC monolayers were wounded by scraping and incubated in duplicate for 24 h with hCG, IL1B, and hCG/IL1B at the indicated concentrations, and cell migration was quantified by microscopy at magnification ×4. Data are from 3 different cultures and expressed as % of closure. *P < 0.05, **P < 0.01, ***P < 0.001 relative to the control medium. †P < 0.05, ††P <

0.01 relative to equivalent hCG dose; +P < 0.05, +++P < 0.001 relative to equivalent IL1B dose.

Figure 3. hCG regulates IL1 receptors' mRNA expression. Confluent cell cultures were starved in the endothelial cell basal medium (MCDB 131) containing 0.2% FBS and incubated in duplicate with this medium alone (control medium) or supplemented with hCG, IL1B or hCG/IL1B at the indicated concentrations for 24 (a, c, e, g, i, k) and 48 (b, d, f, h, j, l) h. Total RNA was extracted and reverse-transcribed. IL1R1, IL1R2, mbIL1RAP, sIL1RAP, IL1RN, LHCGR, and GAPDH (internal control) mRNA levels were quantified by real-time PCR, as described in 'Materials and Methods.' IL1R1 (a, b), IL1R2 (c, d), mbIL1RAP (e, f), sIL1RAP (g, h), IL1RN (i, j), and LHCGR (k, l) mRNA ratio were then determined following normalization to GAPDH mRNA. Data were from eight different cultures and expressed as % of control (ratio of IL1Rs, IL1RN or LHCGR mRNA levels measured in cells exposed to IL1B, hCG or hCG/ILB to those measured in cells exposed to the control medium). *P < 0.05, **P < 0.01 relative to the control medium; †P < 0.05, ††P < 0.01 relative to equivalent IL1B dose.

Figure 4. hCG regulates IL1 receptors' protein expression. Confluent cell cultures were starved in the endothelial cell basal medium (MCDB 131) containing 0.2% FBS and incubated in duplicate with this medium alone (control medium) or supplemented with hCG, IL1B or hCG/IL1B at the indicated concentrations for 48 h. Cells were recovered to extract total proteins and analyze IL1Rs, IL1RN, and LHCGR by Western blotting (a), and culture supernatants were collected to measure sIL1R2 (b), sIL1RAP (c), and IL1RN (d) as described in 'Materials and Methods'. Data were from six (sIL1R2 and sIL1RAP) and four (IL1RN) different cultures and expressed as % of control (ratio of sIL1R2 or IL1RN levels in cells exposed to IL1B, hCG or hCG/ILB to those in cells exposed to the control medium). *P < 0.05, *** P < 0.001 to the control medium; ††P < 0.01 relative to equivalent IL1B dose.

Figure 5. hCG increases the IL1B-induced expression of IL8 in HMVEC. Cells were grown to confluence, starved in the endothelial cell basal medium (MCDB 131) containing 0.2% FBS and incubated in duplicate with this medium alone (control medium) or supplemented with hCG, IL1B or hCG/IL1B at the indicated concentrations for 48 h. Total cell RNA was extracted and reverse-transcribed, and real-time PCR for IL8 and GAPDH was performed as described in the Material and Methods section. IL8 mRNA (a) levels were determined following normalization to GAPDH mRNA levels. IL8 concentration in the culture medium was determined by ELISA (b). Data are from 5 independent HMVEC cultures and expressed as % of control (ratio of IL8 mRNA levels or protein secretion in cells exposed to IL1B, hCG or IL1B/hCG to those in cells exposed to the control medium). *P < 0.05, **P < 0.01 relative to the control medium; †P < 0.05 relative to IL1B control dose.

Figure 6. IL1R2 is a possible target for the hCG-mediated modulation of endothelial cell responsiveness to IL1B. HMVECs were transfected with pcDNA3-IL1R2 or with the empty pcDNA3 vector. Transfected and non-transfected cells were incubated in triplicate and for 48 h with the endothelial cell basal medium (MCDB 131) containing 0.2% FBS (control medium) or supplemented with hCG, IL1B or hCG/IL1B at the indicated concentrations. IL8 (a) and sIL1R2 (b) concentrations in the culture supernatant were measured by ELISA. *P < 0.05, ***P < 0.001 relative to the control medium; †P < 0.05 relative to equivalent IL1B dose; +P < 0.05, ++P < 0.01 relative to equivalent IL1B or hCG/IL1B dose.

4.10 Figures

Figure 1. Immunocytofluorescence analysis of LHCGR, IL1Rs, and IL1RN in HMVECs.

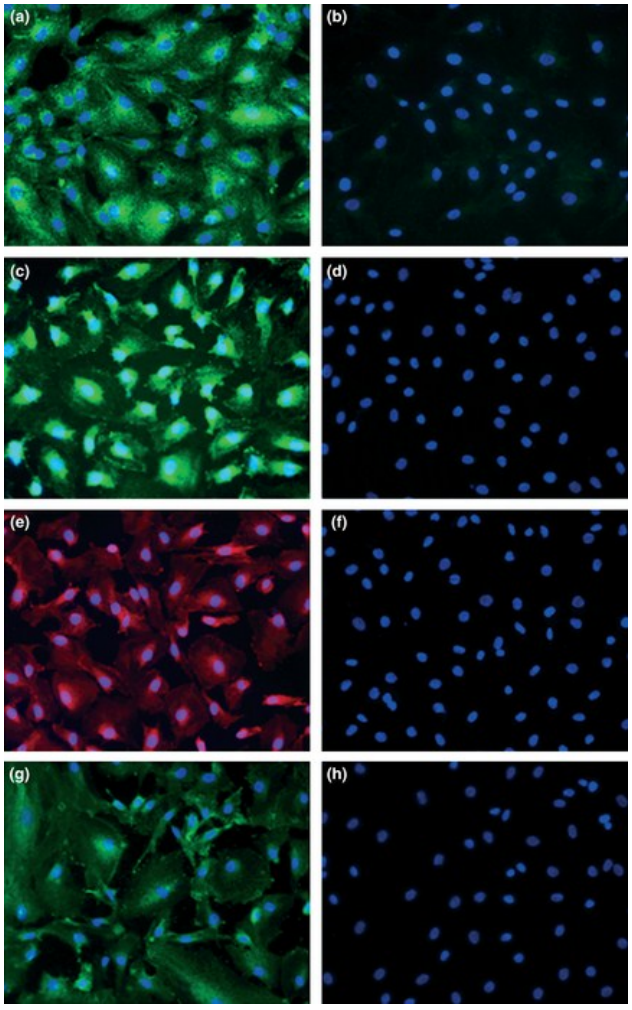


Figure 2. hCG and IL1B stimulate HMVEC proliferation and migration.

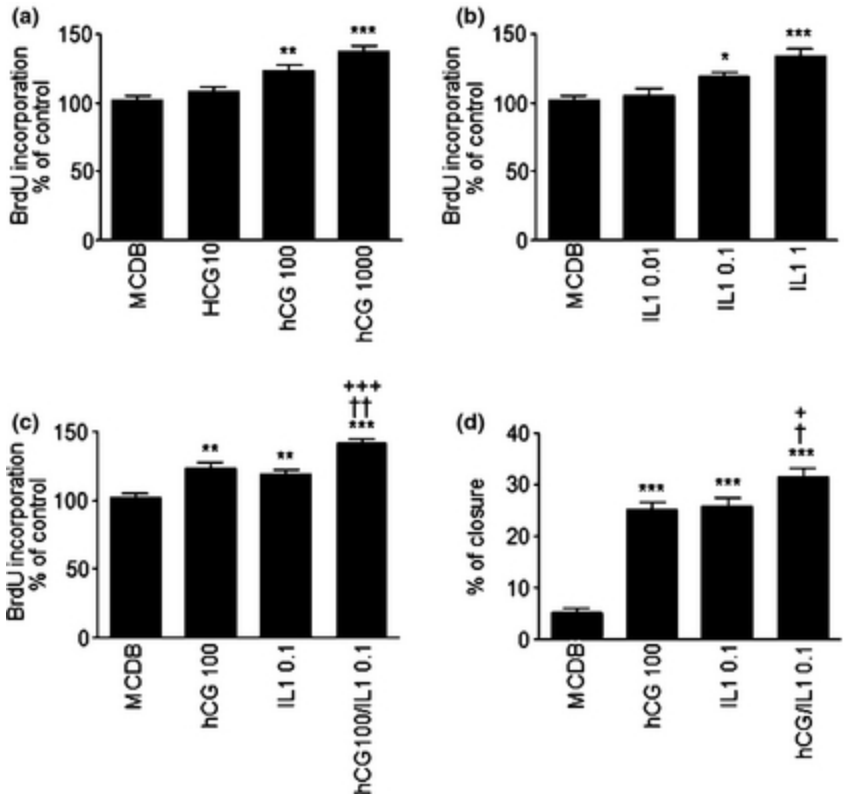


Figure 3. hCG regulates IL1 receptors' mRNA expression.

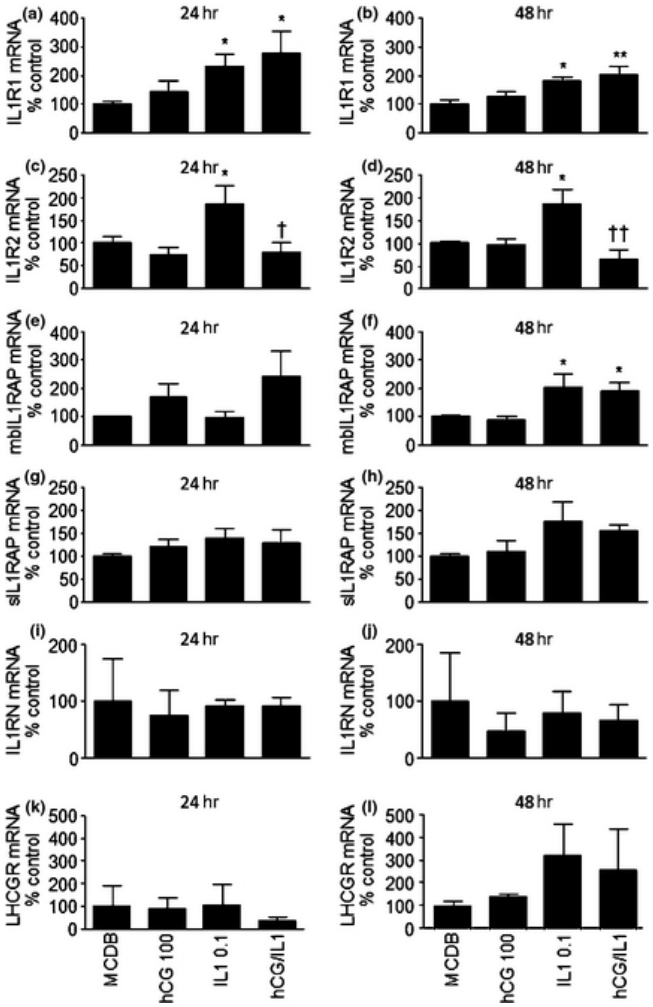


Figure 4. hCG regulates IL1 receptors' protein expression.

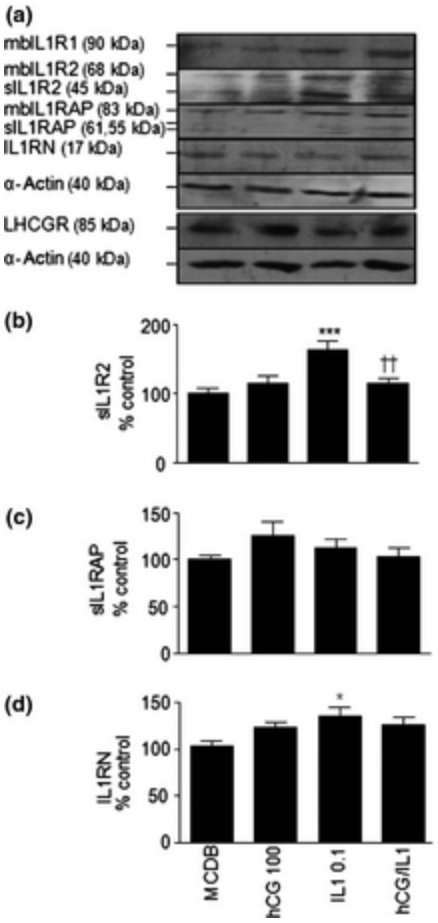


Figure 5. hCG increases the IL1B-induced expression of IL8 in HMVEC.

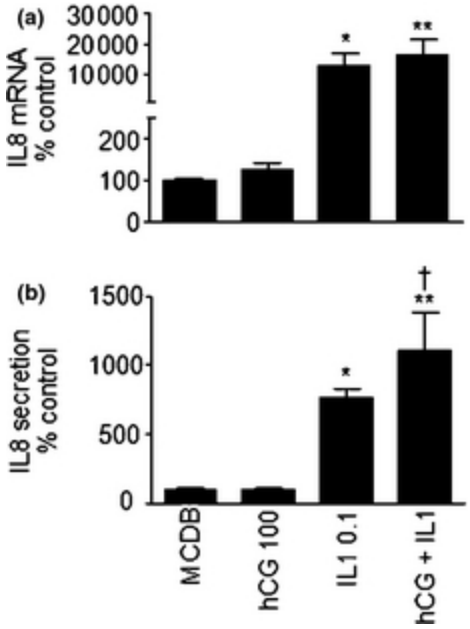
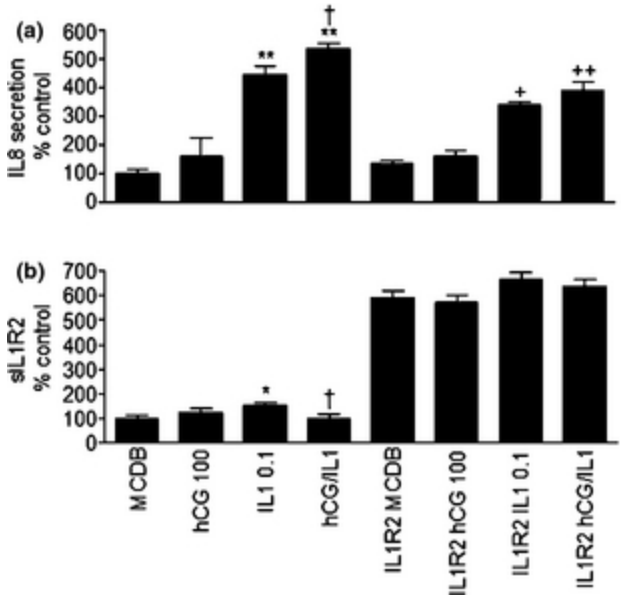


Figure 6. IL1R2 is a possible target for the hCG-mediated modulation of endothelial cell responsiveness to IL1B.



Chapitre 5 : Synchronous regulation of the determinants of endometrial receptivity to interleukin 1 at key stages of early embryo implantation *in vivo*

Amélie Bourdiec,[a] Valéry Martel ,[a] and Ali Akoum.[a,b]

[a]*Endocrinologie de la Reproduction, Centre Hospitalier Universitaire de Québec, Hôpital Saint-François d'Assise, Faculté de Médecine, Université Laval, Québec, Canada,*

[b]Département d'Obstétrique et Gynécologie, Faculté de Médecine, Université Laval, Québec, Canada.

Soumis à Fertility and Sterility.

5.1 Résumé

L'objectif est d'étudier la cinétique d'expression des récepteurs de l'interleukine 1 (Il1rs), du récepteur antagoniste (Il1rn) et la protéine chimiotactique des monocytes 1 (mcp1) tout au long de la gestation précoce chez la souris, soit de l'accouplement au jour 8. Des femelles B6C3F1 âgée de 8 semaines sont placées avec des mâles fertiles de la même souche. Après l'accouplement, du jour 1 au jour 8, les femelles sont sacrifiées et le tissus endométrial est ensuite collecté à la nécropsie. À partir du cinquième jour de gestation la distinction entre endomètre implanté (i) et non implanté (ni) est possible grâce à l'injection intra veineuse de Chicago Blue ®.

L'expression de l'Il1rs 1, 2 et 3, Il1rn et mcp1 est évaluées par PCR quantitative en temps réel ainsi que par immunohistochimie. Les résultats montrent une augmentation considérable de l'expression du récepteur fonctionnel Il1r1 au cours des 2 premiers jours de gestation (GD1-2), qui correspondent à la phase réactive induite par le liquide séminal, avant d'augmenter à nouveau à l'ouverture de la fenêtre d'implantation et se maintient tout au long de l'implantation embryonnaire. L'expression du récepteur leurre Il1r2 et du récepteur inhibiteur concomitamment augmenté en GD1-2, est restée faible au long de la période d'implantation, en particulier dans les sites d'implantation de l'embryon. L'expression du MCP1 est significativement augmentée seulement dans les sites d'implantation de l'embryon et a montré une corrélation positive significative avec l'expression de l'Il1r1.

Nos données ont permis d'identifier pour la première fois la synchronie des changements d'expression des Il1rs de l'endomètre, tout au long de la gestation précoce in vivo. Ils mettent en évidence une modulation profonde de réceptivité de l'endomètre à l'IL1 par des signaux embryonnaires. Cela peut jouer un rôle clé dans

la création d'un phénotype réceptif dans l'endomètre maternel et représenter un mécanisme fondamental qui sous-tend l'implantation de l'embryon et sa croissance.

5.2 Abstract

Objective: To investigate the expression kinetics of interleukin 1 receptors (Il1rs), receptor antagonist (Il1rn) and monocyte chemotactic protein 1 (Mcp1) throughout early gestation in mice.

Design: Assessment of Il1rs, Il1rn and Mcp1 all along early pregnancy.

Setting: Reproduction laboratory.

Animals: B6C3F1 female mice bred with fertile males of the same strain.

Intervention(s): Collection of endometrial tissue at necropsy from non-implanted (ni) and implanted (i) sites.

Main Outcome Measure(s): Il1rs, Il1rn and Mcp1 mRNA expression by qRT-PCR and protein expression by ELISA and immunohistochemistry.

Results: The expression of the signaling Il1r1 significantly increased in the first 2 days of gestation (GD1-2), which correspond to the inflammatory-like period triggered by the seminal fluid, before increasing again at the implantation window and lasting all along embryo implantation. The expression of the inhibitory Il1r2 and Il1rn concomitantly increased in GD1-2, but remained low particularly within the embryo implantation sites and throughout the implantation period. Mcp1 expression significantly increased only in the embryo implantation sites and showed a significant positive correlation with Il1r1 expression.

Conclusions: Our data identified for the first time synchronous changes in endometrial Il1rs all along early gestation in vivo and points to a deep modulation of endometrial receptivity to Il1 by embryo-driven signals. This may play a key role in the creation of a receptive phenotype in the maternal endometrium and represent a key mechanism underlying embryo implantation.

Key words: Interleukin-1, endometrium, embryo implantation, early gestation

5.3 Introduction

Implantation is a critical process where conceptus comes close to and starts its nidation at the maternal endometrium interface (1). A complex network of signaling factors, adhesion molecules and functional effectors may be involved during this process.

Embryonic signaling molecules, such as interleukin 1 (IL1), have been investigated both in human and rodent embryo implantation processes. The IL1 system consists in two agonists (IL1A and IL1B), a functional signaling receptor (IL1R1), which in combination with IL1R accessory protein (IL1RAP), also known as IL1R3, mediates cell activation by IL1, an antagonist (IL1RN), which competes with IL1 for binding to IL1R1, and a decoy inhibitory receptor (IL1R2), which is a potent natural inhibitor of IL1 and acts by capturing IL1 and preventing thereby its interaction with IL1R1 (2). IL1 is considered as one of the earliest signals released by embryonic cells (3-7). The human embryo produces IL1 during the initial stages of its development, and IL1 concentration was positively correlated with successful implantation after in vitro fertilization and transfer to the uterine cavity (8, 9). An essential role of IL1B in implantation was demonstrated by repeated injections of Il1rn into pregnant mice prior to implantation, which caused implantation failure (6, 10). In humans, IL1B was shown to induce the expression of key mediators of embryonic implantation and growth in maternal endometrial, trophoblastic and placental cells. This cytokine appeared to upregulate the endometrial expression of leukemia inhibitory factor (LIF) (11, 12), prostaglandin-endoperoxide synthase 2 (PTGS2), also known as cyclooxygenase 2 (COX2), (13, 14) and leptin (15), potentiate human cytotrophoblast metalloproteinase activity and invasion (16) and induce IL6 production in placental villous core mesenchymal cells (17). Interestingly, IL1 was shown to stimulate human

chorionic gonadotropin (hCG) secretion by first trimester human trophoblasts (18). hCG, one of the earliest key mediators of the embryo-maternal communication network, plays a crucial role in the regulation of endometrial cell differentiation and the coordination of the initial steps of human embryo implantation (19). In women, a decreased expression of IL1B in endometrium during the mid-secretory phase of the menstrual cycle, which corresponds to the implantation window, was associated with habitual abortion (20), suggesting a role for IL1B in the maintenance of pregnancy. Pre-implanted human embryos express IL1B (21), and IL1R1, the signaling activating receptor of IL1, is present in the uterine endometrium (22, 23). Our previous studies carried out on human endometrium showed that the expression of IL1R1 increases during the mid-secretory phase of the menstrual cycle, while that of the inhibitory IL1R2 slightly decreases (24). Our studies further revealed that hCG down-regulates the expression of IL1R2 in human endometrial epithelial cells, without affecting the expression of IL1R1 (25). In pre-decidualized human endometrial stromal cells, hCG appeared to repress the expression of IL1R2, even in presence of IL1B known for upregulating its own receptors (26), and to stimulate in parallel the expression of IL1R1, leading thereby to an imbalance in IL1 receptors at the time of implantation (27). Our studies also led to the finding of similar hCG-mediated bidirectional regulation of IL1Rs in endothelial cells and a possible impact on angiogenesis (28).

Beyond major differences between mice and human embryo implantation, there are many common features including the involvement of the same steroid hormones, a short window of receptivity, stromal decidualization, a hemochorial implantation and an invasive trophoblast, although to a lesser magnitude in rodents than in humans (29-31).

Based on our findings in vitro, showing a deep modulation of endometrial cell receptivity to IL1 driven by major embryonic signals and targeting different endometrial cell types, we thought relevant to assess whether such embryo-maternal interactions occur in vivo and study the kinetics of Il1rs' expression throughout early gestation and embryo implantation.

5.4 Material and Methods

5.4.1 Animals

This study was reviewed and approved by the Animal Care and Use Committee of Laval University and performed in the animal facilities of the Research Center of the CHU de Quebec, Saint-François d'Assise Hospital. Eight-week-old B6C3F1 mice were obtained from Charles River Laboratories, Canada, housed upon arrival (four per cage) and allowed to acclimatize for 1 week. Animals had free access to food and water and were maintained at 22°-24°C on a 12-hour light:12-hour dark cycle. Female mice were checked daily for vaginal smear and those that are in estrus were immediately bred with fertile males of the same strain. The day of the post-coital vaginal plug was designated as gestational day (GD) 1. Implantation sites were visualized by intravenous (iv) injection of 1% Chicago Blue Dye (Sigma-Aldrich Corp., St. Louis, MO) into the jugular vein 5 minutes before necropsy at the end of the day 4 or the beginning of the day 5 of gestation (32) (Fig. 1). Females (5/group) were killed on gestational day 0 (estrus), 1, 2, 4, 5, 6, and 8 of pregnancy at 10:00 am.

At necropsy, one uterus horn was opened longitudinally and the endometrial tissue, from non-implanted (ni) and implanted (i) sites, was gently collected with sterile forceps. Tissue was rinsed twice with sterile PBS and then stored at -80°C.

Subsequently, total RNAs and proteins were extracted using Trizol reagent (Invitrogen Life Technologies, Burlington, ON, Canada). The other uterus horn was stored at -80°C in Tissue-Tek® OCT (Optimal Cutting Temperature) Compound (Sakura Finetek, Torrance, CA) for histology.

5.4.2 RNA Extraction, Reverse Transcription, and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted with Trizol according to the manufacturer's directions (Invitrogen Life Technologies), reverse transcribed and quantitative real time PCR (qRT-PCR) was performed as previously reported (33) using an ABI 7500 Thermal Cycler (Applied Biosystem, Foster City, CA, USA). Each standard PCR reaction contained 2 µL reverse transcriptase (RT) product, 0.5 µL of primer (final concentration, 0.1 mM), 12.5 µL of SYBR Green PCR Master Mix (Invitrogen Life Technologies) consisting of Taq DNA polymerase reaction buffer, Taq DNA polymerase, SYBR green I, deoxynucleotide triphosphate mix and MgCl2. Following a 95°C denaturation for 2 min, the reactions were cycled 45 times with a 95°C denaturation for 15 sec and a 60°C annealing for 60 sec, except for Mcp1 where annealing was performed at 56°C. Primers for Il1r1 (forward, 5'-ATGCACGGCGACACCA-3'; reverse, 5'-AGTTTTGAGGCAGTAAGTTGAGT-3'), Il1r2 (forward, 5'-AGTGCAGCAAGACTCTGGTACCTA-3'; reverse, 5'-AGTTCCACAGACATTTGCTCACA-3'), Il1rap (forward, 5'-ATGGGACTTCTGTGGTATTTGA-3'; reverse, 5'-ATGGGCAGTGCTGTAGTTGTA-3'), Il1rn (forward, 5'-AAGATAGACATGGTGCCTATTGA-3'; reverse, 5'-AGAGCGGATGAAGGTAAAGC-3'), Mcp1 (forward, 5'-GTCCCTGTCATGCTTCTGG-3'; reverse, 5'-AGGTGAGTGGGGCGTTAA-3') and GAPDH (forward, 5'-CCTTCCGTGTTCCTACCCC-3'; reverse, 5'-GCCCAAGATGCCCTTCAGT-3') were designed using Primer Premier 5 software to cross intron-exon boundaries. All samples were

tested in duplicate and for each reaction negative controls without RNA and without reverse transcriptase were included.

