Characterization and Comparison of Intercellular Adherent Junctions Expressed by Human Corneal Endothelial Cells In Vivo and In Vitro

Ying-Ting Zhu, Yasutaka Hayashida, Ahmad Kheirkhah, Hua He, Szu-Yu Chen, and Scheffer C. G. Tseng

PURPOSE. Human corneal endothelial cell (HCEC) proliferation is controlled by HCEC junctions, but the mechanism of proliferation remains unknown. The authors sought to characterize adherent junction components in vivo HCECs and to compare their gene expression and their proliferative potential with those of in vitro counterparts.

METHODS. Stripped human Descemet membranes were digested with collagenase A, and the resultant HCEC aggregates were cultured for 7, 14, and 21 days in supplemented hormonal epithelial medium (SHEM). The growth of HCEC monolayers was monitored by BrdU labeling performed 24 hours before termination. In vivo and in vitro HCECs were subjected to immunostaining to FITC-phalloidin and antibodies to different junction components and BrdU. Their mRNA expressions were determined by RT-PCR.

RESULTS. In vivo HCECs expressed transcripts of N-, VE-, E-, and P-cadherins, α-, β-, γ-, and p120-catenins, and p190. In vitro HCEC counterparts also expressed all these mRNAs except P-cadherin. In vivo HCECs displayed continuous circular F-actin, N-cadherin, β- and p120-catenins, and p190, discontinuous circular VE-cadherin bands at or close to cell junctions, and E-cadherin in the cytoplasm. Such an in vivo pattern was gradually achieved by in vitro HCECs at day 21 and was correlated with a progressive decline of BrdU labeling.

CONCLUSIONS. In vivo and in vitro HCECs displayed distinct protein cytolocalization of N-, VE-, and E-cadherins, β- and p120-catenins, and p190. Progressive maturation of adherent junctions was associated with a decline of the proliferative potential. This information allows us to devise new strategies to engineer in vitro HCECs by targeting these components.

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From Tissue Tech, Inc., Ocular Surface Center, and Ocular Surface Research and Education Foundation, Miami, Florida.

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Corresponding author: Scheffer C. G. Tseng, Ocular Surface Center, Suite 213, 7000 SW 97th Avenue, Miami, FL 33173; tseng@ocularsurface.com.
vitro HCECs and demonstrated that gradual maturation of adherent junctions in HCEC monolayers generated by our cultivation method was associated with a marked decline of the proliferative potential. This baseline characterization was important for devising a strategy to target these components for tissue engineering of HCECs in the future.

MATERIALS AND METHODS

Materials

Dulbecco modified Eagle medium (DMEM), Ham/F12 medium, human epidermal growth factor (bFGF), HEPES buffer, Hanks balanced salt solution (HBSS), phosphate-buffered saline (PBS), amphotericin B, gentamicin, fetal bovine serum (FBS), 0.25% trypsin/0.53 mM EDTA (trypsin/EDTA), and fluorescein phallolidin were purchased from Invitrogen (Carlsbad, CA). Collagenase A was obtained from Roche Applied Science (Indianapolis, IN). Hydrocortisone, dimethyl sulfoxide, cholera toxin, insulin-transferrin-sodium selenite media supplement, bovine serum albumin, agarose, PCR marker, paraformaldehyde, Triton X-100, Hoechst 33342 dye, and FITC-conjugated goat anti–mouse and anti-rabbit IgG were purchased from Sigma-Aldrich (St. Louis, MO). Texas Red dye-conjugated donkey anti–mouse IgG was obtained from Jackson Immunoresearch Laboratories Inc. (West Grove, PA). Specific monoclonal anti–VE-cadherin, -p190, -β-catenin, -ZO-1, -α-Brdu, and polyclonal anti-N-cadherin (type I), -E-cadherin (type I), and -p120-catenin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) or BD Biosciences (San Jose, CA). Mounting medium (Vectashield; Vector Laboratories Inc., Burlingame, CA) and the necessary kits (RNasey Mini Kits [Qiagen, Valencia, CA] and High Capacity Reverse Transcription and TaqMan Universal PCR Master Mix kits [Applied Biosystems, Foster City, CA]) were obtained.

