

Influence of Sp1/Sp3 Expression on Corneal Epithelial Cells Proliferation and Differentiation Properties in Reconstructed Tissues

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PURPOSE. Primary cultured epithelial cells are widely used for the production of tissue-engineered substitutes and are gaining popularity as a model for gene expression studies. However, as such cells are passaged in culture, they often lose their ability to proliferate by progressing toward terminal cell differentiation, a process likely to be determined by altered expression of transcription factors that have functions critical for cell adhesion and differentiation. This study was designed to determine whether the variable life span of primary cultured human corneal epithelial cells (HCECs) might be the consequence of varying expression levels of the well-known transcription factors Sp1 and Sp3 (Sp1/Sp3).

METHODS. HCECs were obtained from donor eyes and cultured on irradiated Swiss-3T3. Sp1/Sp3 expression was monitored by Western blot and electrophoretic mobility shift assay (EMSA). The Sp1/Sp3 regulatory influence was evaluated by transfection of HCECs with a recombinant plasmid bearing the Sp1/Sp3-dependent poly(ADP-ribose) polymerase (rPARP) promoter fused to the CAT reporter gene. HCECs that expressed various levels of Sp1/Sp3 were also used for the production of corneal substitutes.

RESULTS. Expression of Sp1/Sp3 was dramatically inconsistent between HCECs isolated from the eyes of different donors. Both factors were highly expressed during one passage and then totally disappeared as cells terminally differentiated. Proper stratification of HCECs on reconstructed tissue substitutes could be obtained only with cells that also had a delayed peak of Sp1/Sp3 expression when cultured in vitro.

CONCLUSIONS. Expression of Sp1/Sp3 may represent a good predictor for selecting HCECs that are most likely to proliferate, stratify, and differentiate properly when used for the production of reconstructed corneal substitutes. (*Invest Ophthalmol Vis Sci.* 2003;44:1447-1457) DOI:10.1167/iovs.02-0707

Primary cultured cells are gaining in popularity in many areas of both clinical and basic research. They are widely used, either alone or in combination with synthetic biomaterials, in the production of tissue-engineered substitutes, a very active emerging field of medical research (reviewed in Ref. 1). Indeed, new synthetic or bioengineered substitutes capable of upregulating or downregulating biological responses at the tissue-substitute interface are starting to reach clinical application.^{2,3} In addition, primary cultured cells are recognized as being cells much closer to their in vivo counterpart than transformed cells or cell lines are. Because of this characteristic, they became particularly attractive as a source of cellular material for gene expression studies.⁴⁻⁶ However, many technical difficulties render the use of primary cultured cells particularly limiting for such types of studies. These include the inability to grow certain types of cells in vitro, the tendency of many cell types to differentiate with an increasing number of cell passages, and the potential variations that may occur related to the age and health condition of the donor, not to mention the difficulty in obtaining adequate biopsy specimens of some tissues from normal human donors.

The corneal epithelium is a self-renewing tissue, a property that is ensured by the high proliferative potential of stem cells from the limbal area that surrounds the cornea.⁷ After corneal injury, these cells migrate in a swirling circular movement⁸ to the basal layer of the central cornea and then move through the suprabasal compartment toward the corneal surface.⁹ Apart from its protective influence against bacterial infections and the many environmental stresses (reviewed in Ref. 10), an intact corneal epithelium is an absolute requirement for maintaining proper visual acuity.¹¹ Nontransformed, primary cultured human corneal epithelial cells (HCECs) have been used successfully in the production of tissue-engineered human corneal substitutes.¹² The development of a true replacement cornea has several interesting advantages (reviewed in Ref. 13) such as the possibility to harvest corneal tissues from autologous biopsy specimens or from eye banks, nearly no rejection complications, and the absence of transmission of iatrogenic disease. It is also particularly attractive as a model to conduct gene promoter studies, in that HCECs that have been expanded and transfected with recombinant DNA can also be seeded on reconstructed stroma to evaluate a gene's promoter behavior in a cell system much closer to the in vivo context (reviewed in Ref. 14).

Although preliminary reports regarding the transplantation of corneal epithelial stem cells are promising, long-term complications, such as severe corneal dysfunction, occur in many of such treated corneal defects.¹⁵⁻¹⁸ Although the precise mechanism underlying the inefficiency of corneal epithelial

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stem cell transplantation remains obscure, both the adhesive and proliferative properties of the cells selected for grafting are likely to play a pivotal role in the success of this procedure. Indeed, limbal HCECs can sustain, on average, up to 23 population doublings in vitro,¹⁹ which corresponds to four or five passages. We and others have observed a great deal of variability in the ability of HCECs to maintain viability through several passages in culture.^{12,14,20} Most cannot be maintained for more than three or four passages before they terminally differentiate. However, and for yet unknown reasons, a few such cells can be passaged up to seven or eight passages^{12,19,20} which makes them ideal cell sources for the production of tissue-engineered corneal substitutes.

Proper cell adhesion is crucial in obtaining an appropriate histologic structure by the assembly of individual cells into the three-dimensional tissues of animals (reviewed in Ref. 21). It allows cells to link either with one another or with components from the extracellular matrix (ECM), thereby establishing intracellular structural linkages with cytoskeletal proteins that organize themselves into supramolecular complexes. Although cell-cell adhesion through the formation of adherens junctions is primarily determined by cadherins, cell-ECM adhesions, which include the formation of focal adhesions, are primarily based on integrin-type receptors. Expression of the genes encoding these various adhesion receptors is in turn regulated at the transcriptional level by the binding of specific transcription factors to a variety of *cis*-acting regulatory elements present in both the promoter and 5'-flanking sequences of such genes. Among such nuclear proteins, Specificity protein-1 (Sp1) is certainly one of the most interesting candidates. Sp1 belongs to a family of transcription factors that includes three other proteins: Sp2, Sp3, and Sp4.²² Transcription factors from the Sp family are likely to regulate the expression of most, if not all, housekeeping genes, such as that encoding poly(ADP-ribose) polymerase (PARP).²³ In addition, expression of many integrin subunit genes, which include $\alpha 2$,²⁴ $\alpha 5$,²⁵ $\alpha 6$,²⁶ $\beta 2/CD18$,²⁷ $CD11d$,²⁸ αIIb ,²⁹ αv ,³⁰ $\beta 5$,³¹ and $\beta 3$,³² was reported to be regulated by the binding of Sp1 (and often also Sp3^{30,31}) to target sites located in the upstream promoter of these genes. A putative involvement of Sp1 in cell adhesion and migration is therefore not surprising, considering that it probably regulates the expression of most, if not all, integrin subunit genes.

The purpose of this study was to examine the expression of Sp1, as well as that of Sp3, in primary cultured HCECs obtained from different human donors and to establish whether cell passages have any influence on the DNA binding ability and protein concentration of such factors. HCECs from different human donors that express various amounts of these Sp1 family members when subjected to extended cell passages were also examined for their ability to regulate the activity directed by the basal recombinant PARP (rPARP) promoter. This promoter was selected for conducting transfection experiments in HCECs because its transcriptional activity was recently reported by us to be strongly dependent on its recognition by members of the Sp1 family,^{33,34} therefore providing a good molecular tool to investigate how alterations in the levels of Sp1 and Sp3 (Sp1/Sp3) expression translate into functional alterations of Sp1-dependent gene expression. In addition, their differentiation and stratification properties when used for the production of tissue-engineered corneal substitutes were also investigated.

METHODS

This study was conducted in accordance with our institution's guidelines and the Declaration of Helsinki, and the protocols were also approved by the institution's Committee for the Protection of Human Subjects.