5.4.3 Protein Extraction and Desalting

Proteins were extracted with Trizol according to the manufacturer's instructions (Invitrogen Life Technologies) from the same samples that were used to extract RNA. Briefly, After RNA isolation with Trizol, the DNA was precipitated from the organic phase with ethanol and the phenol-ethanol supernatant was then used for protein isolation. Proteins were precipitated with cold acetone (-20°C), washed with guanidine hydrochloride/ethanol/glycerol solution, air-dried and solubilized with 1% SDS. Protein solution was desalted using Amicon® Ultra-0.5 centrifugal filter devices (Millipore Canada, Ltd., Etobicoke, ON, Canada). The concentration of solubilized proteins was determined using the Bio-Rad DC Protein Assay (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada).

5.4.4 Enzyme-Linked Immunosorbent Assay (ELISA)

Mcp1 concentrations were measured using an ELISA procedure developed in the laboratory. Briefly, 96-well plates were coated overnight at 4°C with a rabbit polyclonal anti-hMCP1 antibody (34), which also recognizes mouse Mcp1 □1:1000 dilution in phosphate buffered saline (PBS)□. Plates were washed four times with 0.01 M PBS containing 0.1% Tween 20 (washing buffer), and 100 µL aliquots of recombinant mouse Mcp1 (ProSpec East Brunswick, 08816 NJ), used at concentration ranging from 0.1 to 8.0 ng/mL, or 10 ug of total endometrial protein extracts diluted in PBS containing 0.5% bovine serum albumin (BSA) were then added to the plates. After 60 min incubation at 37°C, the plates were washed and incubated for 60 min at 37°C with biotin-conjugated rabbit polyclonal anti-hMcp1

antibody (1:2000 dilution in PBS containing 0.5% BSA), washed and incubated for 60 min at 37°C with streptavidin-peroxidase (Zymed laboratories, Inc. San Francisco, CA, USA) (1/2000 dilution in PBS/0.5% BSA). After a final wash, 100 µL of TMB (3,3', 5,5',-tetramethylbenzidine)-peroxidase substrate (Bio-Rad laboratories Ltd, Mississauga, Ontario, Canada) were added to each well, the enzymatic reaction was terminated by the addition of 50 µL of 2 N sulfuric acid (H2SO4) and the optical density was determined at 450 nm.

5.4.5 Immunohistochemistry

Serial cryostat sections of 5-µm thickness were cut, mounted, and placed on poly-l-lysine-coated glass microscope slides. After three 5-minute rinses in PBS, sections were incubated with PBS/1% (vol/vol) Triton X-100 (Sigma-Aldrich) for 20 minutes at room temperature to increase cell membrane permeability and treated with PBS containing 5% bovine serum albumin (BSA) and 0.01% Tween 20 (Sigma-Aldrich) for 20 minutes at room temperature. Subsequently, endogenous hydrogen peroxidase activity was quenched using 3% H2O2 in methanol for 20 min at room temperature. Tissue cryosections were then incubated overnight at 4°C and 1 hour at room temperature with 100 µL of an antibody specific to Il1r1 (20 µg/mL in PBS/1% BSA/0.01% Tween 20; sc 689; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Il1r2 (20 µg/mL in PBS/1% BSA/0.01% Tween 20; R&D Systems, Minneapolis, MN, USA), Il1rn (20 µg/mL in PBS/1% BSA/0.01% Tween 20; Genzyme Canada Inc., Mississauga, ON, Canada), Il1rap (20 µg/mL in PBS/1% BSA/0.01% Tween 20; R&D Systems) or Mcp1 (1:10 dilution in PBS/1% BSA/0.01% Tween 20)(34). After three washes in PBS, sections were incubated for 60 minutes with biotin-conjugated goat anti-rabbit IgGs for Il1r1, Il1rap and Mcp1 or biotin-conjugated rabbit anti-goat IgGs for Il12 and Ilrn (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) (1:200 dilution in PBS/0.2% BSA/0.01% Tween 20). Negative controls included tissue sections

incubated without the primary antibody or with an equivalent concentration or dilution of normal rabbit or goat IgGs. Sections were then washed 3 times in PBS, incubated for 45 minutes with a peroxidase-conjugated streptavidin (Jackson Immuno Research Laboratories) (1:500 in PBS/0.2% BSA/0.01% Tween 20), washed in PBS and incubated 20 minutes with 3,3'-diaminobenzidine (DAB), used as chromogen substrate for peroxidase enzyme (3 mg DAB/0.03% H2O2 in PBS). Sections were finally counterstained with hematoxylin (Sigma-Aldrich) and mounted in Mowiol (Calbiochem-Novabiochem Corp., La Jolla, CA).

5.4.6 Statistical Analysis

Statistical analyses were performed using one-way ANOVA, followed by the Bonferroni's test for multiple comparisons (Prism 4.0; GraphPad Software Inc., San Diego, CA) and the Student's t-test for the comparison of two groups. Differences were considered as significant for p values less than 0.05.

5.5 Results

5.5.1 Analysis of Il1rs and Il1rn mRNA expression

Quantitative real-time PCR analysis showed that all Il1rs and antagonists are present in mouse endometrium (Fig. 2). In the first two days preceding embryo implantation, which correspond to the reactive phase where an immune response directed against sperm is known for taking place within the genital tract (35, 36), the functional receptor Il1r1 increased to a first peak at day 2 (p □ 0.01), compared to day 0, but the accessory protein Il1rap presented non-significant variations. At gestation day 3, Il1r1 expression decreased to baseline before increasing again at gestation day 4 (p □ 0.01), which is believed to match with the short implantation window when the

progesterone-primed uterus becomes receptive (35, 37), while no significant changes in Il1rap were seen. Il1r1 expression remained significantly increased only in the embryo implantation sites on day 5 compared to the non-implanted sites or to day 0 (p ≤ 0.01), and throughout the following 3 days of the early gestation period (p ≤ 0.05, p ≤ 0.05 and p ≤ 0.001, respectively). A significant increase in Il1rap expression was only observed at day 7 (p ≤ 0.001).

The inhibitory receptor Il1r2 and the receptor antagonist Il1rn displayed an increased expression in the mouse endometrium at day 1 of gestation (p ≤ 0.001), but declined afterwards at day 2, during the implantation window and throughout embryo implantation, either in the implanted or the non-implanted endometrial sites, before increasing significantly at day 8 (p ≤ 0.001). Il1rn expression followed an expression pattern comparable to that of Il1r2, but further showed a significant decrease in the embryo implantation sites at day 5 compared to the non-implanted sites (p ≤ 0.001) and remained low until day 8 of gestation. Figure 2E summarizes the relative changes in Il1rs and antagonist at different gestational days.

5.5.2 Analysis of Il1rs and IL1rn protein expression

Il1rs' expression during early gestation was then studied by immunohistochemical analyses, which corroborated the mRNA expression pattern. As shown in Fig. 3, during the first two days of pregnancy, Il1r1 immunostaining increased in stromal and epithelial compartments of the mouse endometrium. In addition, the staining was more intense at the apical side of the luminal epithelium. On day 4, Il1r1 immunostaining increased in the stromal and epithelial compartments as well, and such a staining was intense in the glandular epithelium. On day 5, Il1r1 immunostaining was detected both in the inter- and the intra-implantation areas of the maternal endometrium. In the inter-implantation areas, staining was noticeably intense at the apical side of the luminal epithelium, while in the implantation areas,

Il1r1 immunostaining was strongly detected both in epithelial and stromal compartments and remained high throughout the endometrial tissue until gestation day 8. Il1rap immunostaining rather seems to be constitutive and did not show noticeable changes across the mouse endometrium during early gestation.

On the first day of gestation, Il1r2 and Il1rn showed a notable increase of immunostaining in the mouse endometrium, which was strongly present in glandular epithelium and stroma. Il1r2 and Il1rn immunostainning decreased afterwards and remained low at the implantation window on day 4 and during embryo implantation, either in the inter- or the intra-implantation sites. Il1r2 immunostaining increased again at gestation days 7 and particularly 8, where it was prominent in the stromal and vascular compartments, whereas a slight Il1rn immunostaining could be seen. It is worth noting that during preparation for embryo implantation, the endometrium displayed classical histological characteristics, with luminal and glandular epithelium, and stroma. In the implantation sites, however, histological alterations, a loss in the epithelial compartment and marked vascularization were observed. No immunostaining in serial tissue sections incubated without the primary antibody or with an equivalent concentration of normal IgGs of the same species of the primary antibody was observed (data not shown)

5.5.3 Analysis of Mcp1 expression

Our previous studies carried on human stromal cells pointed to Mcp1 as a marker for maternal responsiveness to embryonic factors implicated in maternal receptivity (27). Here we investigated Mcp1 mRNA and protein expression patterns. Interestingly, both Mcp1 mRNA and protein levels significantly increased at the embryo implantation sites, compared to the non-implanted endometrial areas at

day 5 ($p < 0.05$ and $p < 0.001$, respectively) and day 6 ($p < 0.05$ and $p < 0.001$, respectively) of pregnancy (Fig. 4 A and B). Such an increase remained significant in the presence of the embryo at days 7 and 8 compared to day 4, as determined at the mRNA level ($p < 0.001$). Furthermore, a positive correlation between Mcp1 and Il1r1 mRNA levels in the embryo implantation sites at days 5 to 8 was found ($p < 0.0001$) (Fig. 4 C), whereas no correlation in the non-implanted endometrial areas at days 4 to 6 was noted (Fig. 4 D). Mcp1 expression at days 7 and 8 could not be assessed at the protein level by ELISA, because of the difficulty to collect sufficient amounts of endometrial tissues for protein extraction from the implantation sites at the end of the early gestational period. Nevertheless, Mcp1 showed an intense immunostaining in the mouse endometrium during these days of early pregnancy as well as in the embryo implantation sites at days 5 and 6 of gestation, compared to non-implanted endometrial areas (Fig. 4 E). Also of note were the disruption of endometrial structures in the embryo implantations sites and the detection of positive immunostaining for Mcp1 in embryonic cells (Fig. 4 F). No immunostaining in serial tissue sections incubated without the primary anti-Mcp1 antibody or with an equivalent concentration of normal rabbit IgGs was observed (data not shown).

5.6 Discussion

This study first identified significant variations in Il1rs' expression, which occur at key stages throughout early pregnancy in vivo in mice, that is following mating, during the development of endometrial receptivity to the fertilized oocytes and all along the implantation process of the embryos into the maternal endometrium.

The expression of Il1r1, the functional activating receptor of Il1, significantly increased on days 1 and 2 of gestation as well as on day 4, which corresponds to the

short window of uterine receptivity and embryo implantation period (35, 37), and remains high within the embryo implantation sites throughout early pregnancy. The membrane-bound accessory protein of this receptor, Il1rap, which is essential for Il1 signaling via Il1r1, meanwhile showed a slightly variable expression throughout early gestation, but suddenly increased at day 7. This may favor Il1-mediated cell activation via the Il1r1/Il1rap signaling complex. Nevertheless, this result was unexpected as Il1rap rather showed a constitutive expression in the endometrium of women during the menstrual cycle (38). Interestingly, Il1r2, a potent natural specific inhibitor of Il1, displayed a level of expression that was high on the first day of gestation, but then decreased and remained low throughout the period of uterine receptivity and embryo implantation before increasing again on day 7 and significantly on day 8. Il1rn, which also counteracts cell activation by Il1, presented a relatively similar expression pattern with an increase on days 1 and 2 of gestation and a low level of expression throughout the following period of early gestation.

These findings may have important physiological significance. In the first two days after mating, the increased expression of Il1r1 reveals an inflammatory reaction probably initiated by the interaction of the maternal endometrium with seminal fluid. The parallel increase in Il1r2 and Il1rn would represent a local counter-regulatory mechanism presumably deployed to restrain the extent of the maternal inflammatory response. This reaction is well documented in the literature. In mice, seminal fluid was shown to trigger an estrogen-primed inflammation-like response in the female genital tract that activates immune adaptations to the likelihood of conception and pregnancy (35, 36). In women, seminal fluid profoundly influences cervical and uterine immune functions, stimulates synthesis of proinflammatory factors and promotes recruitment of immune cells, which seem to increase sperm

survival and fertilization rates and elicit molecular and cellular changes that help tolerate conceptus and facilitate embryo implantation (39).

Following this post-mating inflammation-like response, which is short-lived and subsides before embryo implantation by day 3 of pregnancy (35), Il1r1 expression dropped to levels comparable to expression at estrus. A fine-tuned balance between inflammatory and immune inhibitory responses at the embryo-maternal interface is required for successful implantation, but little is known about the underlying mechanisms. Such a downregulation of the activating Il1r1 during the peri-implantation period may occur in response to a progesterone-induced shift in the local milieu due to increasing circulating progesterone, since administration of this hormone to ovariectomized mice was shown to downregulate endometrial leukocyte recruitment and expression of proinflammatory factors (35). Nevertheless, the regulation of Il1rs by estradiol and progesterone in the endometrium remains to be determined.

The main purpose of implantation is to ensure that trophoblastic cells firmly anchor into the endometrial stroma (35). In rodents, a sequence of molecular and cellular events initiated at mating culminates in a short window of uterine receptivity on day 4 (31, 35). This receptive period is characterized by complex remodeling events that implicate different types of endometrial and immune cells and play a pivotal role in the induction of an appropriate immune response and the achievement of embryonic implantation. The increase in the expression level of the signaling Il1r1, occurring concurrently with the decrease in those of the decoy inhibitory Il1r2 and the receptor antagonist Il1rn early on and during this crucial period of gestation, reveals an imbalance that would amplify the maternal endometrial tissue receptivity to Il1 and favor thereby the first steps of embryonic interaction with and implantation into the maternal endometrium. It would be of interest to note that

after day 6 of gestation, where implantation is considered as « irreversible » (32), Il1r2 expression in the maternal endometrium started to gradually to increase in the embryo implantation sites. Our present findings in vivo are in keeping with our previous in vitro results showing an imbalance in IL1Rs in human endometrial epithelial and stromal cells triggered by hCG (27, 40), a major embryonic signal, and demonstrate the existence of such a regulatory process in the host maternal endometrial tissue in vivo in contact with the embryo. Using in situ hybridization and immunostaining, a previous study by Zhang et al (41) showed the expression of Il1r2 in decidual cells close to the implanting embryo during days 5 to 8 of pregnancy. However, this study did not detect Il1r2 following mating, neither it quantitatively evaluated the concomitant expression of Il1r1 and its accessory protein and Il1rn.

It is interesting to note that the expression of Mcp1, a potent mediator of angiogenesis and macrophage recruitment and activation (42, 43), was significantly increased within the embryo implantation sites compared to the non-implanted areas of the mouse endometrium, suggesting that Mcp1 expression is driven by the implanting embryo. Also, a significant positive correlation with Il1r1 was observed only in the implantation sites, which may represent a consequence of Il1 embryo-maternal crosstalk. This strengthens the relevance of our findings and further supports a role for Mcp1 during embryo implantation. Actually, these data are consistent with our previous in vitro findings showing a marked increase of MCP1, as well as MCP 2 and 3, in human endometrial stromal cells in response to the synergistic actions of embryonic signals, hCG and IL1, and a role for MCP1 in hCG/IL1-mediated neovascularization and acquisition of an angiogenic phenotype that is capable of sustaining the highly demanding embryonic growth into the maternal endometrium (27, 40). MCP1 is a well-characterized chemokine targeted by IL1 cellular signaling in endometrial stromal cells (44). Chemokines trigger

chemotaxis, promote the response, development, and homeostasis of the immune system, support stem-cell survival, and can stimulate angiogenesis. MCP1 is known for being involved in recruitment of immune cells, particularly macrophages, which remain in substantial numbers for the duration of pregnancy (35). A protective effect of MCP1 may be also related to increased production and maintenance of levels of specialized uterine natural killer cells recruited during pregnancy (45). Interestingly, blastocyst-uterine attachment reaction in mice leads to stromal decidual reaction only at sites of implantation. A direct role for Mcp1 in decidualization is unknown, but this cytokine seems to be produced by decidual stromal cells and to inhibit NK cells cytotoxicity (19).

IL1 is an early embryonic factor. In mice, Il1b mRNA expression was first detected in 4-cell embryos and in embryos at all later stages during embryo development (22). Previous studies showed that Il1r1 is strongly expressed in mouse endometrial luminal epithelium during the peri-implantation period, and Il1r1 blockade interferes with the attachment of mouse blastocysts to maternal endometrium in vivo and induces implantation failure (6, 46). These results, together with the wide variety of IL1 and family members' effects on cell survival and the induction of adhesion, tissue remodeling and growth-promoting factors (40, 47-51), support a role for the IL1 system in the embryo-maternal crosstalk and sustaining early pregnancy.

The mouse has been a valuable animal model for a comprehensive understanding of the molecular and cellular regulation of embryonic development (29, 30, 46, 52) (53). However, alike to humans, mouse uterine preparation for implantation requires the presence of progesterone and estradiol. In the preovulatory phase (proestrus), the endometrial epithelium proliferates under the influence of estrogen. Then, during the estrogen-progestin phase (metaestrus, diestrus), stromal cells

proliferate. Gestation is also characterized by the presence of a phase during which the uterus is receptive to an embryo, called implantation window (31, 54), which, in a fertile estrous cycle, starts at a peak of estradiol on the fourth day after mating (29). By the afternoon of day 5 the uterus becomes refractory to implantation (29). In rodents and other mammals that develop hemochorial or endotheliochorial placentae, embryonic attachment is followed by the invasion of the endometrial connective tissue, though less aggressively than in humans, and transformation of the subepithelial region into decidual tissue in the implantation sites (31). In mice, equivalent gonadotropin hCG is not found yet, but a gonadotropin chorionic-like effect were observed in conditioned media of in vitro cultured mice placenta (55). Indeed, this gonadotropin chorionic-like effect may be mediated by luteinized hormone (Lh) expressed by mouse embryo during implantation (56), since hCG seems to origin from the LH gene (57), and both hCG and LH bind the same LHCG receptor (LHCGR). Actually, a recent study demonstrated that Lhcgr expression in BALB/c mouse endometrial epithelium is increased at the time of implantation and identified a bioactive Lh signal, which suggests that Lhcgr may contribute to the implantation process and provides evidence for cross-talk between the Lhr and Lh during implantation in mice (56).

In conclusion, our data revealed a synchronic regulation of Il1rs with major events occurring throughout early pregnancy and embryonic implantation in mice, a concomitant synthesis of a potent macrophage recruitment and angiogenic factor within the implantation sites, which corroborate our previous in vitro data and support the existence in vivo of an embryo-driven modulation of the maternal endometrial receptivity to the IL1 system. This may represent one of the main embryo-maternal communication mechanisms that underlie successful embryonic implantation and early pregnancy.

5.7 Acknowledgments

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5.8 References

1. King GJ, Atkinson BA, Robertson HA. Implantation and early placentation in domestic ungulates. J Reprod Fertil Suppl 1982;31:17-30.

2. Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood 2011;117:3720-32.

3. Bourdiec A, Shao R, Rao CV, Akoum A. Human Chorionic Gonadotropin Triggers Angiogenesis via the Modulation of Endometrial Stromal Cell Responsiveness to Interleukin 1: A New Possible Mechanism Underlying Embryo Implantation. Biology of reproduction 2012.

4. Paulesu L, Jantra S, Ietta F, Brizzi R, Bigliardi E. Interleukin-1 in reproductive strategies. Evolution & development 2008;10:778-88.

5. Simon C, Frances A, Piquette G, Hendrickson M, Milki A, Polan ML. Interleukin-1 system in the materno-trophoblast unit in human implantation: immunohistochemical evidence for autocrine/paracrine function. The Journal of clinical endocrinology and metabolism 1994;78:847-54.

6. Simon C, Frances A, Piquette GN, el Danasouri I, Zurawski G, Dang W et al. Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist. Endocrinology 1994;134:521-8.

7. Krussel JS, Bielfeld P, Polan ML, Simon C. Regulation of embryonic implantation. European journal of obstetrics, gynecology, and reproductive biology 2003;110 Suppl 1:S2-9.

8. Baranao RI, Piazza A, Rumi LS, Polak de Fried E. Determination of IL-1 and IL-6 levels in human embryo culture-conditioned media. American journal of reproductive immunology 1997;37:191-4.

9. Sheth KV, Roca GL, al-Sedairy ST, Parhar RS, Hamilton CJ, al-Abdul Jabbar F. Prediction of successful embryo implantation by measuring interleukin-1-alpha and immunosuppressive factor(s) in preimplantation embryo culture fluid. Fertil Steril 1991;55:952-7.

10. Simon C, Valbuena D, Krussel J, Bernal A, Murphy CR, Shaw T et al. Interleukin-1 receptor antagonist prevents embryonic implantation by a direct effect on the endometrial epithelium. Fertility and sterility 1998;70:896-906.

11. Sawai K, Matsuzaki N, Okada T, Shimoya K, Koyama M, Azuma C et al. Human decidual cell biosynthesis of leukemia inhibitory factor: regulation by decidual cytokines and steroid hormones. Biology of reproduction 1997;56:1274-80.

12. Gonzalez RR, Rueda BR, Ramos MP, Littell RD, Glasser S, Leavis PC. Leptin-induced increase in leukemia inhibitory factor and its receptor by human endometrium is partially mediated by interleukin 1 receptor signaling. Endocrinology 2004;145:3850-7.

13. Huang JC, Liu DY, Yadollahi S, Wu KK, Dawood MY. Interleukin-1 beta induces cyclooxygenase-2 gene expression in cultured endometrial stromal cells. J Clin Endocrinol Metab 1998;83:538-41.

14. Tamura M, Sebastian S, Yang S, Gurates B, Fang Z, Bulun SE. Interleukin-1beta elevates cyclooxygenase-2 protein level and enzyme activity via increasing its mRNA stability in human endometrial stromal cells: an effect mediated by extracellularly regulated kinases 1 and 2. J Clin Endocrinol Metab 2002;87:3263-73.

15. Dimitriadis E, White CA, Jones RL, Salamonsen LA. Cytokines, chemokines and growth factors in endometrium related to implantation. Hum Reprod Update 2005;11:613-30.

16. Librach CL, Feigenbaum SL, Bass KE, Cui TY, Verastas N, Sadovsky Y et al. Interleukin-1 beta regulates human cytotrophoblast metalloproteinase activity and invasion in vitro. J Biol Chem 1994;269:17125-31.

17. Kauma SW, Turner TT, Harty JR. Interleukin-1 beta stimulates interleukin-6 production in placental villous core mesenchymal cells. Endocrinology 1994;134:457-60.

18. Yagel S, Lala PK, Powell WA, Casper RF. Interleukin-1 stimulates human chorionic gonadotropin secretion by first trimester human trophoblast. J Clin Endocrinol Metab 1989;68:992-5.

19. Cole LA. Hyperglycosylated hCG, a review. Placenta 2010;31:653-64.

20. von Wolff M, Thaler CJ, Strowitzki T, Broome J, Stolz W, Tabibzadeh S. Regulated expression of cytokines in human endometrium throughout the menstrual cycle: dysregulation in habitual abortion. Mol Hum Reprod 2000;6:627-34.

21. Baranao RI, Piazza A, Rumi LS, Polak de Fried E. Determination of IL-1 and IL-6 levels in human embryo culture-conditioned media. Am J Reprod Immunol 1997;37:191-4.

22. Takacs P, Kauma S. The expression of interleukin-1 alpha, interleukin-1 beta, and interleukin-1 receptor type I mRNA during preimplantation mouse development. J Reprod Immunol 1996;32:27-35.

23. Bigonnesse F, Labelle Y, Akoum A. Triphasic expression of interleukin-1 receptor type I in human endometrium throughout the menstrual cycle of fertile women and women with unexplained infertility. Fertility and sterility 2001;75:79-87.

24. Boucher A, Kharfi A, Al-Akoum M, Bossu P, Akoum A. Cycle-dependent expression of interleukin-1 receptor type II in the human endometrium. Biology of reproduction 2001;65:890-8.

25. Herrmann-Lavoie C, Rao CV, Akoum A. Chorionic gonadotropin down-regulates the expression of the decoy inhibitory interleukin 1 receptor type II in human endometrial epithelial cells. Endocrinology 2007;148:5377-84.

26. Bellehumeur C, Blanchet J, Fontaine JY, Bourcier N, Akoum A. Interleukin 1 regulates its own receptors in human endometrial cells via distinct mechanisms. Hum Reprod 2009;24:2193-204.

27. Bourdiec A, Shao R, Rao CV, Akoum A. Human chorionic gonadotropin triggers angiogenesis via the modulation of endometrial stromal cell responsiveness to interleukin 1: a new possible mechanism underlying embryo implantation. Biology of reproduction 2012;87:66.

28. Bourdiec A, Bedard D, Rao CV, Akoum A. Human chorionic gonadotropin regulates endothelial cell responsiveness to interleukin 1 and amplifies the cytokine-mediated effect on cell proliferation, migration and the release of angiogenic factors. Am J Reprod Immunol 2013;70:127-38.

29. Wang H, Dey SK. Roadmap to embryo implantation: clues from mouse models. Nat Rev Genet 2006;7:185-99.

30. Abrahamsohn PA, Zorn TM. Implantation and decidualization in rodents. J Exp Zool 1993;266:603-28.

31. Paria BC, Huet-Hudson YM, Dey SK. Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus. Proc Natl Acad Sci U S A 1993;90:10159-62.

32. Deb K, Reese J, Paria BC. Methodologies to study implantation in mice. Methods Mol Med 2006;121:9-34.

33. Carli C, Metz CN, Al-Abed Y, Naccache PH, Akoum A. Up-regulation of cyclooxygenase-2 expression and prostaglandin E2 production in human endometriotic cells by macrophage migration inhibitory factor: involvement of novel kinase signaling pathways. Endocrinology 2009;150:3128-37.

34. Akoum A, Lemay A, McColl S, Turcot-Lemay L, Maheux R. Elevated concentration and biologic activity of monocyte chemotactic protein-1 in the peritoneal fluid of patients with endometriosis. Fertility and sterility 1996;66:17-23.

35. Robertson SA. Seminal fluid signaling in the female reproductive tract: lessons from rodents and pigs. Journal of animal science 2007;85:E36-44.