HCEC Isolation and Culture

Human corneas were handled in accordance with the tenets of the Declaration of Helsinki. The isolation and culturing of HCECs followed methods recently described.4 In short, human donor corneoscleral tissues, from which their central corneal buttons had been used for corneal transplantation, were obtained from the Florida Lions Eye Bank (Miami, FL). All tissues from donors between 21 and 72 years of age were maintained at 4°C in solution (Optisol; Chiron Vision, Irvine, CA) for less than 5 days before they were rinsed three times with DMEM containing 50 mg/ml gentamicin and 1.25 mg/ml amphotericin B. The trabecular meshwork was cleaned under microscopy, and the rim was trephined within Schwalbe line. Descemet membranes containing HCECs were stripped from the posterior surface of the peripheral corneoscleral tissue under a dissecting microscope and digested at 37°C for 16 hours with 1 mg/ml collagenase A in the SHM, which was made of an equal volume of HEPES-buffered DMEM and Ham F12 supplemented with 5% FBS, 0.5% dimethyl sulfoxide, 2 ng/ml bFGF, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 0.5 μg/ml hydrocortisone, 1 μM cholera toxin, 50 μg/ml gentamicin, and 1.25 μg/ml amphotericin B. After digestion, HCECs formed aggregates, which were collected by centrifugation at 2000 rpm for 3 minutes to remove the digestion solution. The aggregates were directly cultured in 24-well dishes coated with collagen IV for up to 21 days in SHM. Some of the aggregates were pretreated with trypsin/EDTA at 37°C for 5 minutes before the aforementioned cultivation.

Proliferative Activity of HCEC Monolayers

As noted during our experiments, without trypsin/EDTA treatment, each HCEC aggregate attached and expanded from the center of the aggregate to form a monolayer in 5 to 5 days on collagen IV-coated dishes in SHM. To measure the proliferative activity, BrdU was added at a final concentration of 10 μM in the culture medium for 24 hours before termination for each cultivation time point using cultures derived from three different donors aged 44, 53, and 55. For each culture, at least 2000 total nuclei were counted, and the BrdU labeling index, defined as the number of BrdU-labeled nuclei divided by the total number of labeled and unlabeled nuclei, was calculated.

PCR Primer Design, RNA Extraction, Reverse Transcription–Polymerase Chain Reaction

Stripped Descemet membranes and HCEC monolayers cultured at day 2 or day 14 from three donors aged at 22, 67, and 72 were used for this experiment. Some of the cultures were briefly treated with or without trypsin/EDTA. Total RNA from the samples was extracted using mini kits (RNeasy Mini Kits; Qiagen), extracted RNAs were reverse transcribed (High Capacity Reverse Transcription Kits; Applied Biosystems), and respective cDNAs of each junction component were amplified by PCR using specific primers and DNA polymerase in a PCR system (‘7000 Real-Time PCR System; Applied Biosystems). PCR primers for the cDNA from a particular gene, listed in Table 1, were designed by blasting the entire gene bank to ensure the specificity of each primer pair so that they did not overlap with other types or isoforms of cadherins, catenins, or any other cDNAs. To avoid false-positive reactions potentially from contaminated DNA, every primer pair was par intron. We used cDNAs reverse transcribed from miRNAs extracted from ARPE-19 cells known to express most known adherent junction components as the positive control and water as the negative control. The PCR profile consisted of 6 minutes of initial activation at 95°C followed by 35 cycles of 10-second denaturation at 95°C, 1-minute annealing at 60°C, and 1-minute extension at 72°C. We confirmed the genuine identity of each PCR product by the correct size determined by 2% agarose gels using ethidium bromide staining and PCR marker (EC3 Imaging System; BioImaging System, Upland, CA).