Recombinant Plasmids

The recombinant plasmid pCR3, which bears the CAT reporter gene fused to the basal promoter of the rat PARP gene, and the plasmid's mutated derivative pCR3/F2-F3-F4m, which bears mutations in each of its three Sp1 sites, have already been described.³³

Cell Culture and Media

Human corneal epithelial cells (HCECs) were isolated from the limbal area of normal eyes obtained from 3-day-old (L83HCECs), 19-year-old (L81HCECs), 24-year-old (L82HCECs), 49-year-old (L80HCECs), 52-year old (L203HCECs), 55-year old (L200HCECs), 61-year old (L204HCECs), and 65-year-old (L85HCECs) donors by a procedure that we have recently described.¹² They were cultured up to seven passages (referred to as P0 for primary culture [no passage], up to P7 for passage 7), as previously described.^{12,14} All cells were grown under either 5% or 8% CO₂ at 37°C, and culture medium was changed three times a week. Human fibroblasts obtained from the dermal portion of adult breast skin and cultured as described previously³⁵ were used at their sixth passage. Fibroblasts were cultured in Dulbecco-Vogt's modification of Eagle's medium (Life Technologies, Grand Island, NY), supplemented with 10% fetal calf serum (FCS; Hyclone-PDI Bioscience, Aurora, Ontario, Canada), 100 IU/mL penicillin, and 25 µg/mL gentamicin (Sigma, St. Louis, MO).

Preparation and Culture of Reconstructed Human Tissues

Reconstructed substitutes were produced by the autoassembly approach, as previously described.³⁶ Two dermal fibroblast sheets were superimposed and cultured for 7 days to form the reconstructed dermis. HCECs were then seeded on top and allowed to grow to confluence under submerged conditions for 7 days in complete DMEM-HAM supplemented with 10% serum (FetalClone II; Hyclone-PDI Bioscience) and 50 µg/mL ascorbic acid. This dermal-corneal epithelial tissue substitute was brought to the air-liquid interface for 11 days before biopsy specimens were collected.

Histologic Analysis

Biopsy specimens from reconstructed human tissues were fixed with Bouin's solution and embedded in paraffin. Microtome sections were stained with Masson's trichrome for histologic analyses.

Indirect Immunofluorescence

Biopsy specimens from reconstructed human tissues were embedded in frozen tissue embedding medium (OCT compound, Tissue-Tek; Bayer Canada, Etobicoke, Ontario, Canada) frozen in liquid nitrogen, and stored at -70°C until use. Indirect immunofluorescence assays were performed on acetone-fixed cryosections, as previously reported.³⁶ Sections were incubated with the primary antibody (either the AE5 anti-keratin 3 [ICN Biomedicals, Aurora, OH] antibody or the rabbit polyclonal anti-Sp1 antibody [Santa Cruz Biotechnology, Inc., Santa Cruz, CA]) for 45 minutes, followed by incubation with the appropriate conjugated secondary antibody for 30 minutes (goat anti-mouse IgG-IgM conjugated with rhodamine; TRITC; Chemicon, Temecula, CA). Cell nuclei were also labeled with Hoechst reagent 33258 (Sigma) after immunofluorescence staining. They were then observed under a microscope, equipped with epifluorescence (Optiphot; Nikon, Tokyo, Japan) and photographed with a numeric charge-coupled device (CCD) camera.

Nuclear Extract Preparation

Crude nuclear extracts were prepared from each primary culture of HCECs when they reached approximately 80% confluence. Extracts were dialyzed against DNaseI buffer (50 mM KCl, 4 mM MgCl₂, 20 mM K₃PO₄ [pH 7.4], 1 mM β-mercaptoethanol, 20% glycerol), as described^{25,37} and kept frozen in small aliquots at -80°C until use.

Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays (EMSAs) were performed using a synthetic double-stranded oligonucleotide bearing the high-affinity binding site for the positive transcription factor Sp1 (5'-GATCATATCT-GCGGGGCGGGGAGACACAG-3'). The oligonucleotide was chemically synthesized with a commercial apparatus (Biosearch 8700; Millipore, Bedford, MA).³⁸ Approximately 2×10^4 cpm-labeled DNA was incubated with HCEC crude nuclear proteins (5 μ g) in the presence of 500 ng poly(dI-dC).poly(dI-dC) (Pharmacia-LKB; Gaithersburg, MD) in buffer D (5 mM HEPES [pH 7.9], 10% glycerol [vol/vol], 25 mM KCl, 0.05 mM EDTA, 0.5 mM dithiothreitol, and 0.125 mM PhMeSO₂F). Incubation proceeded at room temperature for 15 minutes, at which time DNA-protein complexes were separated by gel electrophoresis through either 6% or 8% native polyacrylamide gels run against Tris-glycine buffer as described.³⁹ Gels were dried and autoradiographed at -80°C to reveal the position of the shifted DNA-protein complexes generated. Competitive EMSA was performed the same, except that a 500-fold molar excess of unlabeled double-stranded oligonucleotides bearing either the Sp1 binding site or the target sequence for human HeLa CCAAT-transcription factor/nuclear factor-I (CTF/NF-I) in adenovirus type 2 (Ad2) (5'-GATCTTATTTGGATTGAAGCCAATATGAG-3'),⁴⁰ or single-stranded oligonucleotides bearing either the top (Sp1a) or the bottom (Sp1b) strand from the Sp1 high-affinity target site, were added to the reaction mix before gel analysis.

SDS-PAGE and Western Blot

The protein concentration from each of the HCEC crude nuclear extracts was evaluated by the Bradford procedure and further validated after Coomassie blue staining of SDS-polyacrylamide fractionated nuclear proteins. Approximately 20 μ g proteins was added to 1 volume of sample buffer and then size fractionated on a 10% SDS-polyacrylamide minigel before transfer onto a nitrocellulose filter under conditions that we have described.²⁵ A full set of protein molecular mass markers (Gibco BRL, Burlington, Ontario, Canada) was also loaded as a control to evaluate protein sizes. The blot was then washed once in TS buffer (150 μ M NaCl, 10 mM Tris-HCl, pH 7.4) and four times in TSM buffer (TS buffer + 5% w/v fat free Carnation milk + 0.1% Tween 20).²⁵ A 1:500 dilution of either a mouse (Sp1) or rabbit (Sp3 and Sp4) polyclonal antibody raised against the transcription factors Sp1, Sp3, or Sp4 (Santa Cruz Biotechnology, Inc.), or a 1:10,000 dilution of a mouse monoclonal antibody (C-2-10⁴¹) raised against the C-terminal end of the DNA binding domain of bovine PARP (kindly provided by Guy G. Poirier, Unit of Health and Environment, University Hospital Center of Laval [CHUL] Research Center, Québec, Canada) was added to the membrane-containing TSM buffer and incubation proceeded for another 4 hours at 22°C. The blot was then washed and incubated an additional 1 hour at 22°C in a 1:1000 dilution of a peroxidase-conjugated goat anti-mouse (Sp1 and PARP) or anti-rabbit (Sp3 and Sp4) IgG (Jackson ImmunoResearch Laboratory, West Grove, PA). The membrane was successively washed in TSM (four times, 5 minutes each) and TS buffers before immunoreactive complexes were revealed using Western blot chemiluminescence reagents (Renaissance, NEN Dupont; Boston, MA) and autoradiographed.

Transient Transfections and CAT Assays

Primary cultured HCECs (5×10^5 cells per 60-mm plate) isolated from the limbal area of normal eyes obtained from three different donors (L200HCECs, L203HCECs, and L204HCECs) were transiently transfected at various cell passages (up to P6, P4, and P3 for L200HCECs, L203HCECs, and L204HCECs, respectively) by the calcium phosphate precipitation method.⁴² Each cell-containing tissue culture well received 15 μ g test plasmid and 5 μ g of the human growth hormone (hGH) gene-encoding plasmid pXGH5.⁴³ Levels of CAT activity for all transfected cells were determined²⁵ and normalized to the amount of hGH secreted into the culture media and assayed using a kit for quantitative measurement of hGH (Immunocorp, Montréal, Québec, Canada). The data for each test plasmid transfected correspond to the

mean \pm SD of results of two separate transfections performed in triplicate.