36. Guerin LR, Moldenhauer LM, Prins JR, Bromfield JJ, Hayball JD, Robertson SA. Seminal fluid regulates accumulation of FOXP3+ regulatory T cells in the preimplantation mouse uterus through expanding the FOXP3+ cell pool and CCL19-mediated recruitment. Biology of reproduction 2011;85:397-408.

37. Reese J, Das SK, Paria BC, Lim H, Song H, Matsumoto H et al. Global gene expression analysis to identify molecular markers of uterine receptivity and embryo implantation. The Journal of biological chemistry 2001;276:44137-45.

38. Bigonnesse F, Marois M, Maheux R, Akoum A. Interleukin-1 receptor accessory protein is constitutively expressed in human endometrium throughout the menstrual cycle. Mol Hum Reprod 2001;7:333-9.

39. Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K, Robertson SA. Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. J Immunol 2012;188:2445-54.

40. Bourdiec A, Calvo E, Rao CV, Akoum A. Transcriptome analysis reveals new insights into the modulation of endometrial stromal cell receptive phenotype by embryo-derived signals interleukin-1 and human chorionic gonadotropin: possible involvement in early embryo implantation. PLoS One 2013;8:e64829.

41. Zhang XH, Tian Z, Lei W, Zhao ZA, Wang TS, Yang ZM. Differential expression of interleukin 1 receptor type II during mouse decidualization. Reprod Sci 2012;19:923-31.

42. Niu J, Azfer A, Zhelyabovska O, Fatma S, Kolattukudy PE. Monocyte chemotactic protein (MCP)-1 promotes angiogenesis via a novel transcription factor, MCP-1-induced protein (MCPIP). The Journal of biological chemistry 2008;283:14542-51.

43. Hong KH, Ryu J, Han KH. Monocyte chemoattractant protein-1-induced angiogenesis is mediated by vascular endothelial growth factor-A. Blood 2005;105:1405-7.

44. Rossi M, Sharkey AM, Vigano P, Fiore G, Furlong R, Florio P et al. Identification of genes regulated by interleukin-1beta in human endometrial stromal cells. Reproduction 2005;130:721-9.

45. Whitcomb BW, Schisterman EF, Klebanoff MA, Baumgarten M, Rhoton-Vlasak A, Luo X et al. Circulating chemokine levels and miscarriage. American journal of epidemiology 2007;166:323-31.

46. Maltepe E, Bakardjiev AI, Fisher SJ. The placenta: transcriptional, epigenetic, and physiological integration during development. J Clin Invest 2010;120:1016-25.

47. Sawai K, Matsuzaki N, Okada T, Shimoya K, Koyama M, Azuma C et al. Human decidual cell biosynthesis of leukemia inhibitory factor: regulation by decidual cytokines and steroid hormones. Biology of reproduction 1997;56:1274-80.

48. Tabibzadeh S, Kaffka KL, Satyaswaroop PG, Kilian PL. Interleukin-1 (IL-1) regulation of human endometrial function: presence of IL-1 receptor correlates with IL-1-stimulated prostaglandin E2 production. The Journal of clinical endocrinology and metabolism 1990;70:1000-6.

49. Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T et al. Molecular cues to implantation. Endocrine reviews 2004;25:341-73.

50. Simon C, Gimeno MJ, Mercader A, O'Connor JE, Remohi J, Polan ML et al. Embryonic regulation of integrins beta 3, alpha 4, and alpha 1 in human endometrial epithelial cells in vitro. The Journal of clinical endocrinology and metabolism 1997;82:2607-16.

51. Hirota Y, Osuga Y, Hasegawa A, Kodama A, Tajima T, Hamasaki K et al. Interleukin (IL)-1beta stimulates migration and survival of first-trimester villous cytotrophoblast cells through endometrial epithelial cell-derived IL-8. Endocrinology 2009;150:350-6.

52. El-Hashash AH, Warburton D, Kimber SJ. Genes and signals regulating murine trophoblast cell development. Mech Dev 2010;127:1-20.

53. Adamson SL, Lu Y, Whiteley KJ, Holmyard D, Hemberger M, Pfarrer C et al. Interactions between trophoblast cells and the maternal and fetal circulation in the mouse placenta. Dev Biol 2002;250:358-73.

54. Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA et al. Embryo implantation. Dev Biol 2000;223:217-37.

55. Rao B, Pointis G, Cedard L. Presence of a chorionic gonadotrophin-like factor in mouse placental cultures during the second half of gestation. J Reprod Fertil 1982;66:341-8.

56. Gridelet V, Tsampalas M, Berndt S, Hagelstein MT, Charlet-Renard C, Conrath V et al. Evidence for cross-talk between the LH receptor and LH during implantation in mice. Reprod Fertil Dev 2013;25:511-22.

57. Maston GA, Ruvolo M. Chorionic gonadotropin has a recent origin within primates and an evolutionary history of selection. Mol Biol Evol 2002;19:320-35.

5.9 Figure legends

Figure 1: A) Diagram schematizing the different embryo implantation phases in the murine endometrium. B) Photomicrograph of mouse uteri at sacrifice following iv injection of Chicago Blue 0.1% in non-pregnant mice and C) in pregnant mice where the arrows indicate the embryo implantation sites. GD, gestational day.

Figure 2: Quantitative real time PCR analysis of Il1rs and Il1rn in the mouse endometrium throughout the first eight days of gestation. Total RNA was extracted and reverse transcribed. Il1r1, Il1rap, Il1r2, Il1rn and Gapdh (internal control) mRNA levels were quantified by qRT-PCR. Il1r1 (A), Il1rap (B), Il1r2 (C) and Il1rn (D) mRNA ratio was then determined following normalization to Gapdh mRNA. The relative changes [fold change as compared to gestation day (GD0)] in Il1rs and antagonist at different GDs are shown (E). Data are duplicate from 5 different mice for each gestational day (GD). *$p < 0.05$, **$p < 0.01$ and *** $p < 0.001$ indicate significant differences with gestation day 0; ++$p < 0.01$ and +++ $p < 0.001$ indicate significant differences between implanted (i; black color) and non-implanted (ni; gray color) endometrial areas at the same GD.

Figure 3: Immunohistochemical analysis of Il1r1, Il1rap, Il1r2 and Il1rn in the mouse endometrium at gestational days (GD) 1, 2, 4, 5 in non-implanted areas (5ni), 5 in implanted areas (5i), 7i and 8i. Note luminal (L) and glandular (G) endometrial epithelium and stroma (S) in GDs 1-4 and the disruption of endometrial structure within the implantation site after GD 5. Note the implantation dependent intensity of Il1r1 staining in the mouse endometrium and the absence of variation in the intensity of Il1rap immunostaining between implanted and non-implanted endometrium site. Note also that Il1r2 and Il1rn immunostaining is intense at GD 1, then decreases at GD 4-6, before noticeably increasing again for Il1r2 at GD7 and slightly increasing for Il1rn (magnification X 400). Immunostaining is representative of that observed in a minimum of 4 tissue sections from 3 different mice per GD.

Figure 4: Mcp1 expression in the mouse endometrium during embryo implantation. Mcp1 mRNA ratio (A) was determined by qRT-PCR followed by normalization to Gapdh mRNA. Mcp1 ELISA (B) was carried out on 10 □g of total proteins extracts of endometrial tissues used for simultaneous extraction of proteins and mRNA with Trizol®. Correlation between Mcp1 and Il1r1 mRNA levels at the embryo implantation sites (i) from days 5 to 8 (C) and the non-implanted (ni) endometrial areas at days 4 to 6 (D). Note the positive correlation depending on embryo implantation ($p < 0.001$). Data are duplicate from 5 different mice for each gestational day (GD). *$p < 0.05$ and *** $p < 0.001$ indicate significant differences with GD4; +$p < 0.05$, ++$p < 0.01$ and +++ $p < 0.001$ indicate significant differences between i and ni endometrial areas at the same GD. Immunohistochemical analysis of Mcp1 in the ni and i areas of the mouse endometrium at GDs 5, 6, 7 and 8 (E and F). Note the intensity of Mcp1 immunostaining in the i compared to the ni sites of the maternal mouse endometrial tissue (E) (magnification X 400), the disruption of endometrial structures in the i sites and positive Mcp1 immunostaining. Immunostaining is representative of that observed in a minimum of 4 tissue sections from 3 different mice per GD.

Figure 1

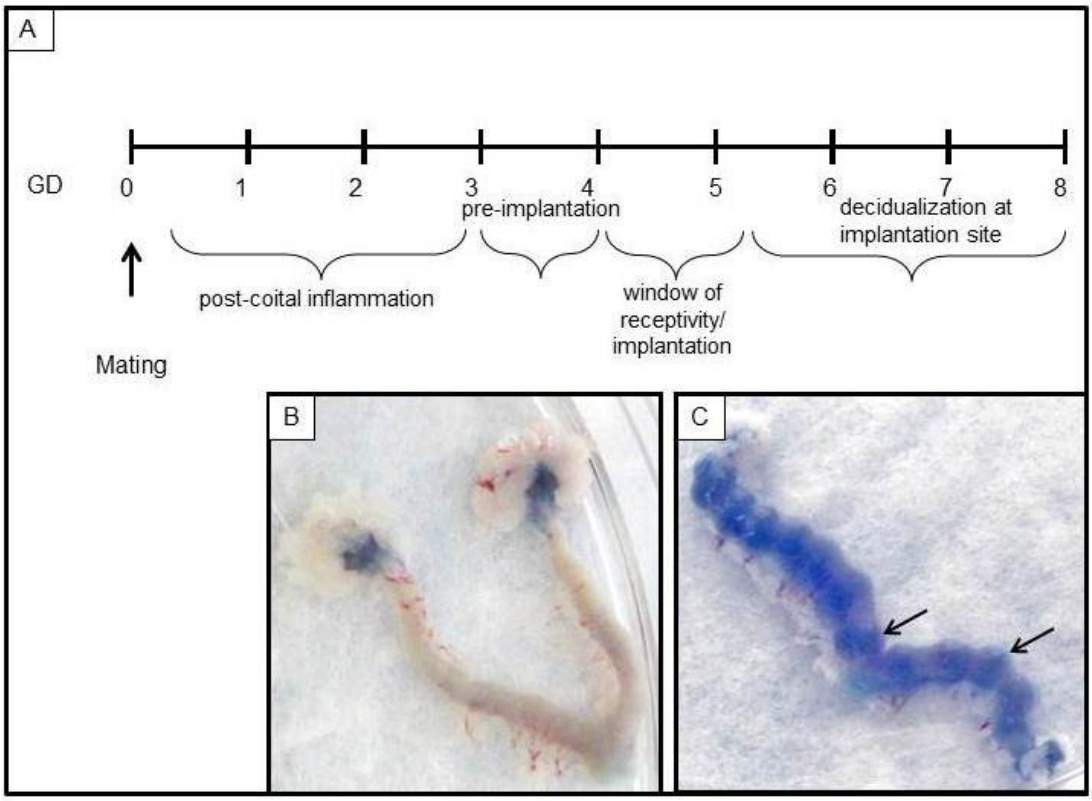

A
GD
0
1
2
3
4
5
6
7
8
pre-implantation
decidualization at implantation site
post-coital inflammation
window of receptivity/ implantation
Mating
B
C

Figure 2

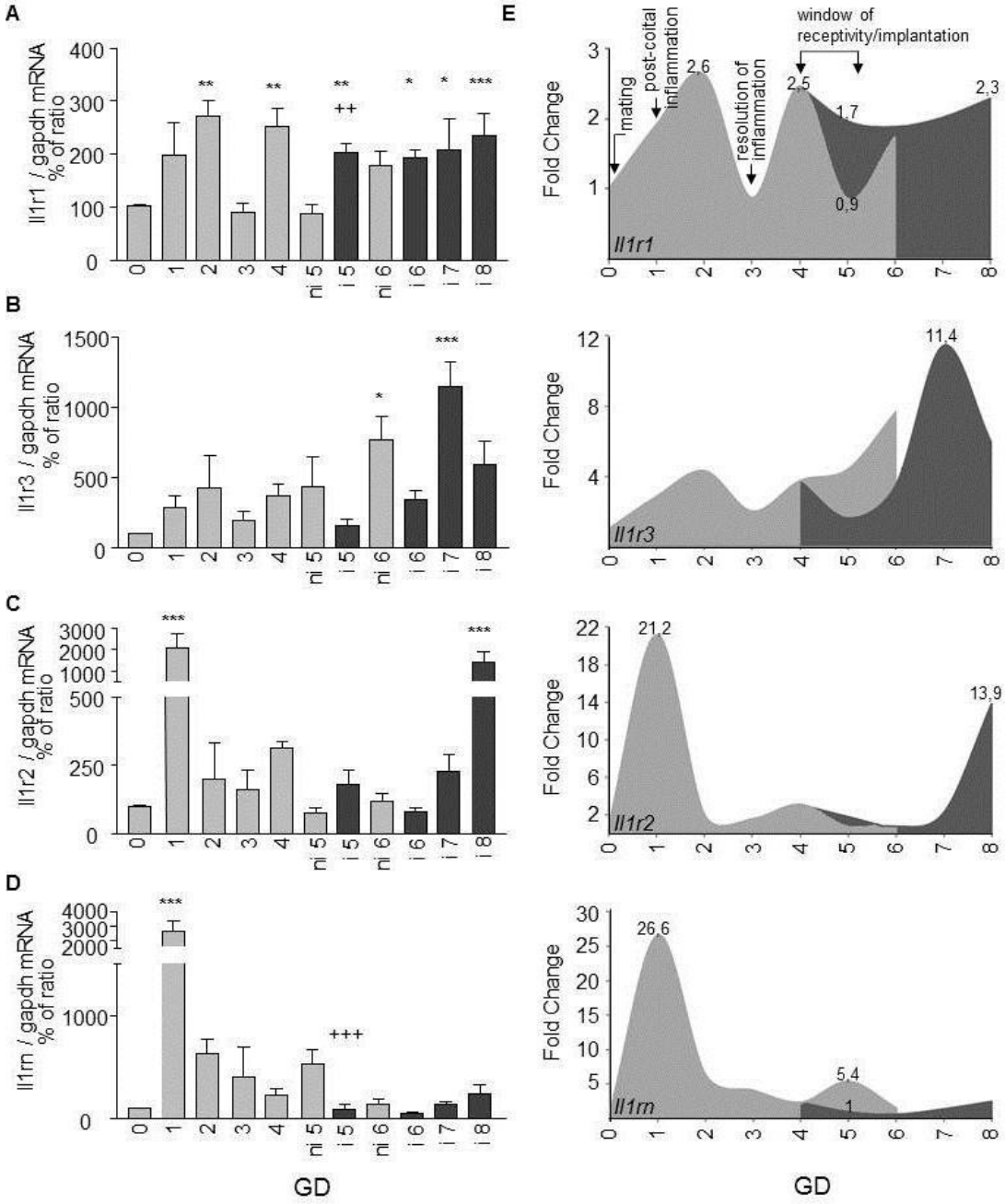

A
Il1r1 / gapdh mRNA % of ratio
B
Il1r3 / gapdh mRNA % of ratio
C
Il1r2 / gapdh mRNA % of ratio
D
Il1rn / gapdh mRNA % of ratio
0 1 2 3 4 ni 5 i 5 ni 6 i 6 i 7 i 8
GD
E
mating
post-coital inflammation
resolution of inflammation
window of receptivity/implantation
Fold Change
Il1r1
Il1r3
Il1r2
Il1rn
2,6
2,5
1,7
2,3
0,9
11,4
21,2
13,9
26,6
5,4
1
GD

Figure 3

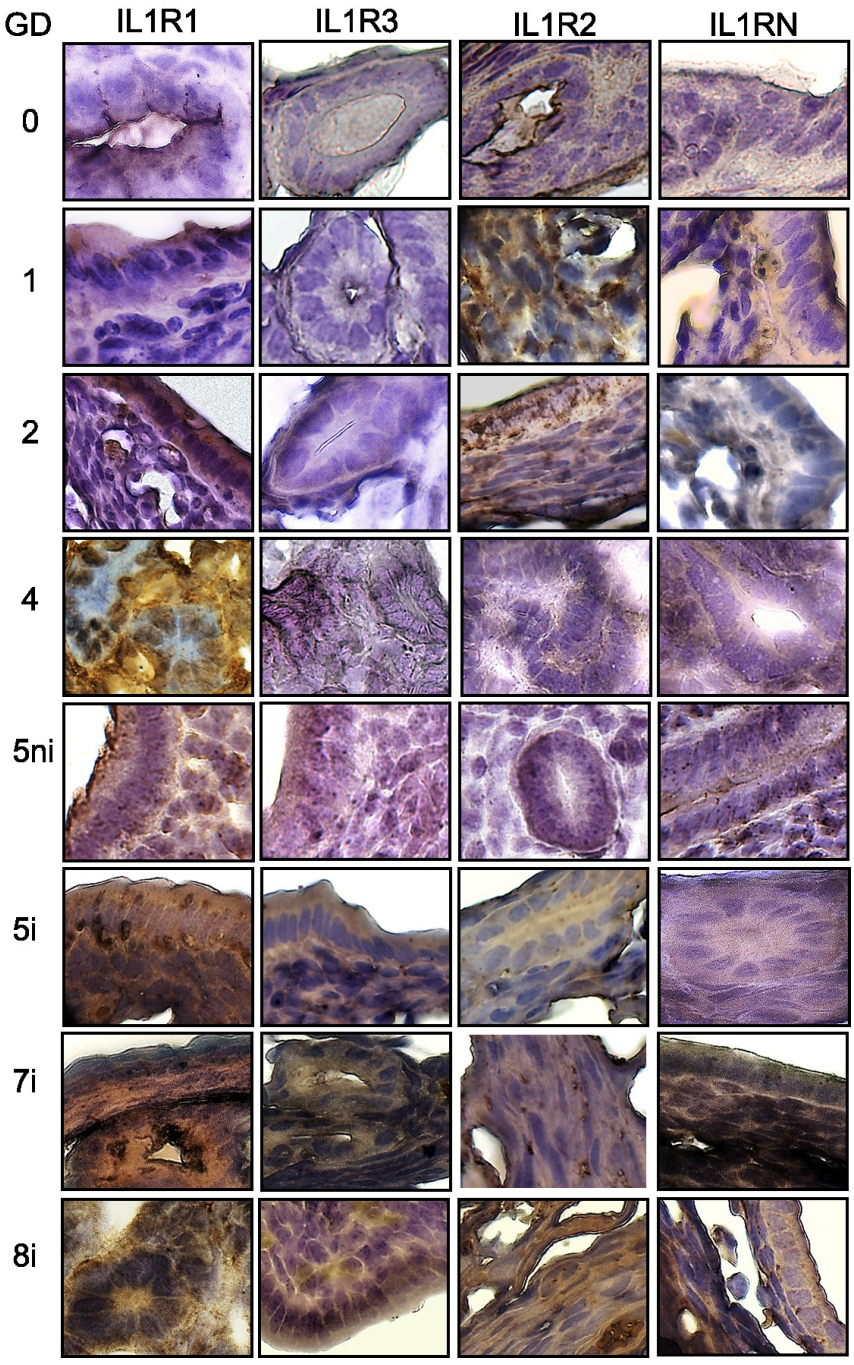
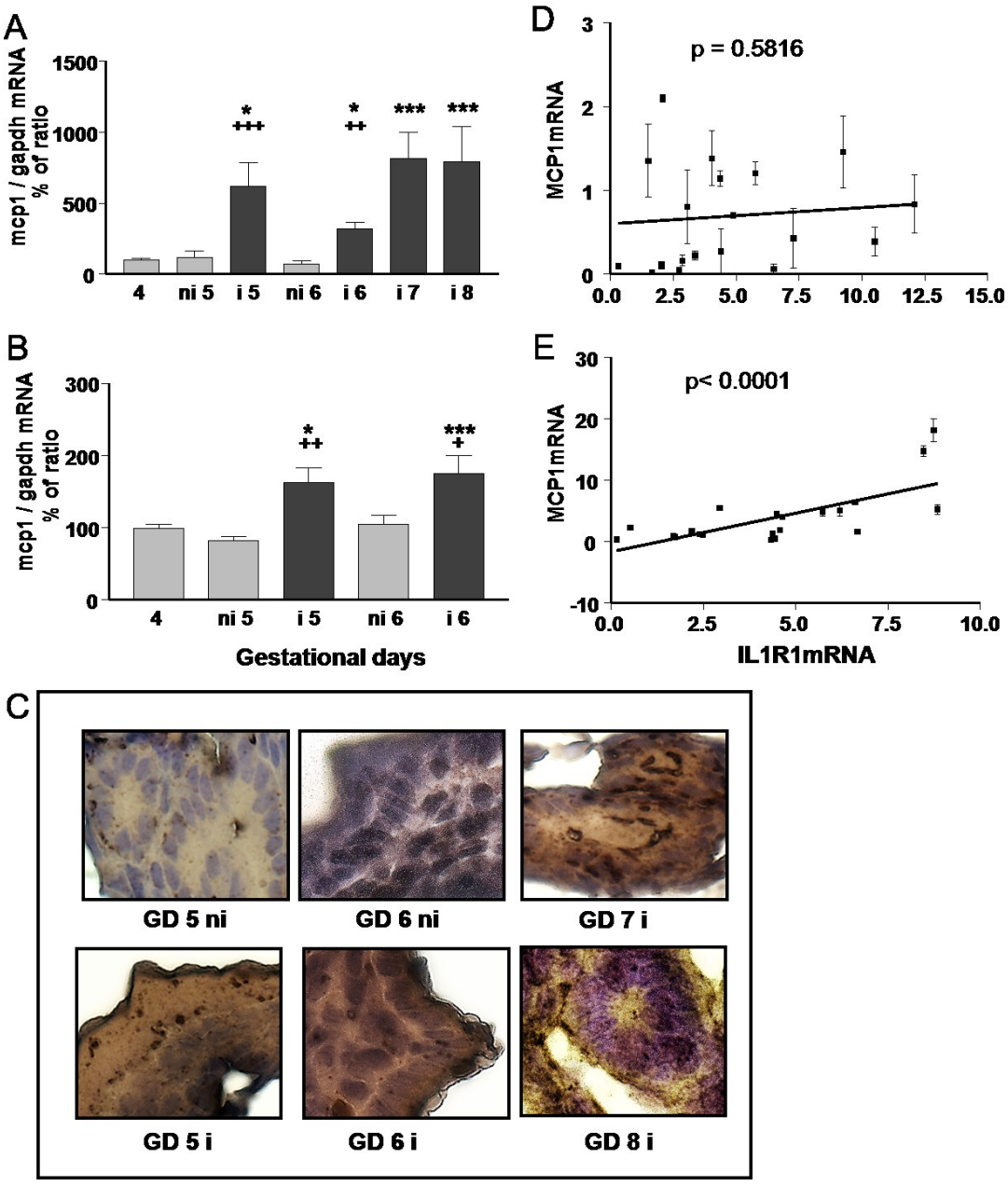


Figure 4


A
mcp1 / gapdh mRNA % of ratio
1500
1000
500
0
4
ni 5
i 5
ni 6
i 6
i 7
i 8
B
mcp1 / gapdh mRNA % of ratio
300
200
100
0
Gestational days
C
GD 5 ni
GD 6 ni
GD 7 i
GD 5 i
GD 6 i
GD 8 i
D
p = 0.5816
MCP1mRNA
IL1R1mRNA
E
p< 0.0001
MCP1mRNA
IL1R1mRNA

Discussion générale

Point de vue du laboratoire

Un dialogue approprié entre les cellules embryonnaires et maternelles doit avoir lieu pour parvenir à une grossesse réussie. Les facteurs maternels dérivés, tels que les hormones stéroïdes, les enzymes dégradant la matrice, les intégrines, les cytokines, les chimiokines et de nombreux facteurs de croissance embryonnaires pourraient être impliqués dans ce phénomène.

Nous croyons que les facteurs embryonnaires précoces, tels que la hCG et l'IL1 exprimés par les cellules de l'embryon, mettent en place un réseau de communication qui atteint les différents types cellulaires de l'endomètre afin de promouvoir l'implantation.

Etat de l'art

La hCG est connue pour orchestrer les changements *in utero* nécessaires au premier trimestre de la gestation. Cette hormone peut avoir plusieurs rôles indirects comme le suggèrent nos travaux. Le soutien de la production de progestérone, *via* le corps jaune, par la hCG prépare l'adaptation de l'endomètre à l'implantation de l'embryon. Cependant, les voies impliquées et les mécanismes sous-jacents (Sherwin, Sharkey et al. 2007) sont encore mal connus. Aussi, la hCG est capable de stimuler la production de cytokines / chimiokines par les cellules épithéliales et stromales de l'endomètre qui jouent un rôle dans la décidualisation et l'angiogenèse (Tang and Gurpide 1993; Jurisicova, Antenos et al. 1999; Carli, Metz et al. 2009). De plus, la présence du récepteur LHCG dans plusieurs types de cellules endométriales suggèrerait que la hCG possède plusieurs cibles (Reshef, Lei et al. 1990).

Des études pointent l'interleukine 1, l'un des premiers signaux embryonnaires, comme un pilier de l'implantation (von Wolff, Thaler et al. 2000; Strakova, Mavrogianis et al. 2005) (Sheth, Roca et al. 1991; Simon, Frances et al. 1994; Baranao, Piazza et al. 1997; Krussel, Bielfeld et al. 2003; Paulesu, Jantra et al. 2008). Les cultures d'embryons humains produisent des quantitées élevées d'IL1α et d'IL1β. De plus, la présence de ces cytokines dans le milieu de culture est corrélée avec une implantation réussie après le transfert d'embryons dans la cavité utérine. De plus, les actions immuno-modulatrices de l'IL1 favorisent la croissance, le remodelage des tissus et l'angiogenèse. La présence de cette cytokine à l'interface embryo-maternelle représente ainsi un pivot qui soutient l'implantation et le développement embryonnaire. En revanche, le fait que les souris déficientes en IL-1RI, IL-1RAcP, l'IL-1α, IL-1β ou doublement déficientes en IL-1α/β soient fertiles est mal compris.