Immunostaining

Flat mounts of stripped human Descemet membranes containing HCECs from three donors aged 55, 61, and 66 and HCEC sphere cultures from three donors aged 33, 58, and 64 were prepared by brief air drying and fixation in 4% formaldehyde, pH 7.0, for 15 minutes at room temperature. They were rehydrated in PBS, incubated with 0.2% Triton X-100 for 15 minutes, and rinsed three times with PBS for 5 minutes each. After incubation with 2% BSA to block nonspecific staining for 30 minutes, they were incubated with monoclonal anti–VE-cadherin, -p190, -β-catenin, -ZO-1, -α-Brdu, and polyclonal anti-N-cadherin (type I), -E-cadherin (type I), and -p120-catenin antibodies (all at 1:50 dilution) for 16 hours at 4°C. After three washes with PBS, they were incubated with FITC-conjugated goat anti–mouse or anti–rabbit IgG for 60 minutes, followed by counterstaining with 10 μg/ml Hoechst 33342 (blue color) for 10 minutes. Specimens were then mounted with the mounting medium (Vectashield; Vector Laboratories) and analyzed under a fluorescence microscope (Eclipse TE2000-U; Nikon, Tokyo, Japan). For BrdU labeling, samples were fixed with 75% methanol plus 25% acetic acid, denatured by 2 M HCl, and neutralized by 0.1 M borate buffer, pH 8.5. Monoclonal anti-Brdu antibody and Texas Red dye-conjugated donkey anti–mouse IgG for 60 minutes, followed by counterstaining with 10 μg/ml Hoechst 33342 and analyzed under a fluorescence microscope (Eclipse TE2000-U; Nikon). Corresponding mouse and rabbit sera were used as negative controls for primary monoclonal and polyclonal antibodies, respectively.

Statistical Analysis

All summary data were reported as mean ± SD calculated for each group and compared using Student’s unpaired t-test (Excel; Microsoft, Redmond, WA). Test results were reported as two-tailed P values, where P < 0.05 was considered statistically significant.
RESULTS

Growth Characteristics of Cultured HCEC Monolayers

After overnight digestion with collagenase A, an intact stripped Descemet membrane of each donor peripheral cornea typically yielded a total of 10 to 12 aggregates. As reported previously,4 without a brief trypsin-EDTA treatment, approximately 80% of these aggregates attached to collagen IV-coated dishes and started to migrate out as a monolayer by day 3 when cultured in SHEM (Fig. 1A). Phase-contrast microscopy revealed that these monolayers, once growing, might or might not have a compacted center corresponding to where the aggregate first attached (Fig. 1B). Monolayers with compact centers continued to expand in size, with nearly all cells tightly associated with one another adopting a hexagonal shape; cells in the periphery tended to be more spindly and migratory (Fig. 1B). These monolayers slowly expanded until day 14 (Fig. 1C) and throughout the entire cultivation time up to day 21 (data not shown). Monolayers without compacted centers tended to be more spindle-like cells cultured at day 7 (Fig. 1D), eventually dying at day 21 (Fig. 1E), or to be fibroblastic-like cells at day 7 (Fig. 1F). The proportion of monolayers with and without a compact center was approximately 2:1.

Figure 1. Growth characteristics of cultured HCEC monolayers. A representative culture from a 35-year-old donor showed how HCECs migrated from an isolated aggregate on a collagen IV-coated dish in SHEM on day 3 (A), generated a monolayer with a compact center on day 7 (B, migratory cells are marked in the periphery), and expanded in size on day 14 (C). HCEC aggregates without a center expanded at day 7 (D) and eventually died at day 21 (E). Some aggregates became fibroblast-like cells at day 7 (F) and grew like fibroblasts (data not shown). Bars represent 100 μm.