RESULTS

Influence of Cell Passage on the Morphology of HCECs

HCECs obtained from the limbus of human donor eyes were seeded with a 3T3 feeder layer on plastic substrate and cultured according to a procedure we recently described¹² and initially designed for culturing human cutaneous keratinocytes. Under these conditions, limbal epithelial cells produced large colonies of small cells in primary culture (Fig. 1A; P0). However, this property could not be maintained with increasing cell passages in most of our patient's HCECs, the colonies being much smaller and essentially made up of large cells that were difficult to subculture to the following passages (Fig. 1A; P1, P2). Many HCEC subcultures could not be maintained beyond passage 3 (Fig. 1A; P3), at which point the culture exhibited a terminally differentiated phenotype, mainly constituted of large nondividing cells.

Patterns of Expression of Sp1 and Sp3

Transcription factors undoubtedly determine the fate of any particular cell by controlling the pattern of genes that should be transcribed to maintain the cell's functionality. Among such genes are those encoding adhesion molecules from the integrin family, which are known to have very critical functions in processes such as cell adhesion and migration. We therefore hypothesized that the morphologic changes occurring in HCECs with increasing cell passages may be the consequence of substantial alterations in the expression of such transcription factors. The positive transcription factors that belong to the Sp1 family are probably those that have been the most studied so far. Most of all, expression of many integrin subunit genes has been reported to be regulated by members of this family.²⁴⁻³²

To investigate whether Sp1, or any other member of this family, is expressed in HCECs, nuclear proteins were obtained from cells at P2 and incubated with a 5'-end-labeled oligonucleotide bearing the sequence of a high-affinity target site for Sp1. Formation of DNA-protein complexes was then monitored by EMSA. As shown on Figure 1B, a number of DNA-protein complexes (designated *a-d*) could be observed when increasing amounts (5–20 μ g) of HCEC crude nuclear proteins were added to the Sp1-labeled probe. Formation of both complexes *a* and *d*, and to some extent that of complex *b*, was prevented by the addition of the unlabeled Sp1 competitor, but not by an oligonucleotide bearing the target sequence for the unrelated transcription factor NF1, providing evidence that formation of all three complexes (*a*, *b*, and *d*) was specific (Fig. 1C). Further competitions using single-stranded oligonucleotides bearing the sequence from either the top (Sp1a) or the bottom (Sp1b) strand of the Sp1 target site revealed that formation of complex *d* resulted from the recognition of the free-labeled Sp1b strand by a yet unknown nuclear protein. We next performed supershift experiments in EMSAs to define more precisely which of the Sp family members yield both complexes *a* and *b*. As revealed on Figure 1D, formation of complex *a*, but not that of any of the remaining complexes, was substantially decreased by the addition of a polyclonal antibody raised against Sp1, which also led to the formation of a new supershifted complex designated *a*/Sp1Ab. The addition of an anti-Sp3 antibody resulted in only a moderate decrease in the formation of complex *a*, but totally prevented formation of a weak complex designated *b*^{*}, often appearing on the gel just above complex *b* when the concentration of the gel was

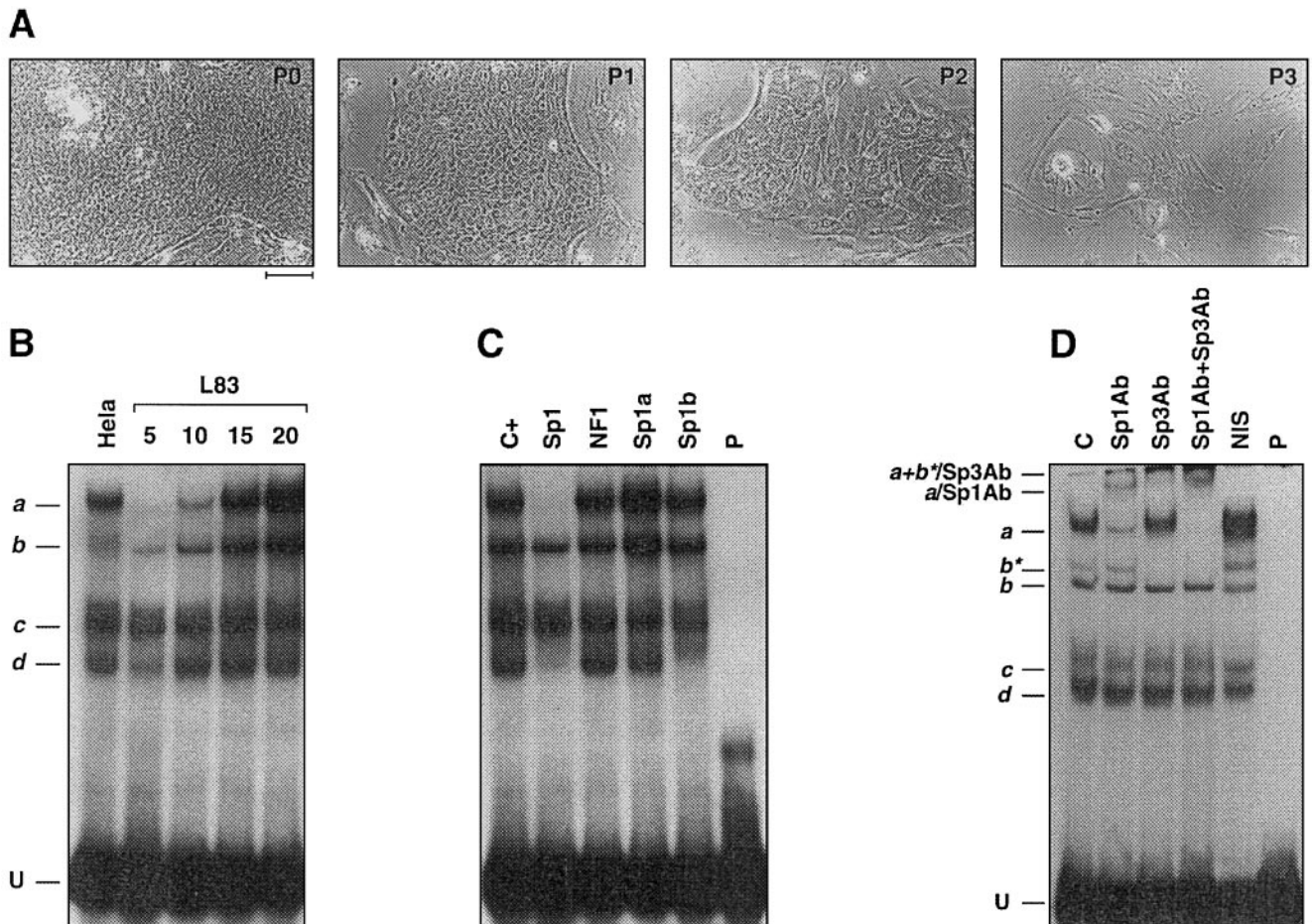


FIGURE 1. Expression of Sp family members in HCECs. **(A)** Phase-contrast images of cultured HCECs in passages indicated (*top right*). Magnification, $\times 200$. Bar, 200 μm . **(B)** Binding of nuclear proteins from HCECs to a high-affinity Sp1 target site. Increasing amounts (5–20 μg) of nuclear proteins from P2 L83HCECs were incubated with the Sp1 probe and formation of DNA-protein complexes analyzed by EMSA on an 8% gel. The Sp1 probe was incubated with 5 μg nuclear proteins from human HeLa cells, as a positive control. The position of four distinct DNA-protein complexes is indicated (*a–d*), along with that of the free probe (*U*). **(C)** Specificity for the formation of complexes *a* to *d* was evaluated by competitive EMSA. Nuclear proteins (10 μg) from L83HCECs were incubated with the Sp1 probe in the presence of a 500-fold molar excess of unlabeled oligonucleotides bearing the binding sites for either Sp1 or NF1 or with unlabeled single-stranded oligonucleotides bearing the sequence of either the top (Sp1a) or the bottom (Sp1b) strand from the Sp1 target site. P, labeled probe alone; C+, labeled probe with nuclear proteins but without competitors. **(D)** Supershift analyses in EMSA. Nuclear proteins (7 μg) from P1 L85HCECs were incubated with the Sp1 probe, either alone or in the presence of antibodies against either Sp1 (2 μL) or Sp3 (4 μL), or both the Sp1 and Sp3 antibodies (2 and 4 μL , respectively). Formation of DNA-protein complexes was then monitored by EMSA on a 6% gel. The position of two supershifted complexes (*a*/Sp1Ab and *a+b**/Sp3Ab) is indicated. C, labeled probe with nuclear proteins but without antibodies; NIS, labeled probe incubated with both nuclear proteins and nonimmune serum as a control.