Contributions scientifiques des projets

Nos travaux mettent en évidence que les récepteurs de l'IL1 sont ciblés par la hCG aux doses correspondant au niveau physiologique relevé lors de la phase d'implantation. D'autre part, c'est l'ensemble de la famille IL1 qui apparaît être modifié par les effets de la hCG. Enfin, *via* la modification de la réceptivité des cellules de l'endomètre à l'IL1, des cibles connues, ou pertinentes, avec l'implantation embryonnaire apparaissent affectées par une relation sélective de la hCG au travers de l'IL1. Ces résultats renforcent l'importance des rôles de cette famille de cytokines du côté maternel de l'implantation.

- **La fenêtre d'implantation**

Nous avons voulu d'abord évaluer l'effet de faibles concentrations de hCG sur la réceptivité des cellules stromales de l'endomètre (ESC) à l'IL1 pendant la fenêtre d'implantation. Nos résultats montrent une augmentation significative de la réceptivité des ESC à l'IL1 associée à une amplification synergique de l'expression de MCP1. Cependant, l'exposition des cellules ESC à l'hCG seule n'a eu aucun effet significatif sur l'expression de l'IL1R1, de l'IL1RN ou de l'IL1R2. Néanmoins, l'hormone de grossesse apparait contre-balancer l'augmentation de l'IL1R2 et de l'IL1RN induite par l'IL1β. L'expression du récepteur fonctionnel de l'IL1 et de ses récepteurs inhibiteurs naturels est modifiée de manières opposées par la hCG. Fonctionnellement, la hCG permet de modifier le ratio des récepteurs en faveur de l'IL1 et explique la synergie observée en sensibilisant les ESC à l'IL1.

De plus, nous avons retrouvé qu'une cible de l'IL1, le MCP1, était induite de façon synergique. Cela suggère fortement que la modulation de la réceptivité des cellules endométriales à IL1, en réponse à la hCG, peut se traduire par une augmentation de la sensibilité des cellules à IL1β et potentialiser la sécrétion de facteurs angiogéniques. La neutralisation du MCP1, contenu dans les milieux conditionnés, par un anticorps spécifique, diminue significativement la prolifération et la migration des cellules endothéliales. Cette observation peut être particulièrement intéressante. Le MCP1 étant classé dans la catégorie des gènes induits dans les ESC lors d'exposition aux stimuli embryonnaires (Rossi, Sharkey et al. 2005; Hess, Hamilton et al. 2007). Cette chimiokine, connue pour ses propriétés chimiotactiques des macrophages et pro-angiogéniques, est pertinente pour la physiologie de l'implantation embryonnaire. MCP1 participe au recrutement de macrophages et de NK lors de l'implantation embryonnaire.

Notre analyse globale par micro array *Affymetrix®* met en évidence d'autres cibles pertinentes à l'angiogenèse locale, telles que le VEGFC, et de nombreuses autres

cytokines induites en synergie, comme RANTES et l'IL6. Ces facteurs pluripotents sont déjà connus pour être impliqués dans la régulation de la réponse immunitaire, la prolifération cellulaire, le remodelage tissulaire et l'angiogenèse. L'IL6 est exprimée par les cellules de l'endomètre et de l'embryon préimplantatoire (Tabibzadeh, Kong et al. 1995) et agit comme une cytokine pléiotropique qui régule la croissance cellulaire, l'angiogenèse, l'inflammation et l'hématopoïèse (Altun, Jindal et al. 2011). En outre, une diminution d'expression d'IL6 par les cellules embryonnaires est associée à un risque d'avortement spontané, expliquant le rôle primordial de l'IL6 dans le maintien de la grossesse (von Wolff, Thaler et al. 2000). Le VEGFC est un facteur de croissance angiogénique puissant et pourrait jouer un rôle important dans la croissance des cellules embryonnaires. De plus, il pourrait induire une tolérance immunitaire (Lund, Duraes et al. 2012) comme le CCL5. En effet, cette cytokine semble supprimer les réponses allogéniques maternelles et favoriser ainsi une implantation réussie (Hornung, Bentzien et al. 2001; Huang, Schatz et al. 2006; Ramhorst, Patel et al. 2006; Ramhorst, Gutierrez et al. 2007). Enfin, l'infusion de la hCG et de l'IL1 *in utero* montre des modifications endométriales imitant les événements précoces de la grossesse chez le babouin qui permettent la création d'un environnement immunotolérant (Butler, Apte et al. 1999).

Notre analyse des résultats de *micro-array* et leurs validations par qRT-PCR ont également montré que plusieurs autres médiateurs du remodelage tissulaire, tels que MMP9, TIMP3 et KRT19 pouvaient être régulés par le duo hCG/IL1. La modulation d'expression de ces cibles, validée par quantification protéique, corrobore le rôle combiné de l'hCG-IL1β dans la modulation de l'angiogenèse, de l'immunité et du remodelage tissulaire des ESC. Ainsi, nos résultats démontrent que la hCG exerce un rôle indirect dans la préparation de l'endomètre, puisqu'elle semble diriger l'implantation embryonnaire par le contrôle du système de l'IL1.

Ce fut intéressant de constater que les effets de l'hCG ciblent d'autres membres du système de l'IL1, tel que les récepteurs de l'IL33 et de l'IL18. Ceci suggère une modulation de la sensibilité des cellules de l'endomètre à l'ensemble de la famille de l'IL1. Nos résultats indiquent que la hCG semble potentialiser l'expression de l'IL1RL1 et de l'IL18R1 induites par l'IL1β. De plus, l'effet modérateur de la hCG touche tous les ligands, notamment les ligands IL33 et IL18. En revanche, l'expression des deux isoformes de l'IL1 reste forte même avec l'effet de la hCG. Ces résultats suggèrent que la hCG induit l'immunotropisme et empêche l'expression excessive de cytokines pro-inflammatoires. Ainsi, nos résultats illustrent le rôle de la hCG dans le contrôle sélectif de la famille de l'IL1. Cependant il serait intéressant de poursuivre vles études avec un modèle de coculture avec des cellules de l'endomètre et des cellules embryonnaire.

Des résultats similaires ont été observés dans une lignée cellulaire endothéliale (HMVEC). En présence d'hCG et d'IL1, les cellules endothéliales présentent une augmentation de l'expression du récepteur fonctionnel de l'IL1, concomitante à une diminution des récepteurs inhibiteurs, créant un déséquilibre fonctionnel de l'IL1. Ces données indiquent que l'hCG module également la réceptivité des cellules endothéliales à IL1 *via* des mécanismes de régulations inconnus, qui peuvent amplifier la sensibilité des cellules endothéliales à IL1 et favoriser l'angiogenèse.

Ce déséquilibre s'est traduit par une induction synergique de l'expression de l'IL8. De manière intéressante, l'expression de l'IL8, renforcée dans l'endomètre en début de grossesse (Milne, Critchley et al. 1999), jouerait un rôle important dans le maintien de la gestation. L'IL8 est impliquée dans le remodelage tissulaire, l'invasion cellulaire et l'angiogenèse (Tsui, Chen et al. 2004), et est ainsi un facteur important dans l'acquisition et le maintien de la réceptivité utérine. D'autre part, le récepteur de cette cytokine (IL8R) a été retrouvé dans les cellules trophoblastiques de

l'embryon en développement (Dame and Juul 2000). Enfin, les cellules de la décidue du premier trimestre de grossesse produisent des quantités considérables d'IL8 (Fluhr, Sauter et al. 2009). En parallèle, nous avons constaté une augmentation significative de la capacité de migration et de prolifération des HMVEC. La synergie IL1β/hCG observée au niveau des cellules endothéliales révèle un nouveau mécanisme qui peut expliquer la capacité de l'embryon à moduler la réceptivité des cellules endométriales maternelles et favoriser sa propre implantation. Ces résultats devront être corroborés avec l'utilisation de culture primaine endothéliale utérine.

Ces premiers résultats suggèrent qu'à l'interface maternelle, les facteurs embryonnaires provoquent des changements importants dans les différents types cellulaires. En effet, ces facteurs modulent profondément la réactivité inflammatoire des cellules endométriales, ainsi que la capacité de ces dernières à agir sur les composants de la matrice extracellulaire. En outre, ces résultats illustrent pour la première fois l'existence d'une interaction étroite entre l'IL1β et la hCG dans la signalisation cellulaire. Ce système pourrait représenter un mécanisme de communication qui cible le remodelage tissulaire et la tolérance immunitaire à l'embryon, lors de la préparation de l'endomètre à l'implantation. Ces effets sont en faveur de l'implantation embryonnaire puisqu'ils préparent les étapes précoces de la gestation.

- **L'endomètre de souris gestante**

Afin de mieux comprendre la dynamique d'expression du système IL1 dans l'endomètre lors de l'implantation embryonnaire, nous avons suivi l'expression des différents récepteurs au cours des huit premiers jours de gestation chez la souris.

L'expression de l'ARNm des récepteurs de l'IL1 varie dans l'endomètre de souris lors de la gestation précoce. Durant la réponse immunitaire classique dirigée contre les

spermatozoïdes, qui a lieu après le coït, le niveau d'expression de l'IL1R1 est renforcé. De plus, lors de la fenêtre d'implantation qui s'ouvre au 4ème jour après l'apparition d'un bouchon vaginal, l'expression de l'IL1R1 atteint un second pic. La cinétique d'expression de l'IL1R1 correspond aux différentes phases de préparation de l'endomètre à l'implantation embryonnaire. Par ailleurs, au jour 5, on constate qu'au site d'implantation de l'embryon, le niveau d'expression d'IL1R1 est maintenu, tandis que les sites adjacents non implantés présentent une expression de l'ARNm de l'IL1R1 plus faible. D'autre part, l'analyse de l'endomètre de souris pseudo-gestantes confirme que la présence des embryons est nécessaire au maintien de l'expression de l'IL1R1 (Fig.12). Ainsi, chez la souris, un relai existe entre la fenêtre d'implantation et la présence des embryons dans la régulation de l'expression du seul récepteur fonctionnel du système IL1.

Un parallélisme dans l'expression de l'IL1R1 entre les souris et les humains existe. En effet, lors de la phase lutéale, le niveau d'expression de l'IL1R1 augmente. Puis, au moment de la fenêtre d'implantation, l'expression de l'IL1R1 est renforcée par les facteurs embryonnaires. Enfin, la décidualisation *in vitro* a montré une augmentation drastique de l'expression de ce récepteur (annexe).

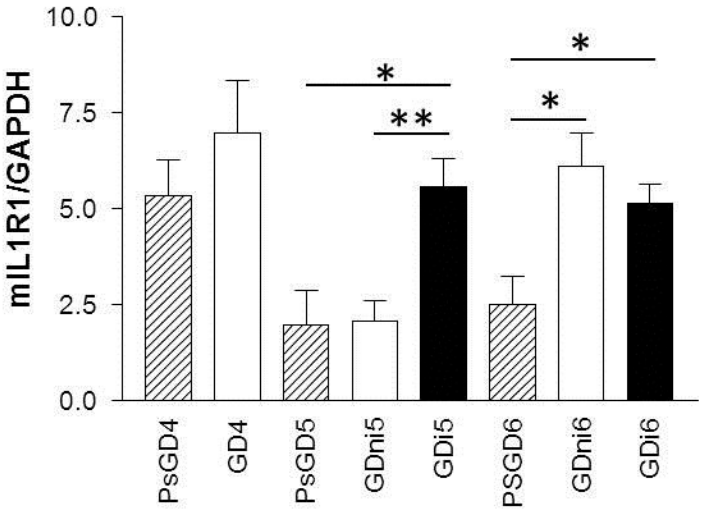


*Figure 12 PCR quantitative de l'IL1R1 dans l'endomètre de souris au jour (GD) 4, 5 et 6 après l'accouplement avec un mâle vasectomisé (souris pseudo-gestante Ps), ou avec un mâle fertile, au site non implanté (blanc) et au site implanté (noir). * P<0.05, ** P<0.01 T test de Student.*

L'expression des récepteurs inhibiteurs IL1RN et IL1R2 est augmentée au jour 1 de la gestation. Ce système permet de réguler la réponse immunitaire locale consécutive à la présence de liquide séminal dans l'endomètre. Il est intéressant de constater que leur niveau d'expression respectif décroit progressivement au cours des jours suivants, puis reste faible tout au long du phénomène implantatoire. Lorsque l'implantation est « irréversible », après le jour 6, leurs expressions paraissent alors augmenter. Nous obtenons des résultats similaires à l'étude menée par Xie-Hong-Zang et al. En effet, d'après leurs résultats, une augmentation de l'expression de l'IL1R2 dans la décidue de souris est visible à partir du $5^{ème}$ jour de gestation (Zhang, Tian et al. 2012). Autrement dit, l'IL1R2 n'est pas détectable dans l'endomètre de souris avant l'implantation embryonnaire, ce qui pourrait correspondre au faible niveau d'expression retrouvé à la même période dans notre modèle. Cependant, les auteurs n'observent aucune augmentation de l'expression du récepteur leurre lors du jour suivant le coït.

Pour notre part, une augmentation de l'IL1R2 est observée dans les ESC humaines décidualisées *in vitro*. L'augmentation de l'IL1R2 après l'implantation pourrait constituer un mécanisme de défense contre les effets tumorigéniques de l'IL1β. D'autre part, Simon. C *et al*. ont observé un défaut d'adhésion des blastocystes dans l'endomètre de souris suite à l'injection répétée d'IL1RN. Ils ont également démontré que l'IL1RN inhibait la transformation de l'épithélium endométrial au moment de l'implantation, via la régulation négative des intégrines αV et β3 [45]. Ces résultats suggèrent que le blocage de l'IL1R1 par l'ILRN empêche l'adhésion des blastocystes de souris dans l'endomètre maternel [44]. Ceci corrobore nos résultats relatifs au faible niveau d'IL1RN observé entre la fenêtre d'implantation et le

moment critique de l'adhésion et de l'implantation du blastocyste dans l'endomètre. Les autres membres de la famille de l'IL1 n'ont pas tous été évalués. Bien que les souris déficientes en IL1 se reproduisent normalement, une étude récente démontre le rôle primordial de l'IL33 dans le phénomène de réceptivité utérine et de décidualisation, tant chez la souris que l'humain (Salker, Nautiyal et al. 2012). L'ensemble de ces résultats permettent de suggérer qu'un phénomène de compensation médié par la redondance de la famille IL1 permettrait à l'implantation de se poursuivre malgré un déficit en IL1β.

Enfin, nous avons évalué l'expression du marqueur MCP1, précédemment étudié dans les ESC humaines. Ainsi, l'augmentation de son niveau d'expression débute avec la présence d'embryons à l'interface maternelle lors de la phase critique de l'implantation embryonnaire. De plus, l'élévation de la transcription de MCP1 corrèle avec celle de l'IL1R1 aux sites d'implantation, aux jours 5, 6, 7 et 8 de la gestation. Enfin, nous observons des similitudes avec les données de nos expériences *in vitro*. En effet, le MCP1 apparaît être un facteur important de l'implantation embryonnaire.

L'analyse de la régulation du système IL1 et du marqueur MCP1 dans l'endomètre de souris gestante a permis d'établir la cinétique spatiale de leurs expressions. Nos résultats, pertinents avec la physiologie de la reproduction, ont permis d'établir un parallèle entre le modèle humain *in vitro* et le modèle murin *in vivo*. En effet, les résultats concordent avec l'augmentation de l'IL1R1 observée lors de la fenêtre d'implantation puis lors de la décidualisation. D'autre part, nous notons une augmentation du niveau d'expression de MCP1. Enfin, les niveaux d'expression des récepteurs inhibiteurs IL1R2 et IL1RN varient durant l'implantation embryonnaire. En effet, lors de la fenêtre d'implantation, leurs niveaux sont faibles, alors qu'ils se retrouvent augmentés dans l'endomètre décidualisé. Ces

résultats suggèrent l'existence d'une fine régulation entre les effets agonistes et antagonistes du système IL1. Ce mécanisme apparait ainsi être crucial lors de l'implantation embryonnaire puisqu'un dérèglement du système de l'IL1 conduit à un échec d'implantation.

Conclusion

Nos résultats montrent une régulation du système IL1 lors du phénomène de l'implantation embryonnaire. De manière fascinante et intrigante, nos données soutiennent le rôle joué par le système IL1 dans l'implantation embryonnaire humaine. Ainsi, le système IL1, contrôlé par la hCG, représente un pivot entre l'implantation embryonnaire et les diverses étapes de préparation de l'endomètre pour l'accueil de l'embryon (stimulation de la réaction déciduale, régulation de l'adhésion et de l'invasion du blastocyste, stimulation de l'angiogénèse et l'infiltration immunitaire). Une fine régulation de l'équilibre du sytème IL1 apparaît nécessaire, d'abord à favoriser la nidation de l'embryon, puis à limiter les effets pseudo-tumoraux liés à l'implantation embryonnaire. Nos résultats illustrent le rôle de chef d'orchestre tenu par la hCG au cours de l'implantation embryonnaire, *via* la modulation du système IL1 et de ces cibles, cependant les mécanismes et les voies de signalisations impliquées dans ces processus restent à élucider.

Annexe 1: L'implantation embryonnaire sous l'angle de l'interleukine 1 et de sa famille

Embryo implantation: role of interleukin 1 family members

Amélie Bourdiec, Ali Akoum

Endocrinologie de la reproduction, Centre de recherche-Hôpital Saint-François d'Assise, Centre Hospitalier Universitaire de Québec, Faculté de médecine, Université Laval, Québec, Canada.

Ali.Akoum@crsfa.ulaval.ca

Soumis à Médceine Science.

Résumé

La réceptivité de l'endomètre à l'embryon est un point fondamental en reproduction. Qu'elle soit naturelle ou assistée, le succès de l'implantation embryonnaire est une étape précoce de la reproduction. Le taux moyen d'implantation des embryons conçus par fécondation in vitro reste faible (20-25%). Même si de nombreux progrès ont été accomplis en procréation médicalement assistée, l'implantation demeure le résultat d'une collaboration étroitement coordonnée entre tissus maternels et embryonnaires, située au seuil de l'endocrinologie et de l'immunologie. Sur le plan scientifique, cette collaboration reste le mystère de la reproduction humaine. L'implantation du blastocyste au sein de l'endomètre dépend d'une synchronisation adéquate entre ces deux derniers. Un dialogue précoce entre la mère et l'embryon est requis dès les premiers instants pour orchestrer des changements mutuels bien synchronisés entre l'embryon en

développement et la réponse maternelle afin d'aboutir à une implantation réussie. Les facteurs maternels, tels que les hormones stéroïdes, les enzymes dégradant la matrice tissulaire, les intégrines, les cytokines, les chimiokines, et de nombreux facteurs de croissance embryonnaires pourraient être impliqués dans ce dialogue fœto-maternel. De ce fait, quelle est la signature moléculaire maternelle compatible avec une implantation embryonnaire ?

Abstract

Endometrial receptivity to embryo implantation is one of the fundamental features of reproduction. Success of natural or assisted embryo implantation is low (20-25%). Implantation remains the result of a successful collaboration, tightly regulated and closely coordinated, between maternal and embryonic tissues located at the crossroads of endocrinology and immunology. In scientific terms, this collaboration is a mystery of human reproduction. The implanted blastocyst within the endometrium is dependent on a fine-tuned synchronization. Therefore, an accurate dialogue between the mother and the embryo is timely required to orchestrate mutual and well-synchronized changes in the developing embryo and maternal responsiveness in order to achieve a successful implantation. Maternal-derived mediators, such as steroid hormones, matrix-degrading enzymes, integrins, cytokines, chemokines, and many embryonic growth factors could be involved in the feto-maternal dialogue. Therefore, what is the maternal molecular signature compatible with embryo implantation?

Introduction

Au cours de chaque cycle menstruel, l'endomètre se prépare à offrir une réceptivité optimale à un embryon, indépendamment de la présence de celui-ci. Cette période, appelée « fenêtre d'implantation », se confine du jour 20 au jour 24 d'un cycle

menstruel normal [1]. A ce moment, sous l'effet de la progestérone et de l'AMP cyclique (AMPc), les cellules stromales de l'endomètre se décidualisent progressivement et acquièrent la propriété unique de réguler l'invasion trophoblastique, de résister aux agressions du stress oxydatif et de développer un environnement de tolérance immunitaire. En effet, concomitante à la décidualisation, l'arrivée massive des cellules de l'immunité participe à la création d'un environnement immunitaire favorable à l'accueil d'un embryon compétant [2, 3]. La fenêtre d'implantation corrèle également avec une réaction vasculaire locale nécessaire à la croissance d'un futur embryon [4]. En parallèle de la préparation endométriale, une transition immunologique de type Th1 vers un type Th2 dans la réponse immunitaire locale est nécessaire pour la réussite de l'implantation embryonnaire [5]. Lors de la fécondation, le liquide séminal aura comme impact d'initier une réaction inflammatoire classique (de type Th1) afin d'éliminer les cellules mortes et les spermatozoïdes restant le jour suivant le coït. Cette réponse fait intervenir des cellules particulières de l'immunité innée [NK utérines (uNK) spécifiques de la décidue principalement, cellules macrophagiques et dendritiques, et cellules T-régulatrices (Treg)], alors que les cellules de l'immunité à médiation humorale (lymphocytes B) semblent fuir l'endomètre [5, 6]. Il s'installe ensuite une réaction anti-inflammatoire de type Th2, qui permet de créer un équilibre Th1/Th2, à l'origine de la tolérance fœto-maternelle et de l'immunotrophisme, permettant l'invasion de l'endomètre par l'embryon. A contrario, un défaut de sécrétion Th2, autant qu'un excès de sécrétion Th1, mène à un échec d'implantation embryonnaire. Il a été rapporté que l'implantation embryonnaire dépend entièrement de l'expression coordonnée de nombreux facteurs incluant les chimiokines, métalloprotéinases (MMPs), molécules d'adhésion, facteurs de croissance précoces et cytokines pro-inflammatoires. L'ensemble de ces médiateurs, cellulaires et moléculaires, permettent et régulent les stades d'apposition,

d'adhésion et d'invasion des cellules trophoblastiques dans l'endomètre [7]. Dès lors, tous les éléments déterminants sont en place pour entamer un dialogue précoce et continu avec un futur embryon. Parmi toutes les molécules présentées par la littérature scientifique comme étant impliquées dans l'implantation embryonnaire, une famille de cytokine, l'interleukine (IL) 1, retient notre attention.

La famille de l'interleukine 1 au cœur de l'interface embryo-maternelle

La famille de l'IL1 comptabilise 11 membres incluant les cytokines IL1, 18, 33, 36, 37 et 38 [8, 9]. Pour chaque cytokine il existe des membres ayant un rôle agoniste et d'autres, un rôle antagoniste, lesquels agissent comme un système tampon finement régulé (Figure 1). L'IL1 se présente sous deux formes. La forme α est principalement intracellulaire, tandis que la forme β est secrétée. L'IL1 se fixe au récepteur membranaire IL1R1 qui nécessite le recrutement d'un corécepteur appelé IL1R3 ou protéine accessoire (IL1RACP) pour transmettre le signal. Ce complexe de signalisation peut être contré de différentes manières: soit par compétition directe entre l'antagoniste IL1RN et l'IL1 au niveau de l'IL1R1, soit par diminution de la biodisponibilité de l'IL1 captée par un récepteur leurre (IL1R2) ou par des récepteurs solubles (sIL1R1, sIL1R2, sIL1RACP). Tandis que l'IL18 forme un complexe avec le récepteur IL18R1 et le corécepteur IL18RACP pour transmettre un signal, celle-ci est régulée par l'IL18BP et la forme soluble de l'IL18R1 qui vont neutraliser l'IL18 et diminuer ainsi sa disponibilité. Enfin, découverte en 2005, l'IL33 se lie au récepteur ST2. La transmission du signal de l'IL33 nécessite également le recrutement du corécepteur IL1RACP. Le seul inhibiteur décrit pour ce système est la forme soluble du récepteur sST2. Chacun de ces systèmes intervient lors du dialogue précoce entre l'endomètre maternel et l'embryon.

En effet, les différents membres de la famille IL1 sont présents à diverses étapes menant à une implantation embryonnaire réussie. L'IL1, ainsi que l'IL18 et l'ILBP

sont exprimés par les cellules de l'endomètre au cours du cycle menstruel [10]. Néanmoins, l'expression de l'IL1 apparaît renforcée au moment de la fenêtre d'implantation, tout comme son récepteur IL1R1, alors qu'on observe une diminution dans l'expression de l'IL1R2 au même moment [11, 12]. Outre ses propriétés immunitaires, l'IL1 est aussi impliquée dans de nombreux processus physiologiques dont l'angiogenèse et le remodelage tissulaire [13]. Cette cytokine est responsable de l'expression de nombreuses molécules clés dans la préparation de l'endomètre à l'implantation embryonnaire tel que l'IL6, l'IL8, LIF, COX2, les intégrines αV/β3, et MCP1 [14, 15]. Il a été démontré que l'IL1 était à l'origine de la vasodilatation locale des artères spiralées via l'induction de la prolifération des cellules endothéliales, et la production de VEGF, de NO et de PGE2 par les cellules de l'endomètre [16]. Cette vasodilatation apparaît primordiale pour l'invasion du compartiment vasculaire par les cellules de l'embryon. En outre, la réaction inflammatoire au sperme augmente fortement le taux utérin d'IL1 qui a pour effet d'activer la sécrétion d'interféron gamma (INFγ) par les cellules uNK [17]. L'adhésion de l'embryon à l'endomètre implique une réaction de type pro-inflammatoire. Cette réaction de type Th1 va activer une cascade moléculaire initiée par l'IL1 et favoriser l'apposition et l'adhésion de l'embryon aux cellules épithéliales de l'endomètre, ainsi que la vasodilatation des artères spiralées par la sécrétion d'INFγ. En réponse à la réaction au sperme, les cellules de l'endomètre vont augmenter l'expression des récepteurs leurres et antagonistes du système IL1. Cette réaction anti-inflammatoire est en parti contrôlée par l'action de faibles doses d'hormone de grossesse (hCG) [18]. L'IL18 occupe également une place majeure dans la préparation de l'endomètre à l'embryon. En effet, cette cytokine qui peut être à la fois, selon sa concentration, Th2 ou Th1 si en excès, est principalement sécrétée par les uNK activées et localisées proches des artères spiralées. Sa présence et son action sont étroitement liées à la régulation de la vasculature de l'endomètre péri-

implantatoire. Ainsi, l'IL18 joue un rôle essentiel dans la déstabilisation des artères spiralées, en plus d'être un facteur pro angiogénique direct. L'IL18 soutient aussi la production d'IFNγ par les cellules uNK [19]. Lors de la poussée anti-inflammatoire secondaire, les cellules uNK vont ensuite exhiber des marqueurs de réponses immunitaire de type Th2, tel que le récepteur ST2 et devenir une source majeure de cytokines anti-inflammatoires (LIF, IL4, IL6, IL18). Lors d'une grossesse normale, ces cytokines vont permettre et contrôler l'invasion de l'embryon dans l'endomètre décidualisé, c'est l'immunotrophisme [20]. La source majeure d'IL1B est l'embryon, ce qui permet de considérer localement cette cytokine comme un signal embryonnaire précoce. Au moment de l'implantation, l'IL1 d'origine embryonnaire, interagit avec ses récepteurs localisés à la surface des différentes cellules de l'endomètre. Cette interaction aura pour effet, entre autres, d'augmenter l'expression d'intégrine spécifique (nécessaire à l'adhésion de l'embryon), et permettre l'invasion des cellules de l'embryon dans la décidue et les artères spiralées via la production de métalloprotéases [18, 21, 22]. De nombreux rapports font état du rôle primordial de l'IL1 et de sa famille dans la réceptivité maternelle et l'implantation embryonnaire. Dans le cadre de la fécondation in vitro, la forte présence de l'IL1B est corrélée avec le succès de l'implantation de l'embryon après son transfert dans la cavité utérine [23]. Aussi, les cas d'avortements spontanés sont corrélés avec une diminution de l'IL1B et de l'IL6 [24]. En outre, l'activation immunitaire résultant d'un excès d'IL18 (déséquilibre pro Th1) transforme les uNK en LAKs et aboutit à un rejet de l'embryon. Ce mécanisme est associé à un faible pronostique d'implantation embryonnaire, d'accouchement prématuré ou de prééclampsie [25].