Table 1. Nucleotide Sequences of Primers Used in PCR

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<th>Human Gene Name</th>
<th>Primer Sequence</th>
<th>Product Size</th>
<th>GenBank No.</th>
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<td>AF240635</td>
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<td>BC014462</td>
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Transcript Expression of Adherent Junction Components by In Vivo and In Vitro HCECs

To know which adherent junction components might be expressed by in vivo HCECs, we first performed RT-PCR with total RNAs extracted from the peripheral corneal endothelia of 22-, 58-, and 67-year-old donors using specific primers listed in Table 1. With GAPDH as a loading control and the results from 22-year-old donor as a representative (Fig. 2), we found that in vivo HCECs from all three donors expressed transcripts of all adherent junction components such as N- (types I, II), VE-, E- (types I, II), and P-cadherins, α-, β-, γ-, and p120-catenins, and p190 (Fig. 2, lane 1). Consistent with previous studies, the ZO-1 transcript was also expressed by in vivo HCECs. After brief treatment with trypsin/EDTA to disrupt intercellular junctions, HCEC aggregates cultured for 2 days lost expression of all transcripts except for p120 (Fig. 2, lane 2). In contrast, without brief trypsin/EDTA treatment, HCEC aggregates still retained the expression of VE-cadherin, N-cadherin (type II), β-catenin, and p120 after 2 days of culture (Fig. 2, lane 3) and expressed all transcripts of these adherent junction components except E- (types I and II) and P-cadherins when HCEC monolayers were cultured up to day 14 (Fig. 2, lane 4). When cultures were extended to day 21, transcripts of E-cadherin (types I, II), but not of P-cadherin, were detected by RT-PCR (data not shown). These results collectively indicated that in vivo HCECs indeed expressed all these adherent junction components and that such expression was largely abolished shortly when adherent junctions were disrupted by brief trypsin/EDTA treatment. When adherent junctions were not intentionally disrupted by trypsin/EDTA, expression of some of these transcripts was retained during the early stage of monolayer growth.

Cytolocalization of Adherent Junction Components Expressed by In Vivo HCECs

To verify that proteins of the aforementioned junction components were indeed expressed by HCECs in vivo, their cytolocalization in reference to the intercellular junction was characterized by immunofluorescence staining. Compared with the characteristic hexagonal configuration of in vivo HCECs (Fig. 3A), phalloidin staining helped localize a preferential circular band of cytoskeletons formed by F-actins close to the intercellular junction (Fig. 3C). Immunostaining to N-cadherin (type I) also showed a continuous circular band (Fig. 3D), but that to VE-cadherin showed a discontinuous circular band (Fig. 3B). N-cadherin distributed similarly at the intercellular junction. In contrast, immunostaining to E-cadherin (type I) was diffuse in the cytoplasm (Fig. 3F). There was no immunostaining to N-cadherin (type II), E-cadherin (type II), and P-cadherin (data not shown), indicating N-cadherin (type II), E-cadherin (type II), and P-cadherin do not play an important role in the adherent junction of HCECs. Immunostaining to β-catenin and p190 also revealed preferential distribution along the intercellular junction (Figs. 3G, 3I), whereas p120 was distributed equally in the intercellular junction and cytoplasm (Fig. 3H). As reported, ZO-1 was primarily distributed to the intercellular junction (Fig. 3J). The same results were obtained in three different donors and from the central cornea of two donors (data not shown). The findings disclosed unique cytolocalization of different adherent junction components at intercellular junctions.

Gradual Maturation of Adherent Junction Components by In Vitro HCEC Monolayers

Because transcript expression of several adherent junction components showed gradual maturation toward the in vivo pattern (Fig. 2), we sought to determine whether their protein expression and cytolocalization were also gradually restored to the in vivo pattern (Fig. 3). To do so, we first performed immunostaining to N-cadherin and p120. Phase-contrast micrographs indicated that HCEC monolayers cultured on collagen IV-coated dishes in SHEM at day 7, day 14, and day 21 adopted similar characteristic hexagonal shapes (Fig. 4, left column). Their intercellular junctions were more visible at days 7 and 14 than at day 21. The linear staining of N-cadherin at the intercellular junction was incomplete at days 7 and 14 but became complete at day 21 (Fig. 4, left column). Staining to p120 was mainly in the cytoplasm at day 7, in both the cytoplasm and the cell junction at day 14, but predominantly in the cell junction at day 21 (Fig. 4, right column). These results suggested that expression and cytolocalization of N-cadherin by in vitro HCEC monolayers gradually reached the in vivo pattern at day 21. Interestingly, p120 in HCECs cultured at day 21 was preferentially distributed, rather than equally distributed in the cell junction and the cytoplasm, compared with the staining pattern of in vivo HCECs. The significance of this p120 distribution change during in vitro culture of HCECs remains to be explored.