lowered to 6%, leading to the formation of a new supershifted complex designated *a+b**/Sp3Ab (Fig. 1D). Altogether, these results suggest that complex *a* is essentially made up of Sp1 with a small amount of Sp3 and that complex *b** results solely from the recognition of the Sp1-labeled probe by Sp3. Indeed, the addition of both the Sp1 and Sp3 antibodies to the reaction mix totally prevented formation of both complexes *a* and *b**, as expected (Fig. 1D). That both Sp1 and Sp3 yield DNA-protein complexes which comigrated in EMSA is not unique and has been reported before.^{4,44,45} The fast-migrating Sp3 complex yielding *b** apparently results from the recognition of the Sp1-labeled probe by an N-terminal truncated form of Sp3 that possess an intact DNA-binding domain but a partially deleted activation domain.^{45–48} Formation of the *b* complex, which in Figures 1B and 1C could not be dissociated from complex *b**, appears to be nonspecific. No influence whatsoever has been observed on the formation of any of these complexes when the reaction was performed in the presence of the anti-Sp4 Ab, even when used at 4 μL (data not shown). In addition, the

formation of neither complex *c* or *d* was altered by any of these antibodies.

Because we observed a great deal of variability in the efficiency of HCECs obtained from different donor eyes in properly stratifying on tissue-engineered corneal substitutes,^{12,14} we investigated whether the formation of complexes *a* to *d* was both quantitatively and qualitatively altered with increasing cell passages in HCECs obtained from different human donors. Crude nuclear proteins were then prepared from limbal HCECs obtained from donor eyes of five different individuals (designated L80HCEC, L81HCEC, L82HCEC, L83HCEC, and L85HCEC) at P1, P2, and P3. When an equal amount of nuclear proteins from each of these extracts was incubated with the Sp1 probe, dramatic age-independent alterations in the formation of both the Sp1/Sp3-dependent complex *a* and the nonspecific complex *b* were observed (Fig. 2A). In contrast, no such alterations were observed in the formation of complexes *c* and *d*. It is interesting to note that the protein yielding complex *a* (essentially Sp1) was obviously more con-

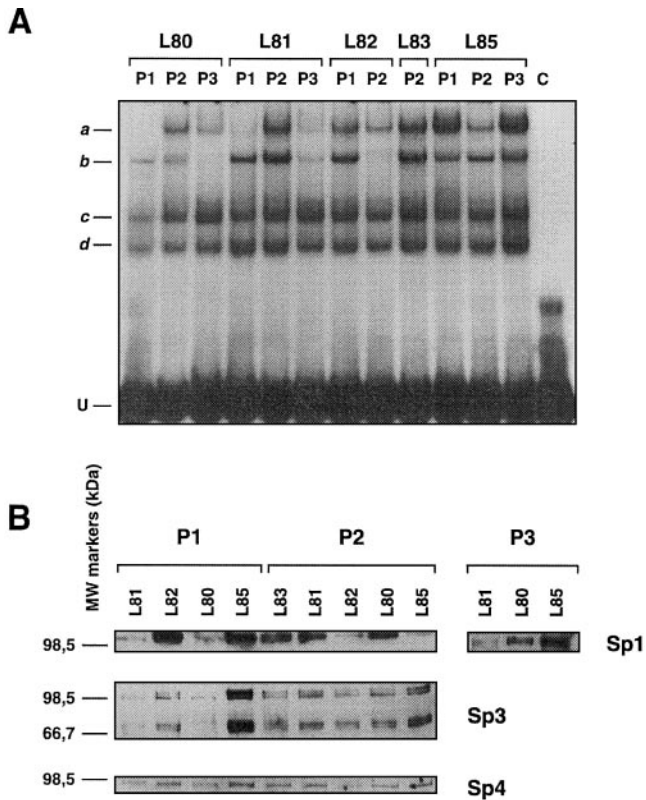


FIGURE 2. Influence of cell passages on the expression of Sp1, Sp3, and Sp4. (A) Nuclear proteins were obtained from L80HCECs, L81HCECs, L82HCECs, L83HCECs, and L85HCECs at P1 to P3 and incubated (20 μ g of each) with the Sp1 probe. Formation of DNA-protein complex *a* (which contain both Sp1 and Sp3) as well as that of complexes *b* to *d* was then monitored by EMSA on an 8% gel. U, unbound fraction of the probe; C, labeled probe with no added proteins. (B) Western blot analysis of Sp family members. Approximately 30 μ g nuclear proteins from the cells used in (A) were examined in Western blot analyses, using antibodies directed against Sp1, Sp3, and Sp4. The position of the specific signals for members of the Sp1 family are indicated, along with that of the nearest molecular mass markers (phosphorylase B; apparent molecular mass, 98.5 kDa) and bovine serum albumin (apparent molecular mass, 66.7 kDa).

sistently expressed at P2 than at P1. Formation of complex *b*, which clearly predominated at P1, progressively shifted toward formation of complex *a* at both P2 and P3 (Fig. 2A).

These results suggest that dramatic alterations, either in the concentration or the DNA binding affinity of both Sp1 and Sp3 for their target site, occurred in HCECs from different donors and from one passage to another. To determine which of these two hypothesis accounts for the alterations in the formation of both complexes *a* and *b*^{*}, expression of both Sp1 and Sp3 (as well as Sp4) was examined by Western blot analyses. As revealed in Figure 2B, the alterations in the formation of complex *a* observed in Figure 2A also perfectly matched with corresponding variations in the absolute concentration of Sp1. Extracts that yielded either no or only a modest formation of complex *a* (e.g., P1 HCECs from L80 and L81) also had barely detectable amounts of Sp1. However, those that yielded complex *a* with strong signal strengths, such as in cells from donors L85HCEC at P1 and L81HCEC at P2 (Fig. 2A), also expressed more of the Sp1 protein (Fig. 2B). Similar alterations were also observed in the expression of Sp3 at both P1 and P2 (Fig. 2B), as well as P3 (result not shown). Expression of Sp4 was found to be much more stable and less subjected to alterations than Sp1 and Sp3 (Fig. 2B). Therefore, the variations

observed in the formation of complexes *a* and *b*^{*} can be accounted for by corresponding alterations in the amount of Sp1/Sp3 expressed in these cells and not by alterations in their affinity for their target sequences, as we recently reported.²⁵

Regulatory Influence of HCEC Sp1/Sp3 on an Sp1-Dependent Promoter

To determine whether the variations observed in the pattern of Sp1/Sp3 expression have any functional significance in vitro, we transiently transfected HCECs isolated from the eyes of three different donors (L200HCEC, L203HCEC, and L204HCEC) at various passages (P1-P6) with a recombinant construct bearing the CAT reporter gene fused to the promoter of the rat PARP (rPARP) gene.^{33,49} The rPARP basal promoter was selected because it has been shown to bear multiple Sp1 binding sites that are critical for basal rPARP promoter activity. Both the recombinant plasmid pCR3, which bears rPARP promoter sequences from position -101 to +13 and has three well-characterized Sp1 sites (F2, F3, and F4), and its corresponding derivative pCR3/F2-F3-F4m, which bears mutations in each of the three Sp1 sites, were selected for this experiment.³³ Crude nuclear extracts were prepared from each of the three HCEC cultures and at each cell passage to determine whether functional correlations can be established between any potential alterations in the level of Sp1 activity and that directed by the rPARP basal promoter.