Des études génotypiques suggèrent l'existence d'une certaine relation entre des polymorphismes du système IL1 et la gestation. Ainsi, les deux polymorphismes maternels IL1B-511C et d'IL1B-31T semblent être associés à une augmentation du

taux de fausses couches récurrentes [26]. L'allèle 1 d'IL1RN (IL-1RN*1) semble être lié à un taux accru d'avortement spontané [27], et l'allèle 2 (IL1RN*2) à un mauvais pronostic de grossesse après FIV [28]. Toutefois, si certaines études ont trouvé qu'IL1RN*2 était trois fois plus fréquent chez les femmes ayant une perte récurrente de grossesse [29] et augmente le risque de naissance prématurée [30], d'autres ont trouvé que ce polymorphisme n'est pas un facteur de risque pour la perte récurrente de grossesse [31]. Les polymorphismes du promoteur du gène de l'IL18 ne semblent pas être pas associés à des avortements spontanés récurrents [32], alors que l'impact du polymorphisme du gène de l'IL33 sur l'implantation embryonnaire et l'établissement de la grossesse demeure inconnu.

L'utilisation de modèles animaux a permis de contribuer à la compréhension de l'implantation embryonnaire et l'établissement de la grossesse. Ainsi il a été observé, chez un modèle de primate non humain, que l'infusion d'hCG et d'IL1B mime les changements qui ont lieu lors de la grossesse et conduisent à l'immunotrophisme [33]. De plus, il a été observé chez un modèle murin que l'injection du récepteur antagoniste à l'IL1 (IL1RN), réduisait considérablement le nombre d'embryons implantés en altérant la réceptivité de l'endomètre à ces derniers. Cependant, il faut noter que ces expériences n'ont jamais pu être reproduites [7, 21]. Aussi, malgré les progrès de la biologie moléculaire, l'utilisation de souris déplétées pour le gène de l'IL1B, l'IL1A, l'IL18 ou l'IL1R1 n'ont pas montré d'altérations majeures de leur fertilité [34, 35]. Cependant, nous savons aujourd'hui qu'un gène ne possède pas qu'une seule fonction, et qu'à la vue des redondances et des interconnections signalétiques des différents membres de la famille de l'IL1, ainsi que du fait que les formes intracellulaires d'IL1A et d'IL33 peuvent exercer leurs rôles comme facteurs de transcription directement au noyau, ces différentes stratégies permettraient de compenser le déficit de l'un d'entre-eux [9, 14, 15].

Dernières avancées

Ces dernières années, l'analyse globale d'endomètre de femmes fertiles en fenêtre d'implantation a révélé de grandes variations selon les différents investigateurs. Seule une poignée de gènes ont conservé une expression commune aux différentes études. Ces minces résultats sont probablement dus aux différents procédés expérimentaux, et finalement aucun marqueur fiable de la réceptivité maternelle n'a vu le jour [36].

Cependant, le concept d'endomètre bio-senseur de l'implantation embryonnaire émerge [37]. De récentes publications démontrent l'existence d'une interaction étroite entre l'IL1B et la hCG, deux signaux embryonnaires précoces, qui sont capables de moduler le phénotype des cellules de l'endomètre. Lors de la fenêtre d'implantation, de faibles quantités d'hCG sont détectables, tant au niveau de l'épithélium utérin que dans le sang circulant de femmes dont la grossesse débute [38-40]. Des expériences in vitro ont révélé un mécanisme de collaboration entre la hCG et l'IL1 qui résulte en une augmentation de la sensibilité des cellules de l'endomètre à l'IL1 et aux autres membres de sa famille. En effet, la hCG serait responsable d'un déséquilibre dans l'expression de la famille IL1, en favorisant l'augmentation des récepteurs fonctionnels tout en diminuant, de façon concomitante, l'expression des récepteurs leurres et antagonistes et en modérant l'expression de l'IL1, de l'IL18 et de l'IL33 [18, 41, 42]. Celle-ci se caractérise par une amplification synergique de certaines cibles moléculaires pertinentes pour la poursuite de l'implantation telle que MCP1, VEGFC, RANTES, VCAM1 et IL6 [18, 41, 43]. Ces facteurs pluripotents sont impliqués dans la régulation de la réponse immunitaire, l'induction d'une tolérance immunitaire, le contrôle de l'angiogenèse locale et de l'immunotrophisme. IL faut également préciser que la présence d'hCG à l'interface embryo-maternelle intervient directement dans l'équilibre Th1/Th2 et la

sécrétion de cytokines Th2 [44] qui contribuent à la tolérance immunitaire et la création d'un environnement favorable à la croissance et au développement du fœtus [45, 46]. Mais au delà du paradigme Th1/Th2 et ses limites pour expliquer le succès ou l'échec de la grossesse, La hCG joue également un rôle dans le recrutement des cellules Treg qui émergent en tant qu'acteurs clés impliqués dans la tolérance immunitaire locale [46][47] et dont l'équilibre avec les cellules Th17 (effectrices proinflammatoires) semble être important pour l'établissement et le succès de la grossesse. Cependant, l'implication et l'impact de la synergie hCG/famille de l'IL1 dans de tels équilibres demeurent inconnus. Il demeure que de faibles quantités d'hCG pourraient modifier les fonctions utérines de l'IL1 en agissant tant sur les récepteurs de la famille, que sur la balance du réseau moléculaire endométrial contrôlé par l'IL1 (Figure 2). De ce fait, l'IL1 et son système de régulation représentent un intermédiaire moléculaire important, pouvant servir de senseur dans l'investigation de la réceptivité maternelle. Une telle hypothèse sur l'expression d'IL18 endométriale comme bio-senseur de l'implantation embryonnaire a été également énoncée [48].

Des données cliniques récentes viennent soutenir l'évaluation de l'expression de la famille de l'IL1 comme marqueurs potentiels de la réceptivité et de l'implantation embryonnaire. En effet, chez certaines patientes présentant un échec d'implantation, un défaut dans la boucle IL18/IL15/uNK empêche les phases d'apposition et d'adhésion de l'embryon en raison de la réaction inadéquate de l'endomètre [25]. À l'inverse, d'autres patientes avec un historique de fausses couches à répétition, présentent, à travers une activation et une cytotoxicité excessive des cellules uNK, une augmentation d'IL18 aux conséquences abortives [49]. D'autre part, la perturbation de l'équilibre dans le système IL33 est positivement corrélée avec l'interruption de la grossesse et le risque de pré-éclampsie [50]. Enfin, l'étude récente de l'expression endométriale de l'IL33 et de

son récepteur a montré que l'interruption de l'axe ST2/IL33 lors de la décidualisation de l'endomètre prédispose aux risques de fausses couches [51].

Conclusion

Cette revue met en évidence que la réorganisation des niveaux d'expression des membres de la famille de l'IL1 dans l'endomètre, via des signaux majeurs et précoces telle que la hCG, fait partie intégrante d'un remaniement moléculaire permettant la création d'un phénotype réceptif à l'implantation et au développement de l'embryon. De plus, il appert que la régulation de l'équilibre délicat au sein de la famille IL1 implique un éventail de types cellulaires endométriaux (Figure 2). Le nouvel environnement induit par le remodelage de l'expression de la famille de l'IL1 par la hCG est compatible avec la physiologie de l'implantation embryonnaire. Ces données in vitro suggèrent l'existence d'une fine régulation entre les effets agonistes et antagonistes de la famille IL1 pour la bonne continuité de la grossesse. Un tel mécanisme s'avère crucial lors de l'implantation embryonnaire puisqu'un dérèglement semble être à l'origine d'un échec d'implantation aussi bien lors des étapes de l'apposition, de l'adhésion et de l'invasion. Avec ces dernières avancées, l'importance de cette famille de cytokines est de plus en plus documentée. Les futures recherches devraient permettre de mieux comprendre les mécanismes sous-jacents à la réorganisation fine et évolutive du dialogue fœto-maternel, ainsi que son impact sur le développement embryonnaire dans un environnement utérin a priori hostile. De telles études devraient aussi permettre de mettre en lumière des marqueurs fiables de la réceptivité maternelle et des senseurs précoces de l'implantation embryonnaire.

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Glossaire

AMPc Adénosine monophosphate cyclique

hCG Gonadotrophine Chorionique humaine

FIV Fertilisation in vitro

IL Interleukine

INF Interféron

ITGB8 Intégrine B8

IRAK Interleukin 1 receptor-associated kinase

JNK c-Jun N-terminal kinase

LAK Lymphocytes activated killers cells

LIF Leukemia inhibitory factor

MAPK Mitogen-activated protein kinases

MCP Monocyte chimoattractant protein

MEKK MAP/ERK kinase kinase

MMP Métalloprotéases

NF-κB Nuclear factor-kappa B

NK Natural killer

PTGS Prostaglandin-endoperoxide synthase

RANTES Regulated on activation, normal T cell expressed and secreted

SDF Stromal-derived facto

TAK Tumor growth factor-beta activated kinase

TIMP Inhibiteurs naturels tissulaires endogènes des métalloprotéases

TRAF Tumor necrosis factor receptor associated factor

VCAM1 Vascular cell adhesion molecule 1

VEGFC Vascular endothelial growth factor C

Références

1. Achache H, Revel A. Endometrial receptivity markers, the journey to successful embryo implantation. Hum Reprod Update 2006; 12: 731-46.

2. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Semin Reprod Med 2007; 25: 445-53.

3. Le Bouteiller P, Tabiasco J. [Immunology of pregnancy: renewed interest]. Medecine sciences : M/S 2006; 22: 745-50.

4. Ledee N, Chaouat G, Serazin V, et al. Endometrial vascularity by three-dimensional power Doppler ultrasound and cytokines: a complementary approach to assess uterine receptivity. Journal of reproductive immunology 2008; 77: 57-62.

5. Chaouat G, Ledee-Bataille N, Dubanchet S, et al. TH1/TH2 paradigm in pregnancy: paradigm lost? Cytokines in pregnancy/early abortion: reexamining the TH1/TH2 paradigm. Int Arch Allergy Immunol 2004; 134: 93-119.

6. Veenstra van Nieuwenhoven AL, Heineman MJ, Faas MM. The immunology of successful pregnancy. Hum Reprod Update 2003; 9: 347-57.

7. Guzeloglu-Kayisli O, Kayisli UA, Taylor HS. The role of growth factors and cytokines during implantation: endocrine and paracrine interactions. Semin Reprod Med 2009; 27: 62-79.

8. Dinarello CA, Novick D, Kim S, Kaplanski G. Interleukin-18 and IL-18 Binding Protein. Front Immunol 2013; 4: 289.

9. Dinarello CA. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. Blood 2011; 117: 3720-32.

10. Yoshino O, Osuga Y, Koga K, et al. Evidence for the expression of interleukin (IL)-18, IL-18 receptor and IL-18 binding protein in the human endometrium. Mol Hum Reprod 2001; 7: 649-54.

11. Bigonnesse F, Labelle Y, Akoum A. Triphasic expression of interleukin-1 receptor type I in human endometrium throughout the menstrual cycle of fertile women and women with unexplained infertility. Fertility and sterility 2001; 75: 79-87.

12. Boucher A, Kharfi A, Al-Akoum M, et al. Cycle-dependent expression of interleukin-1 receptor type II in the human endometrium. Biol Reprod 2001; 65: 890-8.

13. Carmi Y, Voronov E, Dotan S, et al. The role of macrophage-derived IL-1 in induction and maintenance of angiogenesis. J Immunol 2009; 183: 4705-14.

14. Saccani S, Pantano S, Natoli G. p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment. Nat Immunol 2002; 3: 69-75.

15. Rossi M, Sharkey AM, Vigano P, et al. Identification of genes regulated by interleukin-1beta in human endometrial stromal cells. Reproduction 2005; 130: 721-9.

16. Smith SK. Angiogenesis, vascular endothelial growth factor and the endometrium. Hum Reprod Update 1998; 4: 509-19.

17. Edwards RG. Physiological and molecular aspects of human implantation. Hum Reprod 1995; 10 Suppl 2: 1-13.

18. Bourdiec A, Shao R, Rao CV, Akoum A. Human chorionic gonadotropin triggers angiogenesis via the modulation of endometrial stromal cell responsiveness to

interleukin 1: a new possible mechanism underlying embryo implantation. Biol Reprod 2012; 87: 66.

19. Croy BA, Esadeg S, Chantakru S, et al. Update on pathways regulating the activation of uterine Natural Killer cells, their interactions with decidual spiral arteries and homing of their precursors to the uterus. Journal of reproductive immunology 2003; 59: 175-91.

20. Sargent IL, Borzychowski AM, Redman CW. NK cells and human pregnancy--an inflammatory view. Trends Immunol 2006; 27: 399-404.

21. Simon C, Gimeno MJ, Mercader A, et al. Embryonic regulation of integrins beta 3, alpha 4, and alpha 1 in human endometrial epithelial cells in vitro. J Clin Endocrinol Metab 1997; 82: 2607-16.

22. Prutsch N, Fock V, Haslinger P, et al. The role of interleukin-1beta in human trophoblast motility. Placenta 2012; 33: 696-703.

23. Sheth KV, Roca GL, al-Sedairy ST, et al. Prediction of successful embryo implantation by measuring interleukin-1-alpha and immunosuppressive factor(s) in preimplantation embryo culture fluid. Fertility and sterility 1991; 55: 952-7.

24. von Wolff M, Thaler CJ, Strowitzki T, et al. Regulated expression of cytokines in human endometrium throughout the menstrual cycle: dysregulation in habitual abortion. Molecular human reproduction 2000; 6: 627-34.

25. Ledee-Bataille N, Bonnet-Chea K, Hosny G, et al. Role of the endometrial tripod interleukin-18, -15, and -12 in inadequate uterine receptivity in patients with a history of repeated in vitro fertilization-embryo transfer failure. Fertility and sterility 2005; 83: 598-605.

26. Wang ZC, Yunis EJ, De los Santos MJ, et al. T helper 1-type immunity to trophoblast antigens in women with a history of recurrent pregnancy loss is associated with polymorphism of the IL1B promoter region. Genes and immunity 2002; 3: 38-42.

27. Perni SC, Vardhana S, Tuttle SL, et al. Fetal interleukin-1 receptor antagonist gene polymorphism, intra-amniotic interleukin-1beta levels, and history of spontaneous abortion. American journal of obstetrics and gynecology 2004; 191: 1318-23.

28. Gremlich S, Fratta S, Rebellato E, et al. Interleukin-1 receptor antagonist gene (IL-1RN) polymorphism is a predictive factor of clinical pregnancy after IVF. Hum Reprod 2008; 23: 1200-6.

29. Unfried G, Tempfer C, Schneeberger C, et al. Interleukin 1 receptor antagonist polymorphism in women with idiopathic recurrent miscarriage. Fertility and sterility 2001; 75: 683-7.

30. Chaves JH, Babayan A, Bezerra Cde M, et al. Maternal and neonatal interleukin-1 receptor antagonist genotype and pregnancy outcome in a population with a high rate of pre-term birth. Am J Reprod Immunol 2008; 60: 312-7.

31. Levrant S, Coulam CB, Jeyendran RS. Interleukin 1 receptor antagonist gene polymorphisms are not risk factors for recurrent pregnancy loss: evaluation of couples. Am J Reprod Immunol 2008; 60: 224-8.

32. Ostojic S, Volk M, Medica I, et al. Polymorphisms in the interleukin-12/18 genes and recurrent spontaneous abortion. Am J Reprod Immunol 2007; 58: 403-8.

33. Strakova Z, Mavrogianis P, Meng X, et al. In vivo infusion of interleukin-1beta and chorionic gonadotropin induces endometrial changes that mimic early pregnancy events in the baboon. Endocrinology 2005; 146: 4097-104.

34. Horai R, Asano M, Sudo K, et al. Production of mice deficient in genes for interleukin (IL)-1alpha, IL-1beta, IL-1alpha/beta, and IL-1 receptor antagonist shows that IL-1beta is crucial in turpentine-induced fever development and glucocorticoid secretion. J Exp Med 1998; 187: 1463-75.

35. Takeda K, Tsutsui H, Yoshimoto T, et al. Defective NK cell activity and Th1 response in IL-18-deficient mice. Immunity 1998; 8: 383-90.

36. Wang H, Dey SK. Roadmap to embryo implantation: clues from mouse models. Nat Rev Genet 2006; 7: 185-99.

37. Teklenburg G, Salker M, Heijnen C, et al. The molecular basis of recurrent pregnancy loss: impaired natural embryo selection. Mol Hum Reprod 2010; 16: 886-95.

38. Zimmermann G, Ackermann W, Alexander H. Expression and production of human chorionic gonadotropin (hCG) in the normal secretory endometrium: evidence of CGB7 and/or CGB6 beta hCG subunit gene expression. Biol Reprod 2012; 86: 87.

39. Cole LA. hCG, the wonder of today's science. Reprod Biol Endocrinol 2012; 10: 24.

40. Perrier d'Hauterive S, Charlet-Renard C, Dubois M, et al. [What's new at the maternal-foetal interface: role of the hCG/LH-hCG receptor couple during embryo implantation]. Revue medicale de Liege 2006; 61: 705-12.

41. Bourdiec A, Bedard D, Rao CV, Akoum A. Human chorionic gonadotropin regulates endothelial cell responsiveness to interleukin 1 and amplifies the cytokine-mediated effect on cell proliferation, migration and the release of angiogenic factors. Am J Reprod Immunol 2013; 70: 127-38.

42. Herrmann-Lavoie C, Rao CV, Akoum A. Chorionic gonadotropin down-regulates the expression of the decoy inhibitory interleukin 1 receptor type II in human endometrial epithelial cells. Endocrinology 2007; 148: 5377-84.

43. Bourdiec A, Calvo E, Rao CV, Akoum A. Transcriptome analysis reveals new insights into the modulation of endometrial stromal cell receptive phenotype by embryo-derived signals interleukin-1 and human chorionic gonadotropin: possible involvement in early embryo implantation. PLoS One 2013; 8: e64829.

44. Saito S, Nakashima A, Shima T, Ito M. Th1/Th2/Th17 and regulatory T-cell paradigm in pregnancy. Am J Reprod Immunol 2010; 63: 601-10.

45. Lee JY, Lee M, Lee SK. Role of endometrial immune cells in implantation. Clinical and experimental reproductive medicine 2011; 38: 119-25.

46. Tsampalas M, Gridelet V, Berndt S, et al. Human chorionic gonadotropin: a hormone with immunological and angiogenic properties. Journal of reproductive immunology 2010; 85: 93-8.

47. Chaouat G. Inflammation, NK cells and implantation: friend and foe (the good, the bad and the ugly?): replacing placental viviparity in an evolutionary perspective. Journal of reproductive immunology 2013; 97: 2-13.

48. Ledee N, Petitbarat M, Rahmati M, et al. New pre-conception immune biomarkers for clinical practice: interleukin-18, interleukin-15 and TWEAK on the

endometrial side, G-CSF on the follicular side. Journal of reproductive immunology 2011; 88: 118-23.

49. Petitbarat M, Rahmati M, Serazin V, et al. TWEAK appears as a modulator of endometrial IL-18 related cytotoxic activity of uterine natural killers. PLoS One 2011; 6: e14497.

50. Kaitu'u-Lino TJ, Tuohey L, Tong S. Maternal serum interleukin-33 and soluble ST2 across early pregnancy, and their association with miscarriage. Journal of reproductive immunology 2012; 95: 46-9.

51. Salker MS, Nautiyal J, Steel JH, et al. Disordered IL-33/ST2 activation in decidualizing stromal cells prolongs uterine receptivity in women with recurrent pregnancy loss. PLoS One 2012; 7: e52252.

Légendes

Figure 1. Stratégies d'activation et d'inhibition des systèmes IL1, IL18 et IL33. Représentation schématique des mécanismes d'activation et de régulation négative des systèmes IL1, IL18, IL33. La formation d'un complexe de corécepteur après liaison du ligand permet l'activation de voies de signalisation intracellulaire. Une fois assemblée à la membrane, la partie intra-cellulaire du complexe recrute rapidement deux protéines de signalisation, MYD88 et IRAK 4. Ce premier module de signalisation phosphoryle ensuite IRAK1 et IRAK2. Ceci est suivi par le recrutement et l'oligomérisation de TRAF6 et la formation subséquente des complexes de signalisation TAK1 et MEKK3 qui activent les facteurs de transcriptions NF-κB, JNK et p38 MAPK. La translocation nucléaire de NF-κB permet sa liaison à un motif d'ADN conservé que l'on retrouve dans de nombreux gènes cibles de l'IL1. La formation des

complexes de récepteurs à la membrane peut être contrée de différentes manières, soit par le blocage du signal (antagonisme ou récepteurs leurres), soit par neutralisation extra-cellulaire des ligands.

Figure 2. Remaniement de la sensibilité endométriale à l'IL1 et sa famille lors de l'implantation embryonnaire précoce. Représentation schématique de l'endomètre humain lors de l'implantation embryonnaire. Les cellules trophoblastiques (jaunes) envahissent le stroma (orange), et libèrent des concentrations croissantes d'hCG et d'IL1, des signaux embryonnaires précoces. Ces signaux agissent sur les cellules résidentes de l'endomètre en fenêtre d'implantation tel que les cellules stromales et épithéliales (rouge). En réponse, l'équilibre du système IL1 des cellules stromales est rompu, ce qui permet la création d'un nouveau réseau moléculaire favorable à l'implantation embryonnaire. De plus, l'action de la hCG et de l'IL1 s'étend à l'ensemble des membres de la famille IL1, pour augmenter l'expression des récepteurs fonctionnels de l'IL18 et de l'IL33. Les cellules endothéliales qui tapissent les vaisseaux sanguins de l'endomètre peuvent aussi réagir et voir augmenter leur sensibilité à l'IL1, ce qui se traduit par une augmentation de leurs propriétés angiogéniques, notamment via l'IL8. Il a été démontré que la hCG diminuait également le récepteur IL1R2 des cellules épithéliales glandulaires, mais l'information complète sur la famille IL1 n'est pas encore documentée. Symboles : ↓ diminution, ↓↓ diminution en synergie hCG/IL1, ↑ augmentation, ↑↑augmentation en synergie hCG/IL1, = aucune variation.