**FIGURE 2.** Transcript expression of adherent junction components by HCECs in vivo and in vitro. PCR fragments were of the expected sizes (not shown) and were expressed by in vivo HCECs (lane 1), by HCEC aggregates treated by a brief trypsin/EDTA treatment and cultured for 2 days (lane 2), and by those without trypsin/EDTA treatment cultured on day 2 (lane 3) or day 14 (lane 4). The band (lane 2) for E-cadherin (type I) was an artifact.
To further verify whether this was the case for other components, we performed immunofluorescence staining to VE-cadherins, β-catenins, and p190 and compared that to F-actin (by phalloidin) and ZO-1 in HCEC monolayers cultured at day 21. Similar to what has been reported, F-actin–containing cytoskeletons were distributed as continuous circular bands close to the intercellular junction of each cell (Fig. 5A). Staining to VE-cadherin was noted in the junction and the cytoplasm (Fig. 5B), that of E-cadherin predominantly remained in the cytoplasm, similar to that in vivo (Fig. 5C), that of β-catenin remained exclusively in the intercellular junction (Fig. 5D), and that of p190 spread from the junction to the cytoplasm (Fig. 5E). As reported, immunostaining to ZO-1 was clearly outlined mostly at the intercellular junction (Fig. 5F). These results indicated that HCEC monolayers cultured up to day 21 indeed achieved patterns of expression and cytolocalization of all adherent junction components similar to what was observed in vivo.

**Decline of the Proliferative Potential in HCECs In Vitro**

To determine whether the maturation of the cell junction was correlated to the loss of the proliferative activity in HCEC monolayers in vitro, we performed BrdU-labeling experiments at days 7, 14, and 21. Results showed that there were a number of BrdU-labeled nuclei (red) in HCEC monolayers cultured at day 7 (Fig. 6A), but almost none in those cultured at day 21 (Fig. 6C). The average labeling index (N = 3) was 10.2% ± 1.1% at day 7, which was significantly reduced to 2.2% ± 0.1% at day 14 and only 0.1% ± 0.03% at day 21 (Fig. 6C; P < 0.05). These results confirmed that the maturation of intercellular adherent junctions was correlated with a rapid decline of the proliferative potential in HCEC monolayers during cultivation for 21 days.

**FIGURE 3.** Cytolocalization of junction components expressed by in vivo HCECs. Compared with hexagonal HCECs in vivo (A), cytolocalization was detected by immunofluorescence staining to phalloidin (C), N-cadherin (type I) (D), VE-cadherin (E), E-cadherin (type I) (F), β-catenin (G), p120 (H), p190 (I), and ZO-1 (J). Negative control using normal mouse serum or rabbit serum showed the same result as shown in (B). (blue) Nuclear counterstaining. Scale bar, 100 μm.

**FIGURE 4.** Gradual maturation of N-cadherin and p-120 by in vitro HCEC monolayers. Compared with the phase-contrast micrographs of HCEC monolayers at day 21 (A), cytolocalization was examined by immunofluorescence staining to N-cadherin and showed gradual maturation from day 7 (C) to day 14 (E) and day 21 (G). Similar gradual maturation was also noted by immunofluorescence staining to p120 from day 7 (D) to day 14 (F) and day 21 (H). Negative control was shown (B). Blue: nuclear counterstaining. Scale bar, 100 μm.
DISCUSSION