Each of the HCEC primary cultures used for the study exhibited very distinctive growth and proliferative properties. Indeed, the L200HCECs could be cultured up to P7 whereas cells L203HCECs and L204HCECs could be maintained only up to P5 and P4, respectively, before they terminally differentiated. EMSAs revealed that binding of Sp1/Sp3 could not be detected before P4 in L200HCECs, which then totally disappeared in the extracts from P5 and P6 cells (Fig. 3A). A similar pattern was also observed with the extracts from L203HCECs except that the Sp1/Sp3 binding activity appeared earlier, reaching a peak at P2. No Sp1/Sp3 binding activity occurred in the extracts prepared from L204HCECs, which yielded only diffuse, nonspecific signals similar to those observed at P1 and P2 with L200HCECs (Fig. 3A). As Figure 3B indicates, the Sp1 protein was detected by Western blot analyses in the same nuclear extracts (P4 for L200HCECs, P2 and P3 for L203HCECs, and no signal in L204HCECs), which also supported Sp1 binding in the EMSA. The two protein doublets that are typically observed for Sp3 in Western blot were also detected in the Sp1-positive extracts. In addition, a weak but clearly detectable Sp3 signal was observed in the P1 extract from L204HCECs. Although unlikely, extinction of Sp1/Sp3 may have resulted from their proteolytic cleavage by apoptotic caspases as a consequence of passaged HCECs progressing into apoptosis. To test for this hypothesis, proteins from passages P1 to P4 from L203HCECs were blotted and probed with a monoclonal antibody (C-2-10) raised against the bovine PARP protein (bPARP),⁴¹ the cleavage of which by caspase 3 is well recognized as an early marker of apoptosis.⁵⁰ The epitope recognized by the C-2-10 antibody is localized near the C-terminal end of the PARP DNA-binding domain, a region shown to be totally conserved between murine, bovine, and human PARP.⁴¹ Cleavage of the 113-kDa mature PARP by caspase 3 yielded two degradation products with molecular masses of 89 and 24 kDa, of which only the larger, which bears the intact epitope, can be recognized by the C-2-10 mAb.⁵¹ As shown in Figure 3B, bottom, a single clear signal corresponding to the 113-kDa mature PARP protein was observed in the P1 and P2 crude extracts from L203HCEC cells, with a peak expression at P2. Then, as Sp1 decreased at P3, so did the PARP protein, which then became totally absent at P4. Crude nuclear proteins from

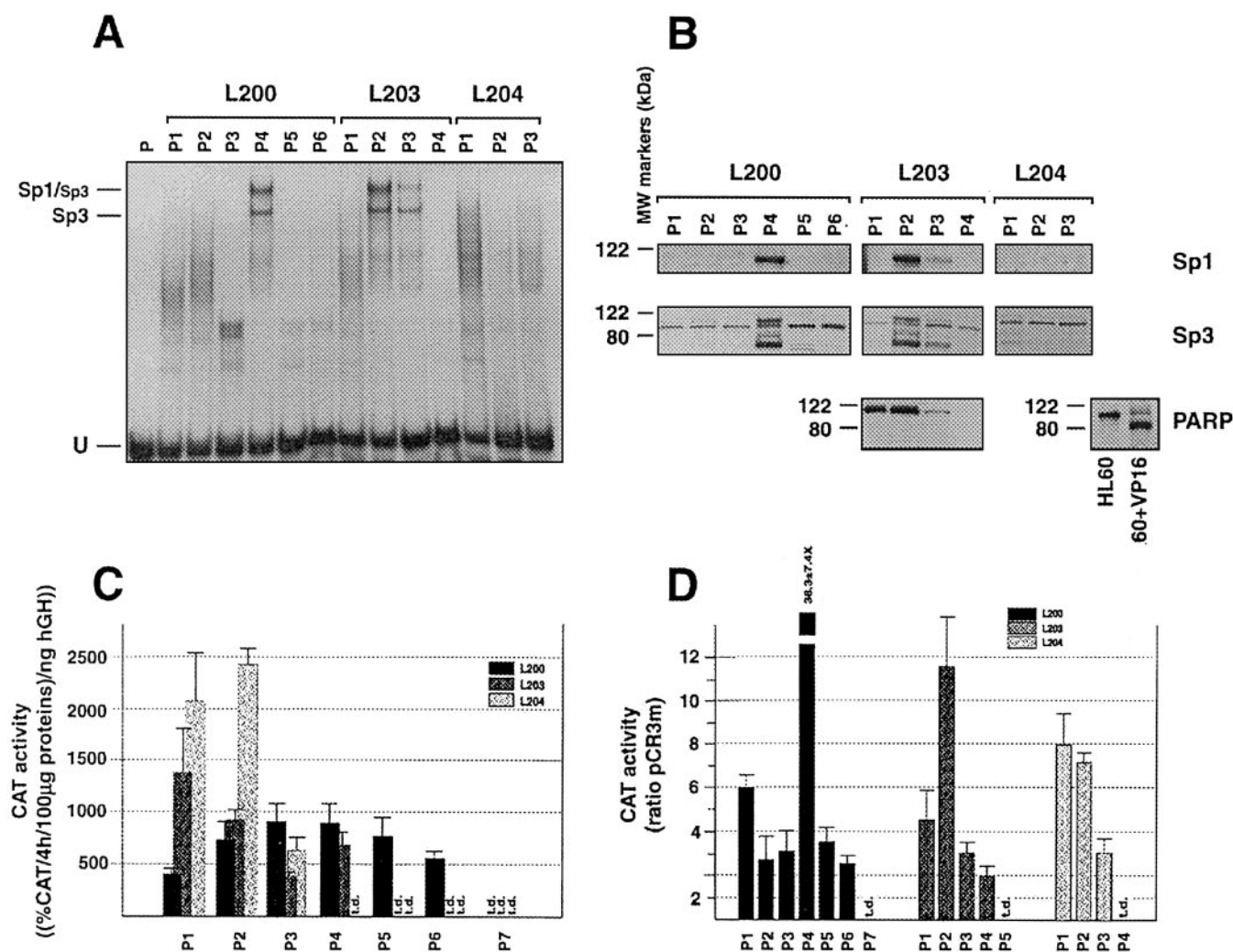


FIGURE 3. Influence of cell passages on Sp1/Sp3 DNA binding and transactivating properties. (A) EMSA analysis of Sp1/Sp3 binding in L200HCECs, L203HCECs, and L204HCECs at various passages. Nuclear proteins were obtained from L200HCECs, L203HCECs, and L204HCECs at different passages (P1–P6 for L200HCECs; P1–P4 for L203HCECs; P1–P3 for L204HCECs). Twenty micrograms of each was incubated with the Sp1 probe and formation of the Sp1/Sp3 DNA–protein complexes was monitored by EMSA on an 8% gel. U, unbound fraction of the probe; P, labeled probe with no added proteins. (B) Approximately 30 µg nuclear proteins from the extracts used in (A) were examined in Western blot analyses, using both Sp1 and Sp3 antisera. Expression of endogenous PARP was also monitored with the C-2-10 mAb in the P1 to P4 extracts from L203HCECs. Nuclear proteins prepared from either untreated or VP16-treated HL60 cells were also included as a control for the expression of the mature 113-kDa PARP protein or its 89-kDa apoptotic cleavage derivative, respectively. The position of the nearest molecular mass markers (β -galactosidase (molecular mass, 122 kDa); bovine serum albumin (molecular mass, 80 kDa) is indicated. (C, D) Transfection analysis in L200HCECs, L203HCECs, and L204HCECs at various passages. The rPARP basal promoter-bearing plasmid pCR3 and its mutated derivative pCR3/F2-F3-F4m were individually transfected into L200HCECs, L203HCECs, and L204HCECs at various passages (P1–P6 for L200HCECs; P1–P4 for L203HCECs; P1–P3 for L204HCECs). Cells were harvested 48 hours later and CAT activities measured and normalized. (C) Results obtained with the wild-type rPARP pCR3 plasmid. (D) Ratio of the activity directed by pCR3 over that of pCR3/F2-F3-F4m. t.d., terminally differentiated.