Figures

Figure 1

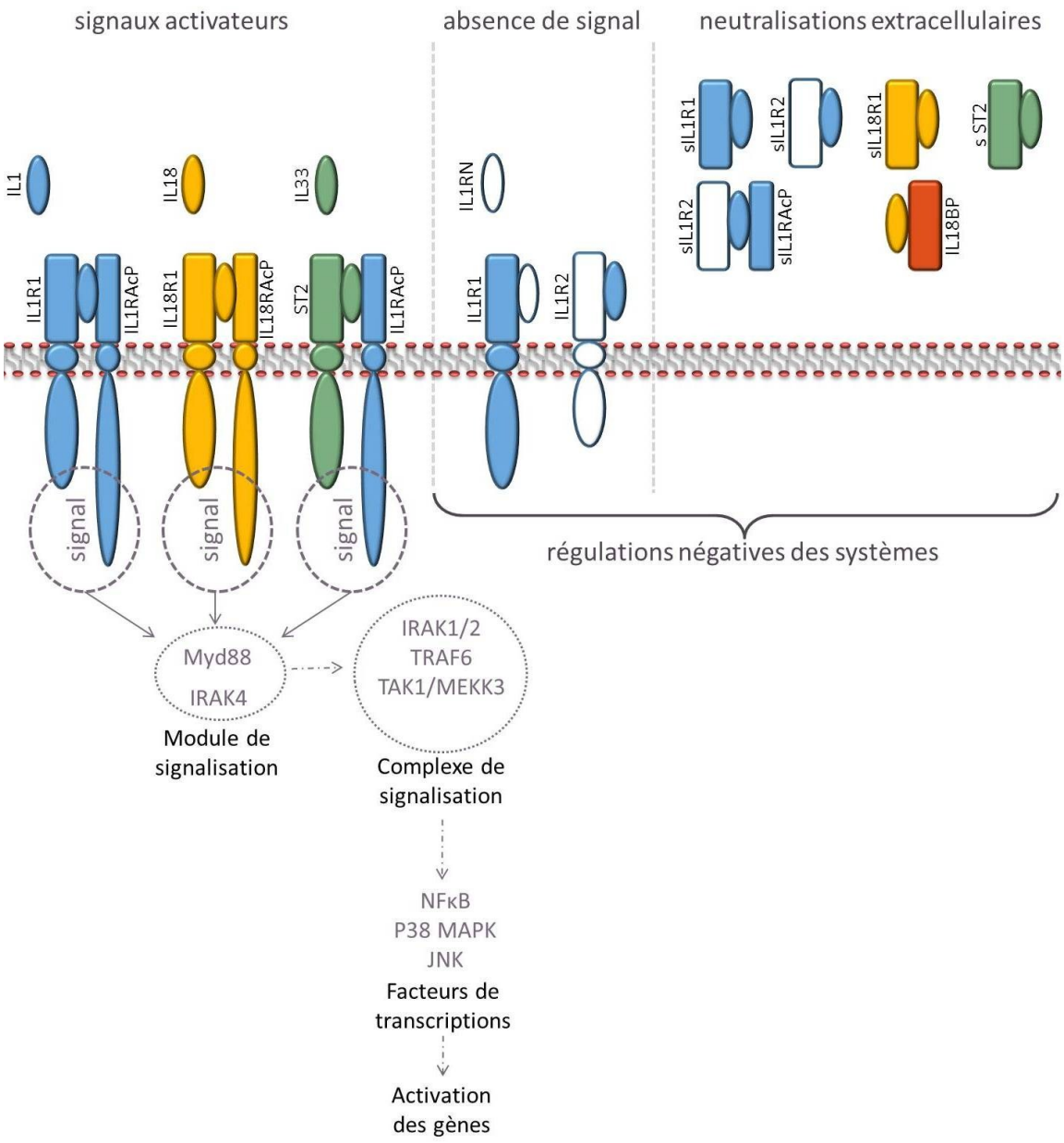
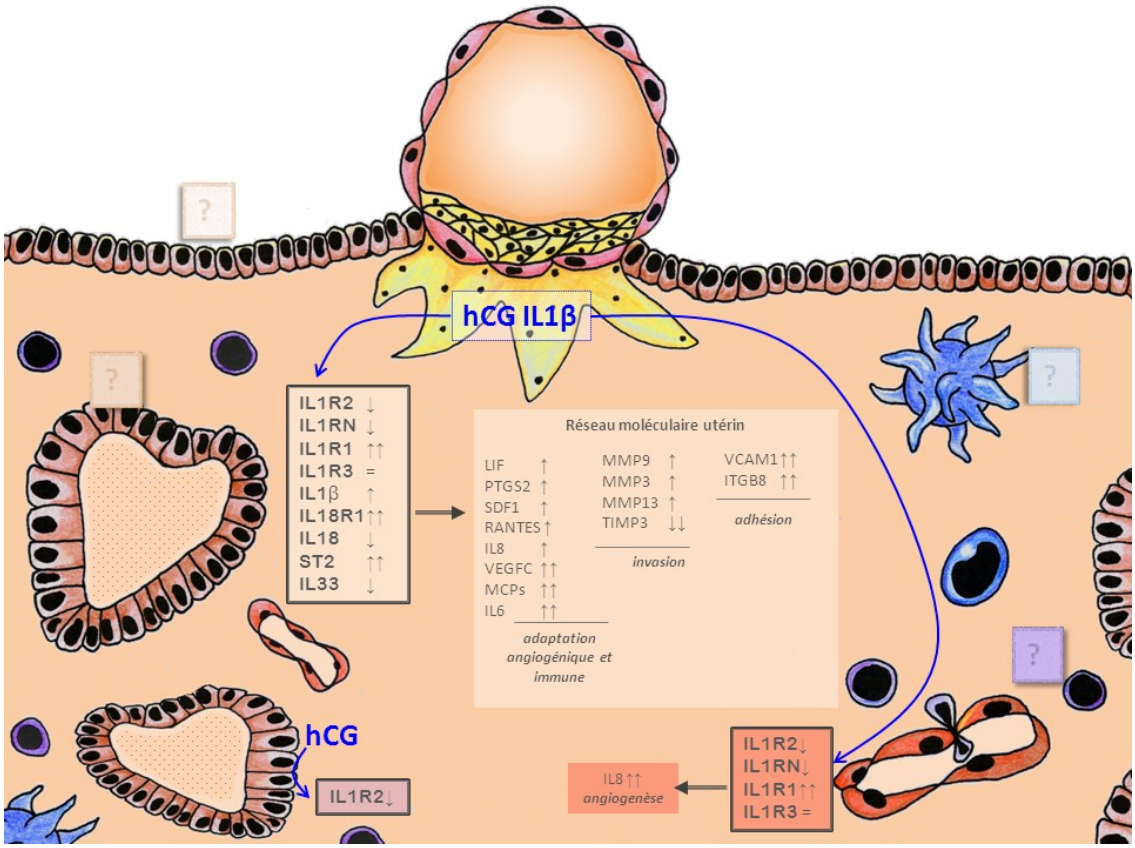

signaux activateurs
absence de signal
neutralisations extracellulaires
IL1
IL18
IL33
IL1RN
sIL1R1
sIL1R2
sIL18R1
s ST2
sIL1R2
sIL1RAcP
IL18BP
IL1R1
IL1RAcP
IL18R1
IL18RAcP
ST2
IL1RAcP
IL1R1
IL1R2
signal
signal
signal
régulations négatives des systèmes
Myd88
IRAK4
Module de signalisation
IRAK1/2
TRAF6
TAK1/MEKK3
Complexe de signalisation
NFκB
P38 MAPK
JNK
Facteurs de transcriptions
Activation des gènes

Figure 2


hCG IL1β
IL1R2 ↓
IL1RN ↓
IL1R1 ↑↑
IL1R3 =
IL1β ↑
IL18R1 ↑↑
IL18 ↓
ST2 ↑↑
IL33 ↓
Réseau moléculaire utérin
LIF ↑
PTGS2 ↑
SDF1 ↑
RANTES ↑
IL8 ↑
VEGFC ↑↑
MCPs ↑↑
IL6 ↑↑
adaptation angiogénique et immune
MMP9 ↑
MMP3 ↑
MMP13 ↑
TIMP3 ↓↓
invasion
VCAM1 ↑↑
ITGB8 ↑↑
adhésion
hCG
IL1R2↓
IL8 ↑↑ angiogenèse
IL1R2↓
IL1RN↓
IL1R1↑↑
IL1R3 =

Annexe 2: Comparaisons d'études globales

Les résultats d'une étude globale micro array *Affymetrix*®, réalisée avec des ESC décidualisées exposées au milieu conditionné de cellules trophoblastiques (TCM 3h et TCM 12h), montrent également la variation des membres de la famille de l'IL1 (Hess, Hamilton et al. 2007). Ainsi des facteurs embryonnaires, présents dans les milieux conditionnés, sont capables de moduler la réceptivité à l'IL1 des cellules stromales décidualisées (Table 4). Nous avons comparé les résultats de nos deux études microarray. L'intersection des listes des gènes régulés à la hausse en réponse aux stimuli hCG/IL1 dans les ESC en fenêtre implantatoire et la liste des gènes régulés à la hausse en réponse aux milieux conditionnés TCM dans les ESC décidualisées *in vitro* met en évidence 167 gènes commun (Table 5), soit plus de 30% des gènes retrouvés dans notre analyse (Fig.15). L'ensemble de ces gènes, conservés entre les deux études, regroupe des facteurs de transcriptions, des facteurs de croissance, des protéines membranaires, des cytokines et des chimiokines. Ces gènes représentent des marqueurs potentiels pour suivre et/ou évaluer la réceptivité endométriale. Cependant des différences existent entre nos deux études au niveau de la régulation de l'IL1R2. En effet dans nos cultures non décidualisées, l'influence de hCG/IL1 diminue l'expression de l'IL1R2, alors que dans les cellules décidualisées, le milieu conditionné augmente l'expression du récepteur leurre. Il faut également noter que nos résultats indiquent une augmentation de l'expression de l'IL1R2 des ESC décidualisées *in vitro*. Ce qui confère à l'IL1R2 un rôle primordial dans le contrôle du système IL1 lors de l'implantation embryonnaire.

	Liste des gènes de la famille IL1			
	Hess et al 2007		Bourdiec et al 2013	
GENE	FC TCM 3h	FC TCM 12h	FC hCG/IL1	q-PCR hCG/IL1
IL1R1	1,70	1,54	-	Up
IL1R2	1,80	2,58	-	Down
IL1RL1	1,40	2,21	2,05	Up
IL18R1	-	-	1,65	Up
IL1RAP	1,70	0,43	-	Down
IL1RN	-	-	-	Down
IL1A	1,61	1,62	2,88	Up
IL1B	4,25	-	13,13	Up

Tableau 4 : Comparaison des résultats de la variation des gènes de la famille de l'IL1.

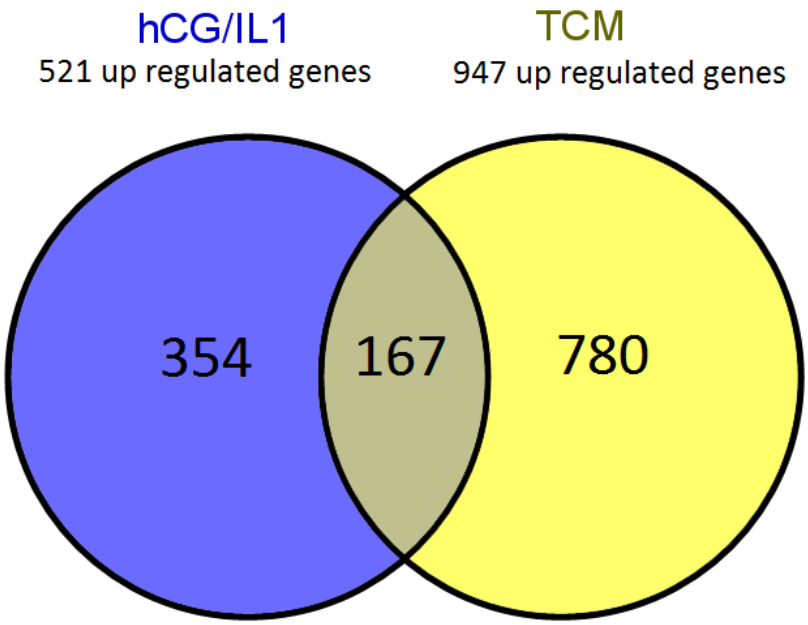


Figure 13 : Diagramme de Venn des résultats obtenus par analyse globale des gènes surexprimés commun à l'étude menée au laboratoire, avec des ESC prélevées en phase d'implantation et stimulées par hCG/IL1β, et l'étude de Hess et al, avec ESC décidualisées et exposées au milieu conditionné de trophoblastes (TCM).

Assignment	Gene name		IPA lf	IPA Pf	IPA Im	IPA Rm	IPA CS	IPA Ap	IPA Ag
NM_016006	ABHD5	abhydrolase domain containing 5							
NM_014945	ABLIM3	actin binding LIM protein family, member 3							
NM_145804	ABTB2	ankyrin repeat and BTB (POZ) domain containing 2							
NM_001124	ADM	adrenomedullin		X	X		X	X	
NM_000693	ALDH1A3	aldehyde dehydrogenase 1 family, member A3						X	
NM_000480	AMPD3	adenosine monophosphate deaminase 3	X						
NM_030641	APOL6	apolipoprotein L, 6							
NM_032199	ARID5B	AT rich interactive domain 5B (MRF1-like)		X					
NM_00104061	ATF3	activating transcription factor 3		X				X	
NM_00119364	ATF5	activating transcription factor 5		X				X	
NM_012342	BAMBI	BMP and activin membrane-bound inhibitor					X		
NM_000710	BDKRB1	bradykinin receptor B1					X	X	
NM_006995	BTN2A2	butyrophilin, subfamily 2, member A2							
NM_017787	C10orf26	chromosome 10 open reading frame 26							
NM_001733	C1R	complement component 1	X						
NM_005623	CCL8	chemokine (C-C motif) ligand 8	X		X		X		
NM_001777	CD47	CD47 molecule		X	X		X	X	
NM_005195	CEBPD	CCAAT		X				X	
NM_003879	CFLAR	CASP8 and FADD-like X regulator	X	X				X	
NM_001304	CPD	carboxypeptidase D							
NM_000757	CSF1	colony stimulating factor 1 (macrophage)	X	X	X	X	X	X	
NM_004079	CTSS	cathepsin S	X	X	X			X	X
NM_001511	CXCL1	chemokine (C-X-C motif) ligand 1	X	X	X		X	X	
NM_148923	CYB5A	cytochrome b5 type A (microsomal)	X					X	
NM_015247	CYLD	cylindromatosis (turban tumor syndrome)	X	X	X			X	
NM_004820	CYP7B1	cytochrome P450, family 7, subfamily B, polypeptide 1		X		X			
NM_001954	DDR1	discoidin domain receptor tyrosine kinase 1	X	X	X		X	X	
NM_006729	DIAPH2	diaphanous homolog 2 (Drosophila)							
NM_00117411	DMXL2	Dmx-like 2							
NM_012100	DNPEP	aspartyl aminopeptidase							
NM_001935	DPP4	dipeptidyl-peptidase 4	X	X	X		X	X	
NM_015177	DTX4	deltex homolog 4 (Drosophila)	X						
NM_001955	EDN1	endothelin 1		X	X	X	X	X	X
NM_005228	EGFR	epidermal growth factor receptor	X	X			X	X	
NM_00100870	EML1	echinoderm microtubule associated protein like 1							
NM_00100226	EPSTI1	epithelial stromal interaction 1 (breast)							
NM_00114382	ETS1	v-ets erythroblastosis virus E26 oncogene homolog 1		X	X		X	X	X
NM_016135	ETV7	ets variant 7	X						
NM_147189	FAM110B	family with sequence similarity 110, member B							
NM_032581	FAM126A	family with sequence similarity 126, member A	X						
AK299337	FAM65C	family with sequence similarity 65, member C							
NM_058229	FBXO32	F-box protein 32						X	
NM_002006	FGF2	fibroblast growth factor 2 (basic)	X	X			X	X	X
NM_175884	FLJ36031	hypothetical protein FLJ36031							
NM_175736	FMNL3	formin-like 3							
NM_032682	FOXP1	forkhead box P1		X			X	X	
NM_005257	GATA6	GATA binding protein 6		X				X	
NM_002053	GBP1	guanylate binding protein 1, interferon-inducible							
NM_004120	GBP2	guanylate binding protein 2, interferon-inducible	X		X		X		
NM_000161	GCH1	GTP cyclohydrolase 1				X	X		X
NM_005110	GFPT2	glutamine-fructose-6-phosphate transaminase 2							
NM_000163	GHR	growth hormone receptor	X	X			X	X	
NM_00108311	GPD2	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	X						
NM_016235	GPRC5B	G protein-coupled receptor, family C, group 5, member B							
NM_020760	HECW2	HECT, C2 and WW domain containing E3 ubiquitin protein lig	X						
NM_006734	HIVEP2	human immunodeficiency virus type I enhancer binding prot				X			

Tableau 5 Gènes régulés à la hausse en réponse aux stimuli hCG/IL1 dans les ESC en fenêtre implantatoire et en réponse aux milieux conditionnés TCM dans les ESC décidualisées in vitro.

Assignment	Gene name		IPA If	IPA Pf	IPA Im	IPA Rm	IPA CS	IPA Ap	IPA Ag
NM_005516	HLA-E	major histocompatibility complex, class I, E	X	X					
NM_018950	HLA-F	major histocompatibility complex, class I, F	X						
NM_005525	HSD11B1	hydroxysteroid (11-beta) dehydrogenase 1	X	X					
NM_000201	ICAM1	intercellular adhesion molecule 1	X	X	X	X	X	X	
NM_003897	IER3	immediate early response 3		X	X			X	
NM_006332	IFI30	interferon, gamma-inducible protein 30	X	X	X				
NM_207585	IFNAR2	interferon (alpha, beta and omega) receptor 2	X	X			X	X	
NM_000416	IFNGR1	interferon gamma receptor 1	X	X			X	X	
NM_000640	IL13RA2	interleukin 13 receptor, alpha 2	X	X					
NM_000575	IL1A	interleukin 1, alpha	X	X	X	X	X	X	X
NM_000576	IL1B	interleukin 1, beta	X	X	X	X	X	X	X
NM_016232	IL1RL1	interleukin 1 receptor-like 1	X	X	X		X	X	
NM_000600	IL6	interleukin 6 (interferon, beta 2)	X	X	X	X	X	X	X
NM_002185	IL7R	interleukin 7 receptor	X	X	X		X	X	
NM_001570	IRAK2	interleukin-1 receptor-associated kinase 2					X		
NM_002198	IRF1	interferon regulatory factor 1		X			X	X	
NM_002214	ITGB8	integrin, beta 8	X	X			X	?	
NM_000215	JAK3	Janus kinase 3	X	X	X		X	X	
NM_014734	KIAA0247	KIAA0247	X						
NM_016531	KLF3	Kruppel-like factor 3 (basic)							
NR_015377	LOC654433	hypothetical LOC654433							
NM_002340	LSS	lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)							
NM_002350	LYN	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	X	X	X		X	X	
NM_005923	MAP3K5	mitogen-activated protein kinase kinase kinase 5				X		X	
NM_005204	MAP3K8	mitogen-activated protein kinase kinase kinase 8					X	X	
NM_004229	MED14	mediator complex subunit 14					X		
NM_002426	MMP12	matrix metallopeptidase 12 (macrophage elastase)	X						
NM_005098	MSC	musculin							
NM_00114497	MTHFD2L	methylenetetrahydrofolate dehydrogenase	X						
NM_005746	NAMPT	nicotinamide phosphoribosyltransferase	X	X			X	X	
NM_181782	NCOA7	nuclear receptor coactivator 7							
NM_003998	NFKB1	nuclear factor of kappa light polypeptide	X	X	X	X	X	X	
NM_002502	NFKB2	nuclear factor of kappa light polypeptide	X	X	X		X	X	
NM_031419	NFKBIZ	nuclear factor of kappa light polypeptide	X					X	
NM_032206	NLRC5	NLR family, CARD domain containing 5							
NM_004688	NMI	N-myc (and STAT) interactor							
NM_201266	NRP2	neuropilin 2		X			X		X
NM_020841	OSBPL8	oxysterol binding protein-like 8	X						
NM_004337	OSGIN2	oxidative stress induced growth inhibitor family member 2							
NM_015368	PANX1	pannexin 1					X		
NM_002581	PAPPA	pregnancy-associated plasma protein A, pappalysin 1	X	X					
NM_017554	PARP14	poly (ADP-ribose) polymerase family, member 14							
NM_003687	PDLIM4	PDZ and LIM domain 4						X	
NM_006474	PDPN	podoplanin		X			X		
NM_00104044	PHF11	PHD finger protein 11							
NM_002648	PIM1	pim-1 oncogene		X				X	
NM_007366	PLA2R1	phospholipase A2 receptor 1, 180kDa	X						
NM_002662	PLD1	phospholipase D1, phosphatidylcholine-specific	X	X			X	X	
NM_033240	PML	promyelocytic leukemia		X			X	X	X
NM_000304	PMP22	peripheral myelin protein 22	X	X			X		
NM_003713	PPAP2B	phosphatidic acid phosphatase type 2B					X		
NM_005605	PPP3CC	protein phosphatase 3, catalytic subunit, gamma isozyme	X						
NM_006902	PRRX1	paired related homeobox 1		X			X		
NM_000447	PSEN2	presenilin 2 (Alzheimer disease 4)	X				X	X	
NM_002801	PSMB10	proteasome (prosome, macropain) subunit, beta type, 10					X		
NM_002818	PSME2	proteasome (prosome, macropain) activator subunit 2			X		X		

Tableau 5 suite

Assignment	Gene name		IPA If	IPA Pf	IPA Im	IPA Rm	IPA CS	IPA Ap	IPA Ag
NM_004878	PTGES	prostaglandin E synthase		X	X		X	X	X
NM_014488	RAB30	RAB30, member RAS oncogene family							
NM_015646	RAP1B	RAP1B, member of RAS oncogene family		X			X		
NM_007211	RASSF8	Ras association	X						
NM_002908	REL	v-rel reticuloendotheliosis viral oncogene homolog (avian)	X	X	X	X	X	X	
NM_003821	RIPK2	receptor-interacting serine-threonine kinase 2		X	X		X	X	
NM_134260	RORA	RAR-related orphan receptor A	X	X			X		
NM_005226	S1PR3	sphingosine-1-phosphate receptor 3		X	X		X	X	
NR_027783	SAT1	spermidine	X	X				X	
NM_00114381	SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin)	X	X			X	X	
NM_00104002	SIRPA	signal-regulatory protein alpha	X	X	X		X	X	
NM_00117412	SLC11A2	solute carrier family 11					X		
NM_015482	SLC22A23	solute carrier family 22							
NM_003059	SLC22A4	solute carrier family 22							
NM_016612	SLC25A37	solute carrier family 25, member 37	X						
AF495725	SLC25A37	solute carrier family 25, member 37	X						
NM_00112843	SLC39A14	solute carrier family 39 (zinc transporter)	X						
NM_022154	SLC39A8	solute carrier family 39 (zinc transporter)							
NM_032148	SLC41A2	solute carrier family 41, member 2	X						
NM_017611	SLC43A3	solute carrier family 43, member 3							
NM_173653	SLC9A9	solute carrier family 9							
NM_013272	SLCO3A1	solute carrier organic anion transporter family							
NM_144975	SLFN5	schlafen family member 5	X						
NM_00102446	SOD2	superoxide dismutase 2, mitochondrial	X	X			X	X	
NM_00108039	SP100	SP100 nuclear antigen							
NM_00116627	SPATA13	spermatogenesis associated 13							
NM_002727	SRGN	serglycin		X				X	
NM_003033	ST3GAL1	ST3 beta-galactoside alpha-2,3-sialyltransferase 1						X	
NM_006278	ST3GAL4	ST3 beta-galactoside alpha-2,3-sialyltransferase 4					X		
NM_005418	ST5	suppression of tumorigenicity 5					X		
NM_005419	STAT2	signal transducer and activator of transcription 2			X		X		
NM_000593	TAP1	transporter 1, ATP-binding cassette, sub-family B	X		X				
NM_000544	TAP2	transporter 2, ATP-binding cassette, sub-family B	X						
NM_015130	TBC1D9	TBC1 domain family, member 9	X						
NM_003692	TMEFF1	transmembrane protein with EGF-like and two follistatin-l							
NM_017870	TMEM132A	transmembrane protein 132A						X	
NM_032021	TMEM133	transmembrane protein 133							
NM_016464	TMEM138	transmembrane protein 138							
NM_018295	TMEM140	transmembrane protein 140							
NM_013390	TMEM2	transmembrane protein 2							
NM_006291	TNFAIP2	tumor necrosis factor, alpha-induced protein 2							
NM_006290	TNFAIP3	tumor necrosis factor, alpha-induced protein 3	X				X	X	
NM_007115	TNFAIP6	tumor necrosis factor, alpha-induced protein 6	X				X		
NM_014350	TNFAIP8	tumor necrosis factor, alpha-induced protein 8		X				X	
NM_001066	TNFRSF1B	tumor necrosis factor receptor superfamily, member 1B	X	X	X	X	X	X	
NM_006058	TNIP1	TNFAIP3 interacting protein 1			X			X	
NR_028338	TRAF3IP2	TRAF3 interacting protein 2	X				X	X	
NM_00111375	TYMP	thymidine phosphorylase					X	X	X
NM_003358	UGCG	UDP-glucose ceramide glucosyltransferase		X			X	X	
NM_001078	VCAM1	vascular cell adhesion molecule 1	X	X	X		X		X
NM_020830	WDFY1	WD repeat and FYVE domain containing 1							
NM_004906	WTAP	Wilms tumor 1 associated protein		X				X	
NM_017523	XAF1	XIAP associated factor 1						X	
NM_033390	ZC3H12C	zinc finger CCCH-type containing 12C							
NM_014795	ZEB2	zinc finger E-box binding homeobox 2	X	X			X	X	
NM_021035	ZNFX1	zinc finger, NFX1-type containing 1							

Tableau 5 Fin

Annexe 3: Travaux sur la décidualisation des cellules stromales

Decidualization disrupts endometrial cell receptivity to interleukin 1 family members: A new possible pathway for embryo implantation

Amélie Bourdiec, M.Sc.a

Ali Akoum, Ph.D.a

aEndocrinologie de la reproduction, Centre de recherche-Hôpital Saint-François d'Assise, Centre Hospitalier Universitaire de Québec, Faculté de médecine, Université Laval, Québec, Canada.

Reprint requests Professor Ali Akoum, Laboratoire d'Endocrinologie de la Reproduction, Centre de Recherche, Hôpital Saint-François d'Assise, 10 rue de l'Espinay, Local D0-711, Québec, Québec, Canada, G1L 3L5. Tel. 418 525-4444 (53329); Fax. 418-525-4495; e-mail: ali.akoum@crsfa.ulaval.ca

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Soumis à Americam Journal of Reproductive Immunology.

Abstract

Problem

To investigate interleukin 1 (IL1) family members' expression in endometrial stromal cells (ESCs) in response to decidualization.

Method of study

Human ESCs were decidualized in culture and the expression of IL1, IL18 and IL33 along with that of their specific receptors, inhibitors and receptors' antagonists was evaluated at the mRNA and protein levels.

Results

Decidualization differentially skewed the balance of IL1 family receptors' expression toward a pattern that increases ESC receptivity to IL1, IL18 and IL33. However, while no significant change in endogenous IL1B secretion was observed, decidualization led to an increased secretion of IL33 and a decreased secretion of IL18.

Conclusion

These findings point to a new pathway by which decidualization may contribute to the creation of a receptive endometrial phenotype and the establishment of a uterine microenvironment that is favorable to embryo implantation.