Cadherin-mediated intercellular adherent junctions are present in all solid tissues of many multicellular organisms (for a review, see Pokutta and Weis\textsuperscript{39}). Therefore, it was not surprising to note such expression in HCECs. Previous studies have shown the cadherins are present at intercellular adherent junctions of in vivo HCECs\textsuperscript{36} but the exact type is not resolved because of the use of a nonspecific pan–cadherin antibody. Although E-cadherin (type I)\textsuperscript{37} and N-cadherin (type I)\textsuperscript{38} transcripts were found later, the detailed characterization and cytolocalization of other components was not disclosed until our study. We noted that in vivo HCECs expressed mRNAs and proteins of N-cadherin (types I and II for its mRNA and type I for its proteins), VE-cadherin, and E-cadherin (types I and II for its mRNA and type I for its proteins) and that both N-cadherin (type I) and VE-cadherin were distributed as continuous and discontinuous circular bands, respectively, along the intercellular junction, whereas E-cadherin (type I) was primarily distributed in the cytoplasm (Figs. 2, 3). Although P-cadherin mRNAs were found only in vivo and N-cadherin (type II) and E-cadherin (type II) were found in vivo and in vitro by RT-PCR, their proteins were not detected by immunostaining in vivo, collectively suggesting that P-cadherin, N-cadherin (type II), and E-cadherin (type II) might not play a significant role in HCECs. In fact, the levels of type II N- and E-cadherin mRNAs were substantially lower than the levels of type I N- and E-cadherin in HCECs in vivo and in vitro, according to our unpublished real-time PCR results (data not shown). Such expression pattern and cytolocalization of cadherins for in vivo HCECs are unique and differ from those of all epithelial cells in which the prototypic E-cadherin is distributed in the intercellular junction, from all vascular endothelia in which the prototypic VE-cadherin is localized at the intercellular junction while N-cadherin is in the cytoplasm,\textsuperscript{40} and from neural cells in which only N-cadherin is present at the cell junction.\textsuperscript{41} Further studies of the mechanism by which such a unique cytolocalization pattern is achieved might help unravel how N-cadherin and VE-cadherin work together in endowing HCECs with the crucial function to control corneal stromal hydration.

Associated with cadherins at the adherent junction are \( \alpha \)-catenin, \( \beta \)-catenin, \( \gamma \)-catenin, and p120-catenin, which collectively help link cadherins to actin filaments (for a review,

FIGURE 5. Maturation of adherent junction components by in vitro HCECs at day 21. Immunofluorescence staining of F-actin (by phalloidin) (A), VE-cadherin (B), E-cadherin (C), \( \beta \)-catenin (D), p190 (E), and ZO-1 (F) also showed gradual maturation. Negative control was similar to Figure 4B (data not shown). Blue: nuclear counterstaining. Scale bar, 100 \( \mu \text{m} \).

FIGURE 6. Decline of proliferative potential in HCEC monolayers. BrdU labeling (red) in the nucleus (blue) was performed to assess the proliferative potential of HCEC monolayers cultured at day 7 (A), day 14 (B), and day 21 (C). The labeling index was 10.2\% \( \pm \) 1.1\% at day 7 and 2.2\% \( \pm \) 0.1\% at day 14 but only 0.1\% \( \pm \) 0.03\% at day 21 (C; \( P < 0.05 \)). \( N = 3 \). Scale bar, 100 \( \mu \text{m} \).
see Perez-Moreno and Fuchs). Herein, we noted that mRNAs of α-, β-, and γ-catenins were expressed by in vivo HCECs (Fig. 2). We did not perform immunostaining of α- and γ-catenins because the staining pattern was reported by Petroll et al. to be located at the intercellular junction by in vivo rabbit corneal endothelium and the staining pattern of γ-catenin was weakly expressed at the same location by in vivo HCECs. We noted that β-catenin was clearly localized at the intercellular junction of in vivo HCECs (Fig. 3). For the first time, we noted that the mRNA and proteins of p120 were also expressed by in vivo HCECs and were distributed in the intercellular junction and the cytoplasm (Figs. 2, 3). Similar to what has been reported by others, we also found that ZO-1 mRNA and proteins were expressed by in vivo HCECs at intercellular junctions (Figs. 2, 3), noticeably localized in the cell junction as continuous circular bands for in vivo HCECs but punctuated bands for in vitro HCECs. Because p190, though not a component of adherent junctions, regulates p120, our study noted that mRNA and proteins of p190 were expressed by in vivo HCEC, and its cytolocalization was close to the intercellular junction but also in the cytoplasm (Figs. 2, 3). The expression pattern and cytolocalization by in vivo HCECs from the peripheral cornea were found to be the same as those from the central cornea (data not shown). Similar to what has been reported for other cell types (for a review, see Erez et al.), such expression patterns of all adherent junction components must play a key role in stabilizing F-actin–based cytoskeletons in vivo HCECs, giving rise to a unique circular pericellular F-actin band reported by earlier studies.