HL60 cells, exposed or not to the cell death inducer VP16, were also included as the control (Fig. 3B, bottom right). The 89-kDa PARP caspase 3 cleavage product, which was easily detected in the extract from apoptotic VP16-treated HL60 cells, was obviously missing in the P1 to P4 extracts from L203HCECs, clear evidence that these cells were never committed to apoptosis at any time. Therefore, that both Sp1 and Sp3 did not form in the extracts from passaged HCECs can be accounted for by the corresponding absence of expression of both Sp1 and Sp3 in these cells and not by their proteolytic cleavage by apoptotic caspases.

Consistent with the highly unstable expression of Sp1/Sp3 between the three HCEC cultures and passages used, transiently transfecting the wild-type construct pCR3 in HCECs also yielded highly variable CAT activity (Fig. 3C). It was

interesting that the lower the PARP promoter activity was at earlier passages, the higher the number of passages the HCECs could sustain. Indeed, L204HCECs had the highest PARP promoter activity at P1 (approximately five times more than that measured with the L200HCECs), closely followed by L203HCECs (3.5 times more than with L200HCECs). Unlike L203- and L204HCECs, HCECs presenting the best proliferative capabilities (e.g., L200HCECs) sustained a more constant PARP promoter activity over a much higher number of passages (up to P6). A detailed examination of the ratio of the CAT activity directed by the recombinant plasmid pCR3 over that directed by its mutated derivative pCR3/F2-F3-F4m, which no longer binds Sp1 and therefore highlights the real Sp1 influence on the PARP promoter, revealed striking differences. As shown in Figure 3D, both L200HCECs and L203HCECs had a peak ratio

of CAT activity that perfectly matched the peak of Sp1 binding activity observed in EMSA (Fig. 3A). Indeed, L200HCECs had detectable Sp1 proteins only at passage 4, which also translated into a dramatic 38-fold increase in the pCR3-to-pCR3/F2-F3-F4m ratio (Fig. 3D). Maximal Sp1 protein expression in L203HCECs was observed at P2. As for L200HCECs, this also perfectly matched the maximal influence both Sp1/Sp3 had on the rPARP promoter in these cells, the pCR3-to-pCR3/F2-F3-F4m ratio reaching a peak (a nearly 12-fold increase) at P2 in L203HCECs (Fig. 3D). As with L200HCECs, the pCR3-to-pCR3/F2-F3-F4m ratio dramatically dropped in the passages beyond that of the peak in L203HCECs. Unlike L200HCECs and L203HCECs, L204HCECs exhibited a moderately high pCR3-to-pCR3/F2-F3-F4m ratio at P1, which then decreased progressively up to P3, suggesting that the peak of Sp1/Sp3 activity must have occurred at the very beginning of the cell culture (P0).

Effect of Various Levels of Sp1/Sp3 on Properties of HCECs

L200HCECs, L203HCECs, and L204HCECs were shown to exhibit clearly distinctive proliferative properties *in vitro* that may be related to the precise moment the peak of Sp1/Sp3 expression appears in these cells. We therefore evaluated the ability of each of these HCECs to attach, grow, differentiate, and stratify properly within reconstructed tissue substitutes.^{12,14} To study the differentiation and stratification abilities of each group of HCECs, we took advantage of the model for tissue reconstruction by the autoassembly approach.³⁶ This approach combines the use of both dermal fibroblasts and corneal epithelial cells, which leads to the formation of a thicker epithelium than when corneal fibroblasts are used (Carrier P, Deschambeault A, Auger FA, Guérin SL, Germain L, ARVO Abstract 1634, 2001) thus facilitating the detection of the effect of Sp1/Sp3 expression on stratification. In contrast, the culture of corneal epithelial cells on reconstructed stroma made with corneal keratocytes leads to the formation of a thin epithelium comprising four- to six-cell layers, as is normally observed with cornea *in situ* (Carrier P, Deschambeault A, Auger FA, Guerin SL, Germain L, ARVO Abstract 1634, 2001). L200HCECs, L203HCECs, and L204HCECs were therefore cultured on top of reconstructed dermis made up of dermal fibroblasts induced to secrete and organize collagen by culturing them in the presence of ascorbic acid. HCEC differentiation was induced by culturing at the air-liquid interface for 11 days.

As shown in Figure 4, the production of reconstructed substitutes with L200HCECs at P1, P2, or P3, yielded properly stratified epithelia constituted of 13 to 15 layers of epithelial cells with a layer of basal cells that exhibited the typical morphology of corneal epithelial basal cells. Substitutes reconstructed with L203HCECs at P1 yielded an epithelium very similar to that composed of L200HCECs. However, when cells were used at P2, the epithelium thickness decreased to only eight to nine layers of cells, yet maintained an intact basal layer. These changes also correlated with the peak of Sp1/Sp3 expression observed in these cells (see Fig. 3). Very little cell stratification was observed when L203HCECs were used in P3, the reconstructed epithelium being constituted of only three or four layers of epithelial cells with no organized basal layer. This reduced efficiency of the HCECs to yield epithelia with proper histologic features was further emphasized when L204HCECs were used. Indeed, even P1 cells yielded poor quality epithelia made up of only a few cell layers. Yet the basal cell layer remained essentially intact up to passage 3, at which point L204HCECs in reconstructed substitutes no longer had the ability to proliferate and differentiate into suprabasal cells. L204HCECs at P3 were also poorly organized in the basal layer.

The state of differentiation reached by cultured cells can be closely examined by observing the expression and accumulation of specific members from the keratin family.⁵² Keratin filaments are formed of a pair of keratins, type I and type II.⁵³ The terminally differentiated cells that constitute the central area of the corneal epithelium express the K3/K12 pair of keratins.⁵⁴ The expression of K3 was therefore examined by immunofluorescence labeling of reconstructed substitutes produced with L203HCECs at P1 to P3. As shown in Figure 5, a proportion of the suprabasal epithelial cells stained positively for K3 when P1 L203HCECs were used. However, the highest level of K3 expression was observed in reconstructed substitutes produced with L203HCECs at P2, which also correlated with the peak of Sp1 expression in these cells, as revealed by EMSA (Fig. 3A), Western blot (Fig. 3B), and transfection (Figs. 3C, 3D) analyses. Only weak staining was observed in the epithelium from the substitute produced with L203HCECs at P3, which is consistent with the dramatic reduction in Sp1/Sp3 activity observed in these cells (see Fig. 3). As further evidence that proper K3 expression is dependent on the presence of Sp1-expressing cells in the culture,^{55,56} endogenous levels of Sp1 were examined by immunofluorescence analyses in the epithelia from the tissue substitutes produced with the L203HCECs of P1 to P3. As shown on Figure 5, the pattern of K3 expression was maximal in reconstructed epithelia with high endogenous expression of Sp1. Indeed, moderate and maximum levels of Sp1 were observed in the epithelium yielded by the L203HCECs of P1 and P2, respectively, whereas no staining was found in those from L203HCECs of P3. These results suggest that proper expression of endogenous Sp1 is absolutely required for HCECs to differentiate properly and express K3 on reconstructed tissue substitutes.