Keywords

Endometrium, decidualization, interleukin 1 family

Introduction

The embryo implantation is associated with a progressive decidualization of endometrium and a large amount of immune cells trafficking into the decidua 1. This differentiation process is dependent on the convergence of cyclic adenosine monophosphate (cAMP) and progesterone signaling pathways that drive integrated changes at both the transcriptome and the proteome level 2. As a consequence, decidualizing endometrial stromal cells (ESCs) acquire the unique ability to regulate trophoblast invasion, resist inflammatory and oxidative stress, and dampen local maternal immune responses 2. Based on the recent available evidence, the modulation of IL1 family members by early embryonic signals may contribute to the establishment of a receptive maternal endometrium and play an important role in embryo implantation 3, 4. IL1, considered as an early embryonic factor, is known for being involved in embryo implantation in many ways 3, 5, 6. It is not clear however whether the endometrium prepares itself to receive the embryo through mechanisms involving changes in the expression of IL1 family members.

Materials and methods

Patients

Endometrial tissue specimens were obtained at the mid-secretory phase of the menstrual cycle from five fertile women with regular menstrual cycles. Women were enrolled in this study after having signed a written informed consent to a research protocol approved by Laval University ethics committee on human research. Tissues were histologically dated, and ESCs were isolated and characterized according to our previously described procedure 5.

Methods

ESCs were incubated for 10 days with decidual mixt (DM) containing 2% charcoal-treated-FBS (Invitrogen Life Technologies, Burlington, ON, Canada), 8-bromo-cAMP (cAMP; 0.5 mM) and progestin MPA (10-6 M) (Sigma-Aldrich Corp., St. Louis, MO). Following incubation with DM devoid of charcoal-treated-FBS for 2 days, IL1 family member's expression was investigated by quantitative real time PCR (q-RT-PCR) an ELISA in both cellular protein extracts and supernatants. Total RNA was extracted with TRIzol™ (Invitrogen), reverse transcribed and analyzed by qRT-PCR according to our previously reported procedure 3. All primers were designed with Primer ExpressTM, version 2.0 (Applied Biosystem, Foster City, CA, USA), spanned intron-exon boundaries to avoid amplification of genomic DNA and were selected for Tm value compatibility (56-61°C). For each experimental sample, IL1R1, IL1R2, IL1RN, IL18R1, and ST2 mRNA levels were normalized to GAPDH mRNA levels. IL1R1, IL1R2 and IL1RN ELISA were performed as previously described 3, 7, prolactin (PRL), IL33 and ST2 were measured using DuoSet kit (DuoSet, R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. IL18 and IL18R1 ELISAs were homemade using monoclonal anti-human IL18 antibody and monoclonal biotin-labeled anti-human IL18 (D044-3; D045-6 R&D Systems, Minneapolis, MN), mouse anti-human IL18R1 monoclonal and polyclonal anti-human IL18R1 (MAB840; AF-840 R&D Systems, Minneapolis, MN).

Results and Discussion

Secreted factors, such as PRL, are widely used to assess the quality of ESC decidual response 8. The decidual phenotype of ESCs was assessed by the release of PRL in conditioned media. A significant increase of PRL ($P<0.001$) was detected in the culture supernatant of ESCs treated with cAMP and MPA (515.5 ± 90.7 pg/mL)

compared to control ESCs incubated with the basic culture medium (88.19 ± 5.5 pg/mL).

Our study showed that the decidualization process affects IL1Rs' expression at both the mRNA and the protein levels. Indeed, IL1R1 mRNA and protein expression levels were significantly increased (Fig.1A and B) in decidualized compared to nondecidualized control ESCs ($P<0.001$ and $P<0.05$ respectively). IL1R1 is the functional receptor of IL1. Upon IL1 binding, IL1R1 can activate signal transduction with the recruitment of a constitutively expressed second subunit termed IL1R accessory protein (IL1RACP)9, 10. Members of the IL1 system also include negative regulators that block the activity of IL1B. The IL1R2 functions as a decoy receptor that binds to IL1B, but does not trigger cell signaling. The soluble form of IL1R2 (sIL1R2) also binds to IL1B and neutralizes its activity 11, 12. The antagonist member IL1RN binds tightly to IL1R1 and prevents IL1 signal transduction 13. Our results showed that IL1 natural inhibitors were upregulated in decidualized ESCs in vitro. IL1R2 mRNA (Fig.1C), membrane-bound form (m) (Fig.1D) and soluble protein (Fig.1E) were significantly upregulated ($P<0.001$, $P<0.05$ and $P<0.05$ respectively). Similar expression patterns for IL1RN were observed at the mRNA level (Fig.1F, $P<0.05$) and the protein level for the intra-cellular form (Fig.1G, $P<0.05$). However, IL1RN secreted form (Fig.1H) did not show noticeable differences. IL1B secretion did not show any significant change (Fig.1I) compared to control ESCs, but the ratio of IL1R1 to IL1R2 (10.3, $P<0.01$) or to ILRN (4.9, $P<0.05$) was significantly increased, thereby suggesting an enhanced sensitivity of decidual ESCs to IL1. Considering the link between IL1 signaling and angiogenesis, cell proliferation, tissue remodeling, inflammation and immunity, increased cell sensitivity to this cytokine may contribute to the creation of an uterine microenvironment that favors embryo implantation 3, 14.

IL18 and IL33 are other members of IL1 family that share similar properties. IL18 signals via IL18R1 and an IL18R accessory protein (IL18RAP). Negative regulators such as IL18BP and soluble IL18R1 antagonize IL18 signaling 15, 16. ST2 was recently identified as a receptor for IL33 17. IL1RACP is required for IL33-mediated signal transduction and soluble ST2 form binds to IL33, thereby regulating its biological activity 18. The decidual process in ESCs distinctly targeted the IL18 and IL33 systems. IL18R1 mRNA (Fig.1J) and protein expression levels (Fig.1K) were significantly upregulated in decidualized ESCs ($P<0.05$), whereas sIL18R1 (Fig.1L) and IL18 secretion (Fig.1M) were significantly downregulated ($P<0.05$ and $P<0.01$ respectively). These results are relevant to normal early pregnancy and consistent with the dual role of IL18 at the embryo-maternal interface in normal pregnancy 19. Actually, IL18 is required for sustained angiogenesis in the maternal endometrium 19, but overexpression of IL18 may contribute to increased local recruitment and activation of natural killer (NK) cells and implantation failure 20. Unlike ST2 soluble form (Fig.1P), ST2 was upregulated in decidualized ESCs (Fig.1 N and O; $P<0.05$ and $P<0.01$ respectively). These results suggest an imbalance that favors IL33-mediated ESC activation. The implication of the IL33 system in the decidual process and embryo implantation was recently described 3, 20. Our results corroborate the fact that IL33 secretion is increased in decidual ESCs (Fig.1Q $P<0.05$) and further provide evidence supporting the role of the ST2-IL33 axis in the process of endometrial preparation for embryonic implantation.

In conclusion, the current study showed that endometrial stromal decidualization leads to a differential regulation of IL1 family members and creates an imbalance that may play an important role in the regulation of endometrial receptivity and the establishment of an uterine environment that is favorable to imminent embryo-maternal crosstalk and embryo implantation.

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Conflict of interest

The authors have no financial conflicts of interest.

References

1 Le Bouteiller P, Tabiasco J: [Immunology of pregnancy: renewed interest]. Medecine sciences : M/S 2006;22:745-750.

2 Gellersen B, Brosens IA, Brosens JJ: Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Semin Reprod Med 2007;25:445-453.

3 Bourdiec A, Calvo E, Rao CV, Akoum A: Transcriptome analysis reveals new insights into the modulation of endometrial stromal cell receptive phenotype by embryo-derived signals interleukin-1 and human chorionic gonadotropin: possible involvement in early embryo implantation. PLoS One 2013;8:e64829.

4 Salker MS, Nautiyal J, Steel JH, Webster Z, Sucurovic S, Nicou M, Singh Y, Lucas ES, Murakami K, Chan YW, James S, Abdallah Y, Christian M, Croy BA, Mulac-Jericevic B, Quenby S, Brosens JJ: Disordered IL-33/ST2 activation in decidualizing

stromal cells prolongs uterine receptivity in women with recurrent pregnancy loss. PloS one 2012;7:e52252.

5 Bourdiec A, Shao R, Rao CV, Akoum A: Human chorionic gonadotropin triggers angiogenesis via the modulation of endometrial stromal cell responsiveness to interleukin 1: a new possible mechanism underlying embryo implantation. Biol Reprod 2012;87:66.

6 Baranao RI, Piazza A, Rumi LS, Polak de Fried E: Determination of IL-1 and IL-6 levels in human embryo culture-conditioned media. Am J Reprod Immunol 1997;37:191-194.

7 Bourdiec A, Bedard D, Rao CV, Akoum A: Human chorionic gonadotropin regulates endothelial cell responsiveness to interleukin 1 and amplifies the cytokine-mediated effect on cell proliferation, migration and the release of angiogenic factors. Am J Reprod Immunol 2013;70:127-138.

8 Al-Sabbagh M, Fusi L, Higham J, Lee Y, Lei K, Hanyaloglu AC, Lam EW, Christian M, Brosens JJ: NADPH oxidase-derived reactive oxygen species mediate decidualization of human endometrial stromal cells in response to cyclic AMP signaling. Endocrinology 2011;152:730-740.

9 Bigonnesse F, Marois M, Maheux R, Akoum A: Interleukin-1 receptor accessory protein is constitutively expressed in human endometrium throughout the menstrual cycle. Mol Hum Reprod 2001;7:333-339.

10 Greenfeder SA, Nunes P, Kwee L, Labow M, Chizzonite RA, Ju G: Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. The Journal of biological chemistry 1995;270:13757-13765.

11 Colotta F, Re F, Muzio M, Bertini R, Polentarutti N, Sironi M, Giri JG, Dower SK, Sims JE, Mantovani A: Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4. Science 1993;261:472-475.

12 Economides AN, Carpenter LR, Rudge JS, Wong V, Koehler-Stec EM, Hartnett C, Pyles EA, Xu X, Daly TJ, Young MR, Fandl JP, Lee F, Carver S, McNay J, Bailey K, Ramakanth S, Hutabarat R, Huang TT, Radziejewski C, Yancopoulos GD, Stahl N: Cytokine traps: multi-component, high-affinity blockers of cytokine action. Nature medicine 2003;9:47-52.

13 Dinarello CA: Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol 2009;27:519-550.

14 Dekel N, Gnainsky Y, Granot I, Mor G: Inflammation and implantation. Am J Reprod Immunol 2010;63:17-21.

15 Dinarello CA, Novick D, Kim S, Kaplanski G: Interleukin-18 and IL-18 Binding Protein. Front Immunol 2013;4:289.

16 Takei S, Hoshino T, Matsunaga K, Sakazaki Y, Sawada M, Oda H, Takenaka S, Imaoka H, Kinoshita T, Honda S, Ida H, Fukuda TA, Aizawa H: Soluble interleukin-18 receptor complex is a novel biomarker in rheumatoid arthritis. Arthritis Res Ther 2011;13:R52.

17 Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, Zurawski G, Moshrefi M, Qin J, Li X, Gorman DM, Bazan JF, Kastelein RA: IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity 2005;23:479-490.

18 Chackerian AA, Oldham ER, Murphy EE, Schmitz J, Pflanz S, Kastelein RA: IL-1 receptor accessory protein and ST2 comprise the IL-33 receptor complex. J Immunol 2007;179:2551-2555.

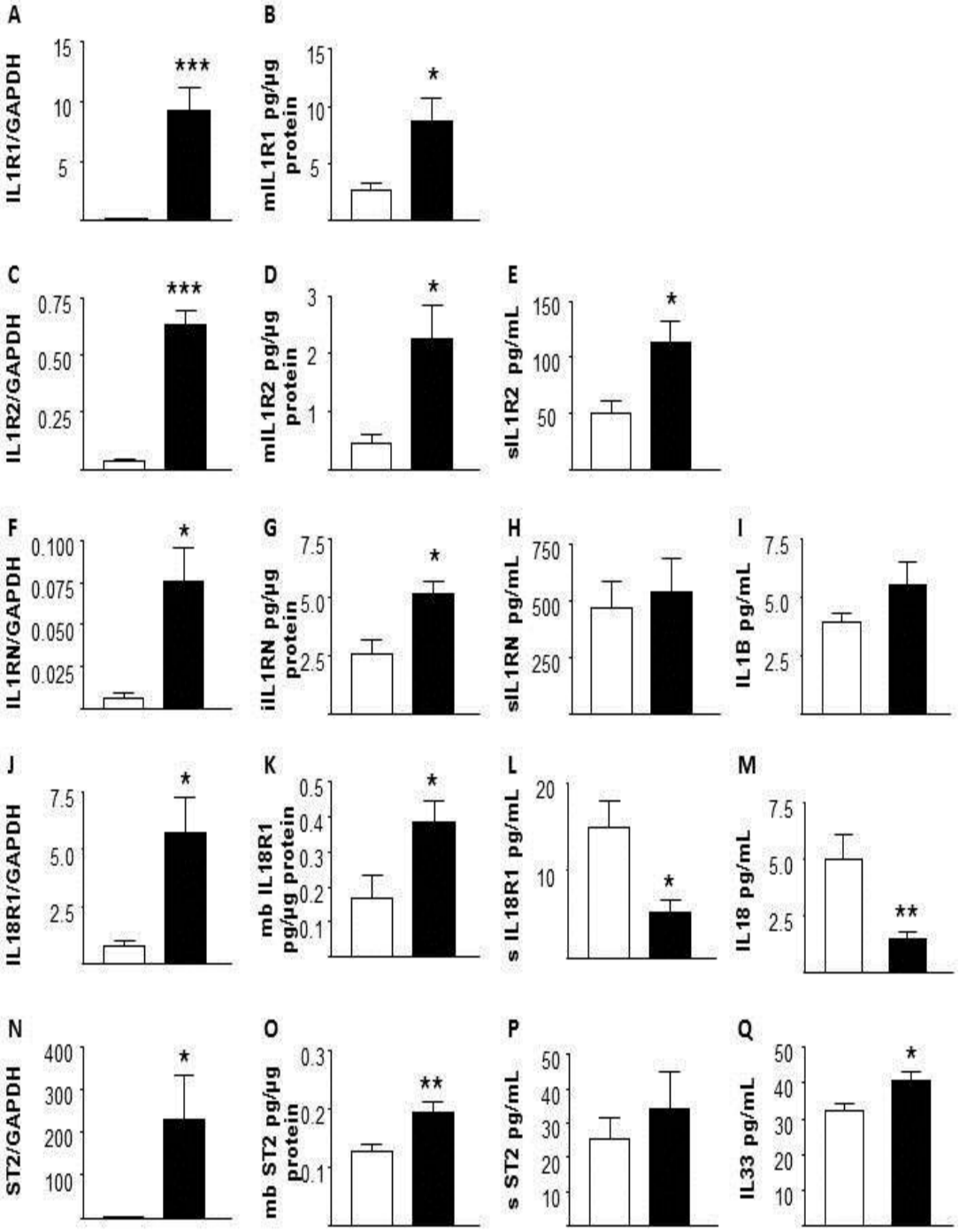
19 Ledee N, Chaouat G, Serazin V, Lombroso R, Dubanchet S, Oger P, Louafi N, Ville Y: Endometrial vascularity by three-dimensional power Doppler ultrasound and cytokines: a complementary approach to assess uterine receptivity. J Reprod Immunol 2008;77:57-62.

20 Ledee-Bataille N, Dubanchet S, Coulomb-L'hermine A, Durand-Gasselin I, Frydman R, Chaouat G: A new role for natural killer cells, interleukin (IL)-12, and IL-18 in repeated implantation failure after in vitro fertilization. Fertility and sterility 2004;81:59-65.

Legend

Fig. 1 Effect of ECS decidualization on the expression of IL1 family members. Statistical analysis was performed with GraphPad Software Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA). As data followed a normal distribution, the Student's t-test was used to compare decidualized (black column) and control (white column) ESCs.

Figure


A
IL1R1/GAPDH
15
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B
mIL1R1 pg/µg protein
15
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C
IL1R2/GAPDH
0.75
0.50
0.25
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mIL1R2 pg/µg protein
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sIL1R2 pg/mL
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IL1RN/GAPDH
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Bibliographie

Abdallah, M. A., Z. M. Lei, et al. (2004). "Human fetal nongonadal tissues contain human chorionic gonadotropin/luteinizing hormone receptors." J Clin Endocrinol Metab **89**(2): 952-956.

Abrahamsohn, P. A. and T. M. Zorn (1993). "Implantation and decidualization in rodents." J Exp Zool **266**(6): 603-628.

Acevedo, H. F. and R. J. Hartsock (1996). "Metastatic phenotype correlates with high expression of membrane-associated complete beta-human chorionic gonadotropin in vivo." Cancer **78**(11): 2388-2399.

Acevedo, H. F., J. Y. Tong, et al. (1995). "Human chorionic gonadotropin-beta subunit gene expression in cultured human fetal and cancer cells of different types and origins." Cancer **76**(8): 1467-1475.

Addison, C. L., T. O. Daniel, et al. (2000). "The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity." J Immunol **165**(9): 5269-5277.

Akoum, A., C. N. Metz, et al. (2005). "Marked increase in macrophage migration inhibitory factor synthesis and secretion in human endometrial cells in response to human chorionic gonadotropin hormone." J Clin Endocrinol Metab **90**(5): 2904-2910.

Altun, T., S. Jindal, et al. (2011). "Low follicular fluid IL-6 levels in IVF patients are associated with increased likelihood of clinical pregnancy." J Assist Reprod Genet **28**(3): 245-251.

Ascoli, M., F. Fanelli, et al. (2002). "The lutropin/choriogonadotropin receptor, a 2002 perspective." Endocr Rev **23**(2): 141-174.

Baranao, R. I., A. Piazza, et al. (1997). "Determination of IL-1 and IL-6 levels in human embryo culture-conditioned media." Am J Reprod Immunol **37**(2): 191-194.

Barry, M. R., S (2005). Embryologie. Elsevier. Paris.

Bavister, B. D. (1995). "Culture of preimplantation embryos: facts and artifacts." Hum Reprod Update **1**(2): 91-148.

Bazan, J. F., J. C. Timans, et al. (1996). "A newly defined interleukin-1?" Nature **379**(6566): 591.

Beckman, C. R. B. (2013). Obstetrics and Gynecology-7th ed.

Bellehumeur, C., J. Blanchet, et al. (2009). "Interleukin 1 regulates its own receptors in human endometrial cells via distinct mechanisms." Hum Reprod **24**(9): 2193-2204.

Bellisario, R., R. B. Carlsen, et al. (1973). "Human chorionic gonadotropin. Linear amino acid sequence of the alpha subunit." J Biol Chem **248**(19): 6796-6809.

Berferon, C. (2006). Histologie et physiologie de l'endomètre.

Bernardini, G., D. Ribatti, et al. (2003). "Analysis of the role of chemokines in angiogenesis." J Immunol Methods **273**(1-2): 83-101.

Berndt, S., S. Blacher, et al. (2009). "Chorionic gonadotropin stimulation of angiogenesis and pericyte recruitment." J Clin Endocrinol Metab **94**(11): 4567-4574.

Bigonnesse, F., Y. Labelle, et al. (2001). "Triphasic expression of interleukin-1 receptor type I in human endometrium throughout the menstrual cycle of fertile women and women with unexplained infertility." Fertil Steril **75**(1): 79-87.

Billington, W. D. (2003). "The immunological problem of pregnancy: 50 years with the hope of progress. A tribute to Peter Medawar." J Reprod Immunol **60**(1): 1-11.

Birkedal-Hansen, H., W. G. Moore, et al. (1993). "Matrix metalloproteinases: a review." Critical reviews in oral biology and medicine : an official publication of the American Association of Oral Biologists **4**(2): 197-250.

Birken, S., Y. Maydelman, et al. (1996). "Isolation and characterization of human pituitary chorionic gonadotropin." Endocrinology **137**(4): 1402-1411.

Bischof, P. and A. Campana (2000). "Molecular mediators of implantation." Baillieres Best Pract Res Clin Obstet Gynaecol **14**(5): 801-814.

Blois, S. M., U. Kammerer, et al. (2007). "Dendritic cells: key to fetal tolerance?" Biol Reprod **77**(4): 590-598.

Boomsma, C. M., A. Kavelaars, et al. (2009). "Endometrial secretion analysis identifies a cytokine profile predictive of pregnancy in IVF." Hum Reprod **24**(6): 1427-1435.

Boucher, A., A. Kharfi, et al. (2001). "Cycle-dependent expression of interleukin-1 receptor type II in the human endometrium." Biol Reprod **65**(3): 890-898.

Butler, G. S., S. S. Apte, et al. (1999). "Human tissue inhibitor of metalloproteinases 3 interacts with both the N- and C-terminal domains of gelatinases A and B. Regulation by polyanions." The Journal of biological chemistry **274**(16): 10846-10851.

Butler, S. A., M. S. Ikram, et al. (2000). "The increase in bladder carcinoma cell population induced by the free beta subunit of human chorionic gonadotrophin is a result of an anti-apoptosis effect and not cell proliferation." Br J Cancer **82**(9): 1553-1556.

Carli, C., C. N. Metz, et al. (2009). "Up-regulation of cyclooxygenase-2 expression and prostaglandin E2 production in human endometriotic cells by macrophage migration inhibitory factor: involvement of novel kinase signaling pathways." Endocrinology **150**(7): 3128-3137.

Carlsen, R. B., O. P. Bahl, et al. (1973). "Human chorionic gonadotropin. Linear amino acid sequence of the beta subunit." J Biol Chem **248**(19): 6810-6827.

Carriere, V., L. Roussel, et al. (2007). "IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo." Proc Natl Acad Sci U S A **104**(1): 282-287.

Carson, D. D., I. Bagchi, et al. (2000). "Embryo implantation." Dev Biol **223**(2): 217-237.

Chaouat, G., N. Ledee-bataille, et al. (2003). "Implantation: can immunological parameters of implantation failure be of interest for pre-eclampsia?" J Reprod Immunol **59**(2): 205-217.

Chaouat, G., N. Ledee-Bataille, et al. (2003). "Cytokines, implantation and early abortion: re-examining the Th1/Th2 paradigm leads to question the single pathway, single therapy concept." Am J Reprod Immunol **50**(3): 177-186.

Chaouat, G., J. Tranchot Diallo, et al. (1997). "Immune suppression and Th1/Th2 balance in pregnancy revisited: a (very) personal tribute to Tom Wegmann." Am J Reprod Immunol **37**(6): 427-434.

Chard, T. (1995). "Cytokines in implantation." Hum Reprod Update **1**(4): 385-396.

Cohen, M., A. Meisser, et al. (2006). "Metalloproteinases and human placental invasiveness." Placenta **27**(8): 783-793.

Cole, L. A. (2009). "New discoveries on the biology and detection of human chorionic gonadotropin." Reprod Biol Endocrinol **7**: 8.

Cole, L. A. (2010). "Biological functions of hCG and hCG-related molecules." Reprod Biol Endocrinol **8**: 102.

Cole, L. A., S. A. Butler, et al. (2006). "Gestational trophoblastic diseases: 2. Hyperglycosylated hCG as a reliable marker of active neoplasia." Gynecol Oncol **102**(2): 151-159.

Cole, L. A., D. Dai, et al. (2006). "Gestational trophoblastic diseases: 1. Pathophysiology of hyperglycosylated hCG." Gynecol Oncol **102**(2): 145-150.

Cole, L. A. and J. M. Gutierrez (2009). "Production of human chorionic gonadotropin during the normal menstrual cycle." J Reprod Med **54**(4): 245-250.

Cole, L. A., A. Tanaka, et al. (1996). "Beta-core fragment (beta-core/UGF/UGP), a tumor marker: a 7-year report." Gynecol Oncol **60**(2): 264-270.

Colotta, F., F. Re, et al. (1993). "Interleukin-1 type II receptor: a decoy target for IL-1 that is regulated by IL-4." Science **261**(5120): 472-475.

Cunningham F.G., L. K. L., Bloom S.L., Hauth J.C., Gilstrap L.C., Wenstrom K.D. (2005). Williams Obstetrics.

Curry, T. E., Jr. and K. G. Osteen (2003). "The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle." Endocrine reviews **24**(4): 428-465.

Dallenbach-Hellweg, G. S., D; Dallenbach, F (2010). Atlas of Endometrial Histopathology. Springer.

Damario, M. A., T. G. Lesnick, et al. (2001). "Endometrial markers of uterine receptivity utilizing the donor oocyte model." Hum Reprod **16**(9): 1893-1899.

Dame, J. B. and S. E. Juul (2000). "The distribution of receptors for the pro-inflammatory cytokines interleukin (IL)-6 and IL-8 in the developing human fetus." Early Hum Dev **58**(1): 25-39.

Damsky, C. H., C. Librach, et al. (1994). "Integrin switching regulates normal trophoblast invasion." Development **120**(12): 3657-3666.

De Brux, J., Ed. (1971). Histopathologie gynécologique. Paris.

Deb, K., J. Reese, et al. (2006). "Methodologies to study implantation in mice." Methods Mol Med **121**: 9-34.

Debets, R., J. C. Timans, et al. (2001). "Two novel IL-1 family members, IL-1 delta and IL-1 epsilon, function as an antagonist and agonist of NF-kappa B activation through the orphan IL-1 receptor-related protein 2." Journal of immunology **167**(3): 1440-1446.

Dinarello, C. A. (2009). "Immunological and inflammatory functions of the interleukin-1 family." Annu Rev Immunol **27**: 519-550.

Dinarello, C. A. (2011). "Interleukin-1 in the pathogenesis and treatment of inflammatory diseases." Blood **117**(14): 3720-3732.

Dinarello, C. A., T. Ikejima, et al. (1987). "Interleukin 1 induces interleukin 1. I. Induction of circulating interleukin 1 in rabbits in vivo and in human mononuclear cells in vitro." J Immunol **139**(6): 1902-1910.

Doheny, H. C., D. D. Houlihan, et al. (2003). "Human chorionic gonadotrophin relaxation of human pregnant myometrium and activation of the BKCa channel." J Clin Endocrinol Metab **88**(9): 4310-4315.

Dufau, M. L. (1998). "The luteinizing hormone receptor." Annu Rev Physiol **60**: 461-496.