As is commonly known, brief treatment of trypsin/EDTA is sufficient to disrupt intercellular adherent junctions. Herein, we observed that such treatment, which was reported to immediately disperse HCECs into separately scattered cells on plastic or collagen IV–coated dishes also dramatically downregulated the transcript expression of all junction components to nothing except for p120 in HCECs cultured for only 2 days (Fig. 2, lane 2). Such rapid downregulation has been known to follow the physical disruption of cadherin junctions to liberate cadherins and β-catenin into the cytoplasm for ubiquitin-mediated proteolysis. Under this circumstance, unlike all other catenins, the p120 transcript remained stable in the cytoplasm, consistent with a previous report. Trypsin/EDTA or EDTA alone is known to promote proliferation for cultured HCECs. We noted that such treated HCECs, though proliferating more quickly, adopted a fibroblastic morphology, resembling the endothelial-mesenchymal transition described by Lee and Kay, especially under the stimulation of bFGF. Herein, we noted that cells treated with trypsin/EDTA eventually died after only one or two passages or became fibroblastic-like even in the absence of bFGF (Fig. 1F). Thus, we suspect that trypsin/EDTA treatment to disrupt intercellular adherent junctions is at odds with the primary objective of maintaining the normal HCEC phenotype.

In contrast, we noted a different transcript expression pattern by HCEC aggregates if not submitted to a brief treatment of trypsin/EDTA before cultivation. Specifically, within the cadherin family, the expression of N-cadherin (type II) and VE-cadherin transcripts was not downregulated, whereas within the catenin family, the expression of β-catenin and p120 transcripts continued at day 2 after cultivation (Fig. 2, lane 3). Such a difference explained why HCECs continued to maintain a monolayer with close cell–cell contacts by day 7 (Fig. 7) when discontinuous intercellular bands of N-cadherin were maintained while p120 was primarily in the cytoplasm (Fig. 4). Disruption of intercellular junctions without complete suppression of all adherent junction components in this cultivation model was sufficient to trigger the active proliferation of HCECs, as evidenced by a high BrdU-labeling index at day 7 (Fig. 6). Such a high proliferative rate during the first 7 days of cultivation resembled what has been reported with brief EDTA treatment. Furthermore, a higher proliferative potential was noted in younger donors (data not shown), similar to what has been noted by others.

By day 14, there was an increase of transcript expression of all adherent junction components to the level of the in vivo counterpart except for E-cadherin (Fig. 2, lane 4). Specifically, the expressions of N-cadherin (type I), α-catenin, γ-catenin, and p190 transcripts were upregulated (Fig. 2, lane 4), whereas the cytolocalization pattern of N-cadherin and p120 (Fig. 4) and all other components did not reach an in vivo pattern until day 21 (Fig. 5), when the proliferative potential markedly declined (Fig. 6). These results strongly suggested that the proliferative potential of in vitro HCECs was greatly affected by gradual maturation of adherent junctions to an in vivo pattern. Taken together, we speculate that the proliferation of HCECs can be promoted while the normal phenotype is maintained by a new strategy to downregulate some, but not all, adherent junction components for achieving ex vivo expansion of HCECs in the future.

References

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