DISCUSSION

It is well known that many primary cultured cells are very difficult to maintain and passage in culture. Over the past few years, we have experienced such difficulties in culturing HCECs *in vitro*.^{12,14} Indeed, many cannot be maintained for more than three or four passages before they terminally differentiate. However, and for as yet unclear reasons, a few such cell cultures reach up to seven or eight passages.^{12,19,20} If these cell cultures are to be used for the engineering of human corneal substitutes, the best cell cultures should be chosen, and our results show that Sp1 expression may become one particularly interesting criterion for selecting them. Cells that terminally differentiate at early passages also stop migrating and proliferating, suggesting that the integrin-mediated cell adhesion mechanisms may be deeply altered during that process. Indeed, corneal wound-healing models have provided evidence that expression of integrins is altered depending on the state reached by corneal epithelial cells during the whole wounding process (Zhu L, Stepp, MA, ARVO Abstract 4743, 1996).^{57,58} Some of them increase their level of expression during the proliferative phase required for the cells to cover the wound. Expression is then stopped while basal corneal epithelial cells terminally differentiate through their vertical stratification. Because such profound changes in integrin expression has been reported to occur at the transcriptional level, we postulated that alterations in the abundance of transcription factors that are required for these genes to be properly expressed may account, at least in part, for these cells' progressing to terminal differentiation. One such likely candidate is Sp1. Indeed, the expression of many integrin subunit genes has been shown to be modulated by this transcription factor or by members of this family.²⁴⁻³² Sp1 belongs to a family of GC-rich binding transcription factors, the Sp family, that includes three other proteins: Sp2, Sp3, and Sp4.^{47,59,60}

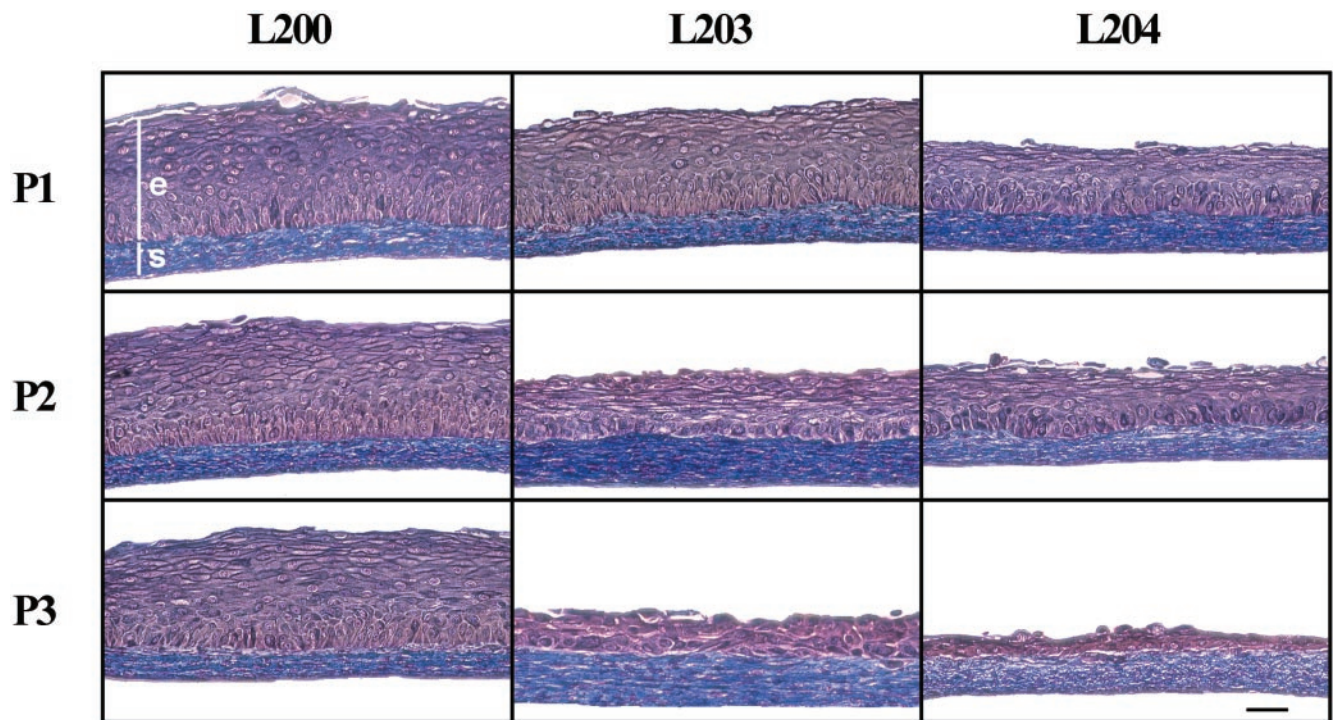


FIGURE 4. Stratification of HCECs in reconstructed tissue substitutes. Histologic cross sections of the reconstructed tissue substitutes after 11 days of culture at the air-liquid interface. L200HCECs, L203HCECs, and L204HCECs were cultured *in vitro* and then seeded on the reconstructed stroma after P1, P2, or P3. e, epithelium; s, stroma. Bar: 100 μm .

The comparison of the three zinc fingers from the DNA-binding domain of each Sp family member reveals that Sp1, Sp3, and Sp4 are more closely related to each other than to Sp2,⁶¹ which is one of the few reasons why we directed our attention solely to Sp1, Sp3, and Sp4. Besides, Sp2 was not reported to recognize the classic GC box that is bound by the remaining mem-

bers of this family, but rather targets GT-rich elements.⁶² The Sp family has been recognized recently as a subgroup within the larger Sp/XKLF family, which comprises at least 16 members that possess affinity for GC/GT-rich elements.⁶¹

In the present study, we investigated whether the frequent terminal differentiation of primary cultured HCECs is also ac-