Eblen, A., S. Bao, et al. (2001). "The presence of functional luteinizing hormone/chorionic gonadotropin receptors in human sperm." J Clin Endocrinol Metab **86**(6): 2643-2648.

Ebner, T., M. Moser, et al. (2003). "Selection based on morphological assessment of oocytes and embryos at different stages of preimplantation development: a review." Hum Reprod Update **9**(3): 251-262.

Eta, E., G. Ambrus, et al. (1994). "Direct regulation of human myometrial contractions by human chorionic gonadotropin." J Clin Endocrinol Metab **79**(6): 1582-1586.

Fahey, J. V., T. M. Schaefer, et al. (2005). "Secretion of cytokines and chemokines by polarized human epithelial cells from the female reproductive tract." Hum Reprod **20**(6): 1439-1446.

Fazleabas, A. T., J. J. Kim, et al. (2004). "Implantation: embryonic signals and the modulation of the uterine environment--a review." Placenta **25 Suppl A**: S26-31.

Fluhr, H., D. Bischof-Islami, et al. (2008). "Human chorionic gonadotropin stimulates matrix metalloproteinases-2 and -9 in cytotrophoblastic cells and decreases tissue inhibitor of metalloproteinases-1, -2, and -3 in decidualized endometrial stromal cells." Fertil Steril **90**(4 Suppl): 1390-1395.

Fluhr, H., G. Sauter, et al. (2009). "Nonapoptotic effects of tumor necrosis factor-related apoptosis-inducing ligand on interleukin-6, leukemia inhibitory factor, interleukin-8, and monocyte chemoattractant protein 1 vary between undifferentiated and decidualized human endometrial stromal cells." Fertil Steril **92**(4): 1420-1423.

Franco, S. J. and A. Huttenlocher (2005). "Regulating cell migration: calpains make the cut." J Cell Sci **118**(Pt 17): 3829-3838.

Gaestel, M., A. Kotlyarov, et al. (2009). "Targeting innate immunity protein kinase signalling in inflammation." Nat Rev Drug Discov **8**(6): 480-499.

Gargett, C. E. and P. A. Rogers (2001). "Human endometrial angiogenesis." Reproduction **121**(2): 181-186.

Gellersen, B., I. A. Brosens, et al. (2007). "Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives." Semin Reprod Med **25**(6): 445-453.

Gellersen, B. and J. Brosens (2003). "Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair." The Journal of endocrinology **178**(3): 357-372.

Gellersen, B. and J. Brosens (2003). "Cyclic AMP and progesterone receptor cross-talk in human endometrium: a decidualizing affair." J Endocrinol **178**(3): 357-372.

Goldsmith, P. C., W. G. McGregor, et al. (1983). "Cellular localization of chorionic gonadotropin in human fetal kidney and liver." J Clin Endocrinol Metab **57**(3): 654-661.

Granowitz, E. V., B. D. Clark, et al. (1992). "Effect of interleukin-1 (IL-1) blockade on cytokine synthesis: I. IL-1 receptor antagonist inhibits IL-1-induced cytokine synthesis and blocks the binding of IL-1 to its type II receptor on human monocytes." Blood **79**(9): 2356-2363.

Greenfeder, S. A., P. Nunes, et al. (1995). "Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex." J Biol Chem **270**(23): 13757-13765.

Haig, D. (1993). "Genetic conflicts in human pregnancy." Q Rev Biol **68**(4): 495-532.

Hamada, A. L., K. Nakabayashi, et al. (2005). "Transfection of antisense chorionic gonadotropin beta gene into choriocarcinoma cells suppresses the cell proliferation and induces apoptosis." J Clin Endocrinol Metab **90**(8): 4873-4879.

Hampton, A. L. and L. A. Salamonsen (1994). "Expression of messenger ribonucleic acid encoding matrix metalloproteinases and their tissue inhibitors is related to menstruation." The Journal of endocrinology **141**(1): R1-3.

Herrmann-Lavoie, C., C. V. Rao, et al. (2007). "Chorionic gonadotropin down-regulates the expression of the decoy inhibitory interleukin 1 receptor type II in human endometrial epithelial cells." Endocrinology **148**(11): 5377-5384.

Hess, A. P., A. E. Hamilton, et al. (2007). "Decidual stromal cell response to paracrine signals from the trophoblast: amplification of immune and angiogenic modulators." Biol Reprod **76**(1): 102-117.

Hitschmann, F. a. A., L (1908). "Der bau der uterusschleimhaut des geschlechtsreifen weibes mit besonderer berucksichtigung der menstruation." Monatsschr Geburtshilfe Gynaekol **27**: 1–82.

Hoffmann, E., O. Dittrich-Breiholz, et al. (2002). "Multiple control of interleukin-8 gene expression." J Leukoc Biol **72**(5): 847-855.

Hornung, D., F. Bentzien, et al. (2001). "Chemokine bioactivity of RANTES in endometriotic and normal endometrial stromal cells and peritoneal fluid." Molecular human reproduction **7**(2): 163-168.

Hou, Q., B. C. Paria, et al. (1996). "Immunolocalization of estrogen receptor protein in the mouse blastocyst during normal and delayed implantation." Proc Natl Acad Sci U S A **93**(6): 2376-2381.

Huang, S. J., F. Schatz, et al. (2006). "Regulation of chemokine production in response to pro-inflammatory cytokines in first trimester decidual cells." Journal of reproductive immunology **72**(1-2): 60-73.

Hulboy, D. L., L. A. Rudolph, et al. (1997). "Matrix metalloproteinases as mediators of reproductive function." Molecular human reproduction **3**(1): 27-45.

Juan Felipe Velez de la Calle, N. C. (2012). "Prolonged culture: results for embryo co-culture on human endometrial cells." MT / médecine de la reproduction, gynécologie et endocrinologie **14**.

Jurisicova, A., M. Antenos, et al. (1999). "Variability in the expression of trophectodermal markers beta-human chorionic gonadotrophin, human leukocyte antigen-G and pregnancy specific beta-1 glycoprotein by the human blastocyst." Hum Reprod **14**(7): 1852-1858.

Kalluri, R. and R. A. Weinberg (2009). "The basics of epithelial-mesenchymal transition." J Clin Invest **119**(6): 1420-1428.

Kamijo, T., M. R. Rajabi, et al. (1998). "Biochemical evidence for autocrine/paracrine regulation of apoptosis in cultured uterine epithelial cells during mouse embryo implantation in vitro." Mol Hum Reprod **4**(10): 990-998.

Kane, N., R. Kelly, et al. (2009). "Proliferation of uterine natural killer cells is induced by human chorionic gonadotropin and mediated via the mannose receptor." Endocrinology **150**(6): 2882-2888.

Kardana, A. and L. A. Cole (1994). "Human chorionic gonadotropin beta-subunit nicking enzymes in pregnancy and cancer patient serum." J Clin Endocrinol Metab **79**(3): 761-767.

Keeley, E. C., B. Mehrad, et al. (2008). "Chemokines as mediators of neovascularization." Arterioscler Thromb Vasc Biol **28**(11): 1928-1936.

Kovalevskaya, G., O. Genbacev, et al. (2002). "Trophoblast origin of hCG isoforms: cytotrophoblasts are the primary source of choriocarcinoma-like hCG." Mol Cell Endocrinol **194**(1-2): 147-155.

Krussel, J. S., P. Bielfeld, et al. (2003). "Regulation of embryonic implantation." Eur J Obstet Gynecol Reprod Biol **110 Suppl 1**: S2-9.

Kwak-Kim, J. and A. Gilman-Sachs (2008). "Clinical implication of natural killer cells and reproduction." Am J Reprod Immunol **59**(5): 388-400.

Le Bouteiller, P. and J. Tabiasco (2006). "Killers become builders during pregnancy." Nat Med **12**(9): 991-992.

Ledee-Bataille, N., K. Bonnet-Chea, et al. (2005). "Role of the endometrial tripod interleukin-18, -15, and -12 in inadequate uterine receptivity in patients with a history of repeated in vitro fertilization-embryo transfer failure." Fertil Steril **83**(3): 598-605.

Ledee-Bataille, N., G. Lapree-Delage, et al. (2002). "Concentration of leukaemia inhibitory factor (LIF) in uterine flushing fluid is highly predictive of embryo implantation." Hum Reprod **17**(1): 213-218.

Ledee-Bataille, N., F. Olivennes, et al. (2004). "Detectable levels of interleukin-18 in uterine luminal secretions at oocyte retrieval predict failure of the embryo transfer." Hum Reprod **19**(9): 1968-1973.

Lei, Z. M., C. V. Rao, et al. (1993). "Novel expression of human chorionic gonadotropin/luteinizing hormone receptor gene in brain." Endocrinology **132**(5): 2262-2270.

Lei, Z. M., E. Reshef, et al. (1992). "The expression of human chorionic gonadotropin/luteinizing hormone receptors in human endometrial and myometrial blood vessels." J Clin Endocrinol Metab **75**(2): 651-659.

Lei, Z. M., P. Toth, et al. (1993). "Novel coexpression of human chorionic gonadotropin (hCG)/human luteinizing hormone receptors and their ligand hCG in human fallopian tubes." J Clin Endocrinol Metab **77**(3): 863-872.

Loke, Y. W., A. King, et al. (1995). "Decidua in human implantation." Hum Reprod **10 Suppl 2**: 14-21.

Lopata, A. and D. L. Hay (1989). "The potential of early human embryos to form blastocysts, hatch from their zona and secrete HCG in culture." Hum Reprod **4**(8 Suppl): 87-94.

Lund, A. W., F. V. Duraes, et al. (2012). "VEGF-C promotes immune tolerance in B16 melanomas and cross-presentation of tumor antigen by lymph node lymphatics." Cell Rep **1**(3): 191-199.

Majumdar, S., B. C. Bapna, et al. (1982). "Pregnancy specific proteins: suppression of in vitro blastogenic response to mitogen by these proteins." Int J Fertil **27**(2): 66-69.

Martins-Green, M. and H. Hanafusa (1997). "The 9E3/CEF4 gene and its product the chicken chemotactic and angiogenic factor (cCAF): potential roles in wound healing and tumor development." Cytokine Growth Factor Rev **8**(3): 221-232.

Matzinger, P. (2002). "The danger model: a renewed sense of self." Science **296**(5566): 301-305.

Mazur, M. T. K., R .J (2005). Diagnosis of endometrial biopsies and curettings: a practical approach c. New York : Springer.

McIntush, E. W. and M. F. Smith (1998). "Matrix metalloproteinases and tissue inhibitors of metalloproteinases in ovarian function." Reviews of reproduction **3**(1): 23-30.

McMaster, M. T., R. C. Newton, et al. (1992). "Activation and distribution of inflammatory cells in the mouse uterus during the preimplantation period." J Immunol **148**(6): 1699-1705.

Medawar, P. B. (1953). "Some immunological and endocrinological problems raised by the evolution of viviparity in vertebrate." Symp Soc Exp Biol **7**: 320-338.

Meseguer, M., J. D. Aplin, et al. (2001). "Human endometrial mucin MUC1 is up-regulated by progesterone and down-regulated in vitro by the human blastocyst." Biol Reprod **64**(2): 590-601.

Milne, S. A., H. O. Critchley, et al. (1999). "Perivascular interleukin-8 messenger ribonucleic acid expression in human endometrium varies across the menstrual cycle and in early pregnancy decidua." J Clin Endocrinol Metab **84**(7): 2563-2567.

Moffett, A. and C. Loke (2006). "Immunology of placentation in eutherian mammals." Nat Rev Immunol **6**(8): 584-594.

Morgan, F. J., S. Birken, et al. (1975). "The amino acid sequence of human chorionic gonadotropin. The alpha subunit and beta subunit." J Biol Chem **250**(13): 5247-5258.

Morrish, D. W., H. Marusyk, et al. (1987). "Demonstration of specific secretory granules for human chorionic gonadotropin in placenta." J Histochem Cytochem **35**(1): 93-101.

Moyle, W. R., M. M. Matzuk, et al. (1990). "Localization of residues that confer antibody binding specificity using human chorionic gonadotropin/luteinizing hormone beta subunit chimeras and mutants." J Biol Chem **265**(15): 8511-8518.

Muller, C. Y. and L. A. Cole (2009). "The quagmire of hCG and hCG testing in gynecologic oncology." Gynecol Oncol **112**(3): 663-672.

Naldini, A. and F. Carraro (2005). "Role of inflammatory mediators in angiogenesis." Curr Drug Targets Inflamm Allergy **4**(1): 3-8.

Noonan, F. P., W. J. Halliday, et al. (1979). "Early pregnancy factor is immunosuppressive." Nature **278**(5705): 649-651.

Novick, D., S. H. Kim, et al. (1999). "Interleukin-18 binding protein: a novel modulator of the Th1 cytokine response." Immunity **10**(1): 127-136.

Noyes, R. (1950). "Dating the endometrial biopsy." Fertil Steril **1**: 3-25.

Otrock, Z. K., R. A. Mahfouz, et al. (2007). "Understanding the biology of angiogenesis: review of the most important molecular mechanisms." Blood Cells Mol Dis **39**(2): 212-220.

Papetti, M. and I. M. Herman (2002). "Mechanisms of normal and tumor-derived angiogenesis." Am J Physiol Cell Physiol **282**(5): C947-970.

Paria, B. C., Y. M. Huet-Hudson, et al. (1993). "Blastocyst's state of activity determines the "window" of implantation in the receptive mouse uterus." Proc Natl Acad Sci U S A **90**(21): 10159-10162.

Paria, B. C., J. Reese, et al. (2002). "Deciphering the cross-talk of implantation: advances and challenges." Science **296**(5576): 2185-2188.

Paulesu, L., S. Jantra, et al. (2008). "Interleukin-1 in reproductive strategies." Evol Dev **10**(6): 778-788.

Perrier d'Hauterive, S., S. Berndt, et al. (2007). "Dialogue between blastocyst hCG and endometrial LH/hCG receptor: which role in implantation?" Gynecol Obstet Invest **64**(3): 156-160.

Psychoyos, A. (1973). "Hormonal control of ovoimplantation." Vitam Horm **31**: 201-256.

Ramhorst, R., G. Gutierrez, et al. (2007). "Implication of RANTES in the modulation of alloimmune response by progesterone during pregnancy." American journal of reproductive immunology **57**(2): 147-152.

Ramhorst, R., R. Patel, et al. (2006). "Induction of maternal tolerance to fetal alloantigens by RANTES production." American journal of reproductive immunology **56**(5-6): 302-311.

Rao, C. V. and Z. M. Lei (2007). "The past, present and future of nongonadal LH/hCG actions in reproductive biology and medicine." Mol Cell Endocrinol **269**(1-2): 2-8.

Rao, C. V., X. Li, et al. (1993). "Novel expression of functional human chorionic gonadotropin/luteinizing hormone receptor gene in human umbilical cords." J Clin Endocrinol Metab **77**(6): 1706-1714.

Ray, P. F., N. Gigarel, et al. (2000). "First specific preimplantation genetic diagnosis for ornithine transcarbamylase deficiency." Prenatal diagnosis **20**(13): 1048-1054.

Reisinger, K., N. Baal, et al. (2007). "The gonadotropins: tissue-specific angiogenic factors?" Mol Cell Endocrinol **269**(1-2): 65-80.

Reshef, E., Z. M. Lei, et al. (1990). "The presence of gonadotropin receptors in nonpregnant human uterus, human placenta, fetal membranes, and decidua." J Clin Endocrinol Metab **70**(2): 421-430.

Reznikov, L. L., J. Waksman, et al. (2004). "Effect of advanced glycation end products on endotoxin-induced TNF-alpha, IL-1beta and IL-8 in human peripheral blood mononuclear cells." Clin Nephrol **61**(5): 324-336.

Romagnani, P., F. Annunziato, et al. (2001). "Cell cycle-dependent expression of CXC chemokine receptor 3 by endothelial cells mediates angiostatic activity." J Clin Invest **107**(1): 53-63.

Rosenkilde, M. M. and T. W. Schwartz (2004). "The chemokine system -- a major regulator of angiogenesis in health and disease." Apmis **112**(7-8): 481-495.

Rossi, D. and A. Zlotnik (2000). "The biology of chemokines and their receptors." Annu Rev Immunol **18**: 217-242.

Rossi, M., A. M. Sharkey, et al. (2005). "Identification of genes regulated by interleukin-1beta in human endometrial stromal cells." Reproduction **130**(5): 721-729.

Roy, A. and P. E. Kolattukudy (2012). "Monocyte chemotactic protein-induced protein (MCPIP) promotes inflammatory angiogenesis via sequential induction of oxidative stress, endoplasmic reticulum stress and autophagy." Cell Signal **24**(11): 2123-2131.

Ruddon, R. W., C. A. Hanson, et al. (1979). "Synthesis and processing of human chorionic gonadotropin subunits in cultured choriocarcinoma cells." Proc Natl Acad Sci U S A **76**(10): 5143-5147.

Ryan, R. J., M. C. Charlesworth, et al. (1988). "The glycoprotein hormones: recent studies of structure-function relationships." FASEB J **2**(11): 2661-2669.

S Aschheim , B. Z. (1927). "Das Hormon des hypophysenvorderlappens: testobjekt zum Nachweis des hormons. ." Klin Wochenschr **6**: 248-252.

Saccani, S., S. Pantano, et al. (2002). "p38-Dependent marking of inflammatory genes for increased NF-kappa B recruitment." Nat Immunol **3**(1): 69-75.

Saito, S. (2001). "Cytokine cross-talk between mother and the embryo/placenta." J Reprod Immunol **52**(1-2): 15-33.

Salamonsen, L. A. (1999). "Role of proteases in implantation." Rev Reprod **4**(1): 11-22.

Salcedo, R., K. Wasserman, et al. (1999). "Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: In vivo neovascularization induced by stromal-derived factor-1alpha." Am J Pathol **154**(4): 1125-1135.

Salker, M. S., J. Nautiyal, et al. (2012). "Disordered IL-33/ST2 activation in decidualizing stromal cells prolongs uterine receptivity in women with recurrent pregnancy loss." PLoS One **7**(12): e52252.

Sasaki, Y., D. G. Ladner, et al. (2008). "Hyperglycosylated human chorionic gonadotropin and the source of pregnancy failures." Fertil Steril **89**(6): 1781-1786.

Sawai, K., N. Matsuzaki, et al. (1997). "Human decidual cell biosynthesis of leukemia inhibitory factor: regulation by decidual cytokines and steroid hormones." Biol Reprod **56**(5): 1274-1280.

Sayegh, R., J. T. Awwad, et al. (1995). "Alpha 2-macroglobulin production by the human endometrium." J Clin Endocrinol Metab **80**(3): 1021-1026.

Schafer-Somi, S. (2003). "Cytokines during early pregnancy of mammals: a review." Anim Reprod Sci **75**(1-2): 73-94.

Schmitz, J., A. Owyang, et al. (2005). "IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines." Immunity **23**(5): 479-490.

Sherwin, J. R., A. M. Sharkey, et al. (2007). "Identification of novel genes regulated by chorionic gonadotropin in baboon endometrium during the window of implantation." Endocrinology **148**(2): 618-626.

Sheth, K. V., G. L. Roca, et al. (1991). "Prediction of successful embryo implantation by measuring interleukin-1-alpha and immunosuppressive factor(s) in preimplantation embryo culture fluid." Fertil Steril **55**(5): 952-957.

Shi, Q. J., Z. M. Lei, et al. (1993). "Novel role of human chorionic gonadotropin in differentiation of human cytotrophoblasts." Endocrinology **132**(3): 1387-1395.

Simon, C., A. Frances, et al. (1994). "Interleukin-1 system in the materno-trophoblast unit in human implantation: immunohistochemical evidence for autocrine/paracrine function." J Clin Endocrinol Metab **78**(4): 847-854.

Simon, C., A. Frances, et al. (1994). "Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist." Endocrinology **134**(2): 521-528.

Simon, C., M. J. Gimeno, et al. (1997). "Embryonic regulation of integrins beta 3, alpha 4, and alpha 1 in human endometrial epithelial cells in vitro." J Clin Endocrinol Metab **82**(8): 2607-2616.

Simon, C., D. Valbuena, et al. (1998). "Interleukin-1 receptor antagonist prevents embryonic implantation by a direct effect on the endometrial epithelium." Fertil Steril **70**(5): 896-906.

Snyder, J. A., S. Haymond, et al. (2005). "Diagnostic considerations in the measurement of human chorionic gonadotropin in aging women." Clin Chem **51**(10): 1830-1835.

Sophie Perrier d'Hauterive, M. T. J.-M., Foidart Vincent Geenen (2007). "Y a-t-il un marqueur décisif de l'implantation embryonnaire ?" médecine de la reproduction **9**(6): 389-398.

Stavreus-Evers, A., L. Aghajanova, et al. (2002). "Co-existence of heparin-binding epidermal growth factor-like growth factor and pinopodes in human endometrium at the time of implantation." Mol Hum Reprod **8**(8): 765-769.

Strakova, Z., P. Mavrogianis, et al. (2005). "In vivo infusion of interleukin-1beta and chorionic gonadotropin induces endometrial changes that mimic early pregnancy events in the baboon." Endocrinology **146**(9): 4097-4104.

Strieter, R. M., P. J. Polverini, et al. (1995). "The role of CXC chemokines as regulators of angiogenesis." Shock **4**(3): 155-160.

Strott, C. A., T. Yoshimi, et al. (1969). "Ovarian physiology: relationship between plasma LH and steroidogenesis by the follicle and corpus luteum; effect of HCG." J Clin Endocrinol Metab **29**(9): 1157-1167.

Tabibzadeh, S., Q. F. Kong, et al. (1995). "Progressive rise in the expression of interleukin-6 in human endometrium during menstrual cycle is initiated during the implantation window." Hum Reprod **10**(10): 2793-2799.

Tang, B. and E. Gurpide (1993). "Direct effect of gonadotropins on decidualization of human endometrial stroma cells." J Steroid Biochem Mol Biol **47**(1-6): 115-121.

Thibault, C. L., MC (2001). La reproduction chez les mammifères et l'homme c. Paris : INRA : Ellipses

Toth, P., X. Li, et al. (1994). "Expression of functional human chorionic gonadotropin/human luteinizing hormone receptor gene in human uterine arteries." J Clin Endocrinol Metab **79**(1): 307-315.

Trévoux, R., Ed. (2009). L'Endomètre Présent Et Avenir

Tsampalas, M., V. Gridelet, et al. (2010). "Human chorionic gonadotropin: a hormone with immunological and angiogenic properties." J Reprod Immunol **85**(1): 93-98.

Tsui, K. H., L. Y. Chen, et al. (2004). "Interleukin-8 can stimulate progesterone secretion from a human trophoblast cell line, BeWo." In Vitro Cell Dev Biol Anim **40**(10): 331-336.

Van Royen, E., K. Mangelschots, et al. (1999). "Characterization of a top quality embryo, a step towards single-embryo transfer." Hum Reprod **14**(9): 2345-2349.

Velez de la Calle JF, C. N. (2012). " La culture prolongée : résultats de la co-culture embryonnaire sur des cellules endométriales humaines." Médecine de la Reproduction, Gynécologie Endocrinologie **14**(4): 311-318.

Vilcek, J. T. (1996). "Cytokines in 1995." Cytokine Growth Factor Rev **7**(1): 103-106.

von Wolff, M., C. J. Thaler, et al. (2000). "Regulated expression of cytokines in human endometrium throughout the menstrual cycle: dysregulation in habitual abortion." Molecular human reproduction **6**(7): 627-634.

von Wolff, M., C. J. Thaler, et al. (2000). "Regulated expression of cytokines in human endometrium throughout the menstrual cycle: dysregulation in habitual abortion." Mol Hum Reprod **6**(7): 627-634.

Wallace, A. E., R. Fraser, et al. (2012). "Extravillous trophoblast and decidual natural killer cells: a remodelling partnership." Human reproduction update **18**(4): 458-471.

Wallace, A. E., R. Fraser, et al. (2012). "Extravillous trophoblast and decidual natural killer cells: a remodelling partnership." Hum Reprod Update **18**(4): 458-471.

Wang, H. and S. K. Dey (2006). "Roadmap to embryo implantation: clues from mouse models." Nat Rev Genet **7**(3): 185-199.

Weber, A., P. Wasiliew, et al. (2010). "Interleukin-1 (IL-1) pathway." Sci Signal **3**(105): cm1.

Werb, Z. (1997). "ECM and cell surface proteolysis: regulating cellular ecology." Cell **91**(4): 439-442.

Wheater, P. R. (2004). Histologie fonctionnelle.

Wilcox, A. J., D. D. Baird, et al. (1999). "Time of implantation of the conceptus and loss of pregnancy." N Engl J Med **340**(23): 1796-1799.

Wolf, K. and P. Friedl (2011). "Extracellular matrix determinants of proteolytic and non-proteolytic cell migration." Trends in cell biology **21**(12): 736-744.

Wolter, S., A. Doerrie, et al. (2008). "c-Jun controls histone modifications, NF-kappaB recruitment, and RNA polymerase II function to activate the ccl2 gene." Mol Cell Biol **28**(13): 4407-4423.

Zhang, J., B. A. Croy, et al. (2005). "Uterine natural killer cells: their choices, their missions." Cell Mol Immunol **2**(2): 123-129.

Zhang, X. H., Z. Tian, et al. (2012). "Differential expression of interleukin 1 receptor type II during mouse decidualization." Reprod Sci **19**(9): 923-931.

Zimmermann, G., W. Ackermann, et al. (2012). "Expression and production of human chorionic gonadotropin (hCG) in the normal secretory endometrium: evidence of CGB7 and/or CGB6 beta hCG subunit gene expression." Biol Reprod **86**(3): 87.

Zuo, J., Z. M. Lei, et al. (1994). "Human myometrial chorionic gonadotropin/luteinizing hormone receptors in preterm and term deliveries." J Clin Endocrinol Metab **79**(3): 907-911.

Zygmunt, M., F. Herr, et al. (2002). "Characterization of human chorionic gonadotropin as a novel angiogenic factor." J Clin Endocrinol Metab **87**(11): 5290-5296.