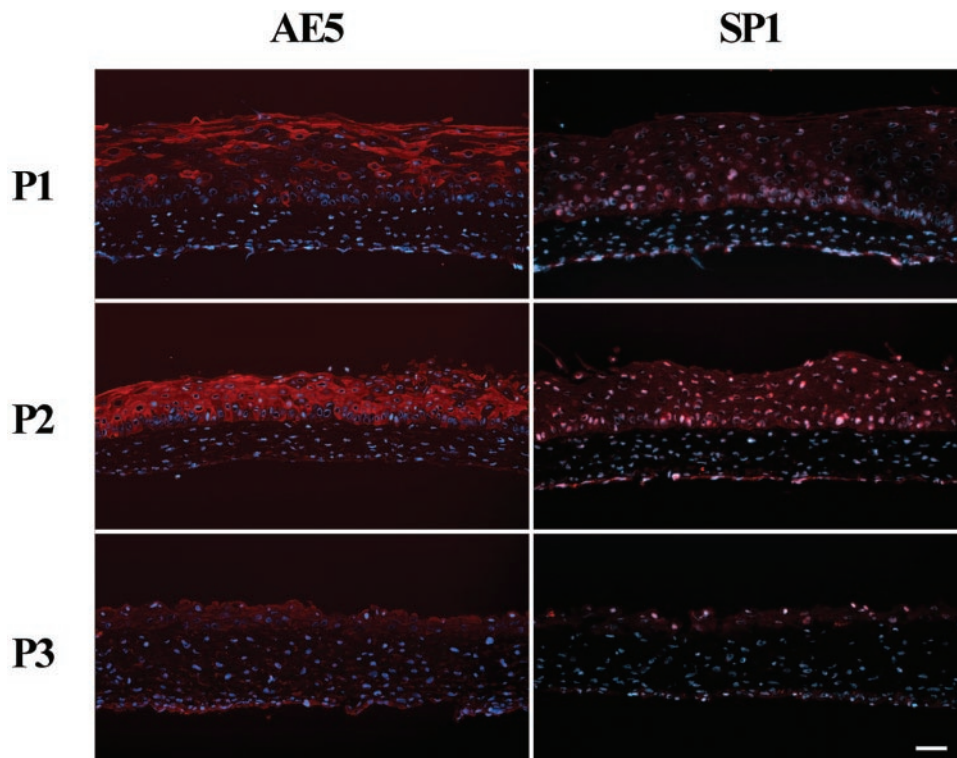


FIGURE 5. Immunofluorescence analysis of K3 and Sp1 expression in the epithelia of the reconstructed tissue substitutes. L203HCECs were expanded *in vitro* and seeded on the reconstructed stroma after P1, P2, or P3. Immunolocalization of both keratin K3 and Sp1 was performed using the AE5 and Sp1 mAbs and further revealed with TRITC (red). Nuclei were counterstained with Hoechst reagent 33258 (blue). Pink (blue+red) fluorescence of the nuclei represents double labeling of Sp1 and nuclei. Bar: 100 μm .

accompanied by various expression levels of the members of the Sp1 family. In our results, the DNA binding ability of Sp1 varied dramatically in HCECs isolated from the eyes of different human donors. These alterations are clearly not accounted for by alterations in the state of phosphorylation of Sp1, as has been recently reported^{25,63,64} but resulted from corresponding changes in the absolute amount of Sp1 protein. Furthermore, these alterations are not related to the age of the individuals from whom corneas were excised. There was a clear surge in the expression of Sp1 at P2 which then, in most HCEC cultures, decreased when cells were passaged beyond P2 (as occurred with L80HCECs and L81HCECs). HCECs that expressed substantial amounts of Sp1 at early passages (e.g., P1), also corresponded to those cells that terminally differentiated early. That low levels of Sp1 are found in differentiated or nonproliferating human corneal epithelial cells has also been reported in a study by Whitelock et al.,⁶⁵ who examined expression of a panel of transcription factors in the corneal epithelium of normal individuals and in patients with keratoconus. This noninflammatory disease progressively thins and distorts the central portion of the cornea, ultimately leading to visual impairment. Through the use of several procedures, they found only a low basal level of Sp1 expression in corneal epithelia from normal individuals. A dramatic surge in Sp1 expression was observed in the corneal epithelium of those patients affected by keratoconus.⁶⁵ These results are of particular interest, in that they link aberrant expression of Sp1 to upregulation of Sp1-regulated genes that encode enzymes and inhibitor genes affected in keratoconus.^{66,67} Most of all, they also bridge corneal epithelial wound healing, a process that is typical of keratoconus and that requires active proliferation and migration of the basal cells from the corneal epithelium to high levels of Sp1 expression.

The progressive reduction of the positive influence exerted by Sp1 on the rPARP gene promoter when HCECs were passaged is consistent with the reduction in Sp1 binding observed by Chen et al.⁵⁵ in differentiated rabbit (R)CECs. Although the rPARP promoter has been shown to bear a putative AP-2 binding site,²³ no evidence has been provided as to whether this transcription factor exerts any regulatory influence at all on rPARP gene transcription. However, it is interesting that undifferentiated RCECs have been shown to express high levels of the AP-2 transcription factor.⁵⁵ This may explain, at least in part, the activity driven by the rPARP promoter in the L200HCECs and L203HCECs at low passages (P1 to P3 for L200HCECs and P1 for L203HCECs) where no Sp1/Sp3 proteins are observed. Other transcription factors for which binding sites were identified in the rPARP basal promoter, such as members from the nuclear factor 1 (NF1) family,^{33,49} may contribute to maintain low basal promoter activity under Sp1-deficient culture conditions. Furthermore, the reduced transcriptional activity directed by the rPARP promoter when HCECs progress toward terminal differentiation is consistent with the decrease in PARP-1 mRNA levels that is typically associated with cellular differentiation^{68,69} and senescence.⁷⁰ Extinction of rPARP gene expression in differentiated cells is therefore likely to be the consequence of reduced expression of both Sp1 and Sp3.

The results presented in this study suggest that alterations in the level of expression of both Sp1 and Sp3 may trigger the cells to commit themselves to terminal differentiation. Undifferentiated basal epithelial cells from rabbit cornea have been reported to express both keratins K5 and K14.⁷¹ Keratins belong to a family of intermediate filament proteins that comprises more than 20 members with expression that is confined to epithelial cells. As cells grow exponentially, expression of the hyperproliferation markers K6 and K16 keratins is

switched on. Then, as the cells progress toward terminal differentiation through vertical stratification, expression of both K6 and K16 is turned off and that of both K3 and K12 is turned on.⁷² Expression of the K3 gene is heavily dependent on the recognition of a regulatory element from the K3 gene promoter designated the E site by Sp1.^{55,56} Furthermore, the ratio between Sp1 and the transcription factor activator-protein 2 (AP-2) has recently been found to be very critical for the expression of the K3 gene in RCECs, which, along with the K12 keratin, have been reported as markers of RCEC differentiation.⁵⁵ The dependence of K3 gene expression on the presence of Sp1/Sp3 was well demonstrated in the corneal epithelia from the tissue substitutes constructed in our study. Indeed, maximal expression of K3 in reconstructed epithelia produced with L203HCECs was observed with cells seeded at P2 on the dermal substitute, which is the precise condition under which maximal Sp1 expression was also observed. The immunologic detection of K3 is recognized as a marker of fully differentiated corneal epithelial cells. The fact that we detected only low levels of K3 expression in the epithelium reconstructed with P3 L203HCECs is puzzling. This could be explained by the fact that the nonproliferative, K3-positive, fully differentiated cells contained in the subcultured L203HCECs at P3 could not be maintained on the dermal substitutes. The thin epithelium observed may have resulted solely from the low proliferative properties of a few epithelial cells still present in L203HCECs at P3. Such cells would be expected to express only low amounts of Sp1 and to progress rapidly toward terminal differentiation after only a few cell doublings, therefore producing poorly stratified epithelium in reconstructed substitutes as those seen with L203HCECs and L204HCECs at P3. Under such conditions, epithelial cells probably had no time to accumulate sufficient amounts of K3 to be detected by the usual immunologic procedures.

Because they are recognized as being much closer to normal cells than transformed cells or cell lines are, primary cultured cells represent a very powerful model for gene promoter studies.⁴⁻⁶ However, the results yielded by the transfection of the rPARP promoter, which has a transcriptional activity largely dependent on Sp1,³³ highlighted how critical it is to conduct such analyses in cells that reach the same number of passages when primary cultures are used as a model. As pointed out by Apt et al.,⁴⁶ research on Sp1 might be biased by the fact that Sp1 is apparently unspecifically upregulated in transformed cultured cells, whereas its expression in different tissues varies significantly *in vivo*, as reported by Saffer et al.⁷⁴ It is noteworthy that, so far, virtually all gene-promoter studies have been conducted in transformed cultured cells and not in primary cultures of normal (untransformed) cells, most likely for the reasons stated previously (refer to the introduction section). The results presented herein add further to the complexity of the Sp1 picture by providing evidence that its expression is also subjected to higher levels of regulation that depend on the state of differentiation reached by the cell system used. The use of primary cultured cells may be entering a new era as a tool to study and characterize gene promoter and regulatory sequences, as well as providing living starting material for clinical applications through tissue engineering. It is then vital to have a better understanding of how parameters such as cell passages and the human donor's origin influences the results of such studies.

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