The Effects of Different Culture Media on Human Corneal Endothelial Cells

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Purpose. To investigate the most appropriate media condition for the proliferation and functional maintenance of human corneal endothelial cells (HCECs).

Methods. We cultured HCECs in traditional media (medium A or D) and in stem cell media (medium E or N). The morphology of the cells was evaluated by inverted microscopy. Collagen, type VIII, alpha 2 and sodium-potassium adenosine triphosphatase (Na^+-K^+ ATPase) expression were analyzed as differentiation markers. Octamer-binding transcription factor 3/4, glial fibrillary acidic protein, nestin and β-catenin expression were evaluated as stem cell associated proteins. The cell proliferation rate was evaluated with a cell counting kit-8 assay. Wound healing assays were also performed. The transendothelial electrical potential difference (TEPD) value was used to estimate the endothelial cell permeability in vitro.

Results. The proliferation and morphology analyses demonstrated that there were significant differences between the media. The expression of differentiation markers and stem cell–associated proteins was different between the media. Medium D resulted in higher proliferation rates compared with the other media, while still maintaining the differentiation potential and surface marker expression profile characteristic of HCECs. Compared with other media, TEPD was higher in medium N.

Conclusions. Culture medium D was superior to the other media with regard to the expression of stem cell–associated proteins, proliferation, and cell migration. However, medium N was more appropriate than the other three media with regard to maintaining the proper cell shape and function.

Keywords: human corneal endothelial cells, nestin, COL8A2, Na^+-K^+ ATPase, OCT3/4, GFAP, media

There are three types of cells in the human cornea: corneal epithelial, stromal, and endothelial cells. Although corneal epithelial cells have been reported to originate from the surface ectoderm,[1] corneal stromal cells and endothelial cells originate from neural crest origin.[2] Corneal endothelial stem cells have been suggested to be located at the transition zone adjacent to the trabecular meshwork.[3,4] Thus, proteins associated with neural stem cells may be expressed in cultured corneal endothelial cells.

Human corneal endothelial cells (HCECs) form a monolayer on the posterior surface of the cornea; they maintain the barrier between the cornea and aqueous humor and are responsible for pumping fluid out of the cornea.[5] Human corneal endothelial cells play an important role in maintaining the clarity and thickness of the cornea.[6] When a large number of cells are damaged due to disease or trauma, the endothelium loses both its barrier and pump functions resulting in corneal edema and blindness.[7] Although HCECs have limited regenerative potential in vivo, they have been reported to have proliferative capacity and to be able to proliferate in vitro.[5] Thus, cultured HCEC transplantation could be a treatment for corneal endothelial diseases or for the repair of corneal damages. For the culturing of HCECs, several different media have been developed.[5,8,9] However, the effects of different media on the HCECs have not been thoroughly studied. The type of medium used regulate cell phenotype including survival, differentiation, transdifferentiation, migration, and proliferation,[10,11] as well as providing nutrient molecules to the cells.[11] The expression of proteins associated with differentiation or with proliferation depend on the medium and has effects on cell phenotype.[10–12] It is desirable to develop media that maintain cell functions and induce proliferation without transdifferentiation. This is important in regenerative medicine as well as in the experiment that utilize cell culture.

In this study, we investigated the most appropriate media conditions for the proliferation and maintenance of function of HCECs.

Methods

This study was performed according to the tenets of the Declaration of Helsinki and was reviewed and approved by the

institutional review board/ethics committee of Hallym University Medical Center. The corneas were purchased from Lions Eye Bank (Portland, OR, USA), which obtained informed consent for all tissue samples held, and cultured. HCECs were obtained from discarded corneal–scleral rings after penetrating keratoplasty. These tissues were stored in media (Optisol-GS; Bausch and Lomb, Inc., Rochester, NY, USA) at 4°C until processed for culture.

Isolation and Culture of HCECs

Cells were cultured in accordance with previously published methods.[4,13] Human corneal endothelial cells obtained from the remnant donor tissue after corneal transplantation were harvested on or before the seventh day after death. All of the cells remained attached to the Descemet's membrane. The endothelial cells and Descemet's membrane complex were incubated for 16 to 18 hours in 0.02% collagenase I. The cells were then plated into the bottom of 6 well plates coated with fibronectin-collagen combination (FNC) coating mix (Athena Environmental Sciences, Inc., Baltimore, MD, USA). Cells were cultured for 10 to 14 days until they were confluent. The cells were passaged at 1:2 ratios using 0.25% trypsin/0.02% ethylenediaminetetraacetic acid solution after reaching confluency.[14] After six passages, the culture medium was changed to one of four different media types:

1. Medium A, a traditional medium for HCEC culture consisting of reduced serum medium (Opti-MEM-I; Life Technologies, Inc., Carlsbad, CA, USA) supplemented with 8% fetal bovine serum (FBS), chondroitin sulfate (0.08%), ascorbic acid (20 μg/mL), $CaCl_2$ (200 mg/L), multivitamin solution (1:100), epidermal growth factor (EGF) (5 ng/mL), nerve growth factor (20 ng/mL), and penicillin/streptomycin[8];
2. Medium D, a second type of traditional medium for HCEC culture, consisted of Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with 20% FBS, chondroitin sulfate (0.08%), basic fibroblast growth factor (bFGF; 10 ng/mL), EGF (10 ng/mL), and penicillin/streptomycin;
3. Medium E, which has been used for the culture of embryonic stem cells, consisted of DMEM/F12 supplemented with cell culture media (GlutaMAX; Life Technologies, Inc.), serum-free medium (StemPro; Life Technologies, Inc.) hESC SFM growth supplement, bovine serum albumin (BSA; 1.8%), bFGF (8 ng/mL), 2-mercaptoethanol (0.1 mM), and penicillin/streptomycin[15]; and
4. Medium N, which has been used to culture neural stem cells, consisted of knockout DMEM/F12 supplemented with serum-free medium (Life Technologies, Inc.) neural supplement, bFGF (20 ng/mL), EGF (20 ng/mL), cell culture media (200 mM; Life Tehcnologies, Inc.), and penicillin/streptomycin.[16]

Inverted Microscope

Cells (5×10^4 cells/mL) were cultured in medium A, D, E, or N for 1 week and then the morphology of the cells was assessed using phase-contrast microscopy. To quantify cell morphological changes, the boundaries of cells were outlined using microscopy software (AxioVision, Rel. 4.7; Carl Zeiss Meditec, Jena, Germany).[11,17] At least 20 cells from more than three fields were used for cell morphology analysis. The cells were characterized with respect to their elongation and alignment. The lengths of the short and long axis of the cells were measured using microscopy software (Carl Zeiss Meditec), and the ratio of the long axis to the short axis was calculated.

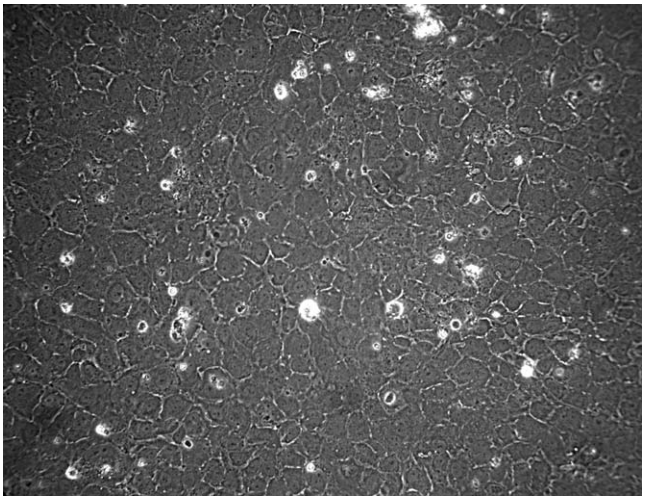

FIGURE 1. Cultured primary HCECs at p0 showed a mosaic pattern. The cells had a hexagonal morphology similar to that seen in vivo.

Immunohistochemistry of HCECs

Human corneal endothelial cells were cultured on cover glasses in 12-well plates, washed with PBS, and fixed for 20 minutes in 3.7% formaldehyde solution. The cells were permeabilized for 10 minutes with 0.5% Triton X-100 and blocked for 1 hour with 1% BSA at room temperature. After washing, the cells were incubated overnight with either goat anti-human Collagen VIII Alpha2 (COL8A2) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse anti-human sodium-potassium adenosine triphosphatase (Na^+-K^+ ATPase) antibody (Abcam, Cambridge, MA, USA); goat anti-human octamer-binding transcription factor 3/4 (OCT 3/4; Santa Cruz Biotechnology); or goat anti-human nestin antibody (Santa Cruz Biotechnology) at 4°C, and then washed with PBS. The cells were incubated with either fluorescein isothiocyanate-conjugated goat anti-rabbit IgG antibody, rabbit anti-goat antibody, or rabbit anti-mouse antibody (1:100) for 1 hour at 37°C in the dark, and then counterstained with Hoechst nuclear staining dye (1:2000; Molecular Probes, Eugene, OR, USA), in accordance with the manufacturer's recommendations. After extensive washing with PBS, the slides were mounted in a drop of mounting medium to reduce photobleaching. Negative control staining was conducted in parallel with the omission of the primary antibodies.

Western Blotting for COL8A2, Na^+-K^+ ATPase, OCT 3/4, Glial Fibrillary Acidic Protein (GFAP), Nestin, and β-Catenin

Human corneal endothelial cells were grown under various culture conditions and then extracted in radioimmunoprecipitation assay buffer (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl, [pH, 7.4], 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.03 TIU/mL aprotinin), which included protease inhibitor cocktail (Sigma-Aldrich Corp., St. Louis, MO, USA) and phosphatase inhibitor cocktail (PhosSTOP; Hoffmann-La Roche, Basel, Switzerland), after the culture medium was removed. The supernatant was collected after centrifugation at 13,000*g* for 20

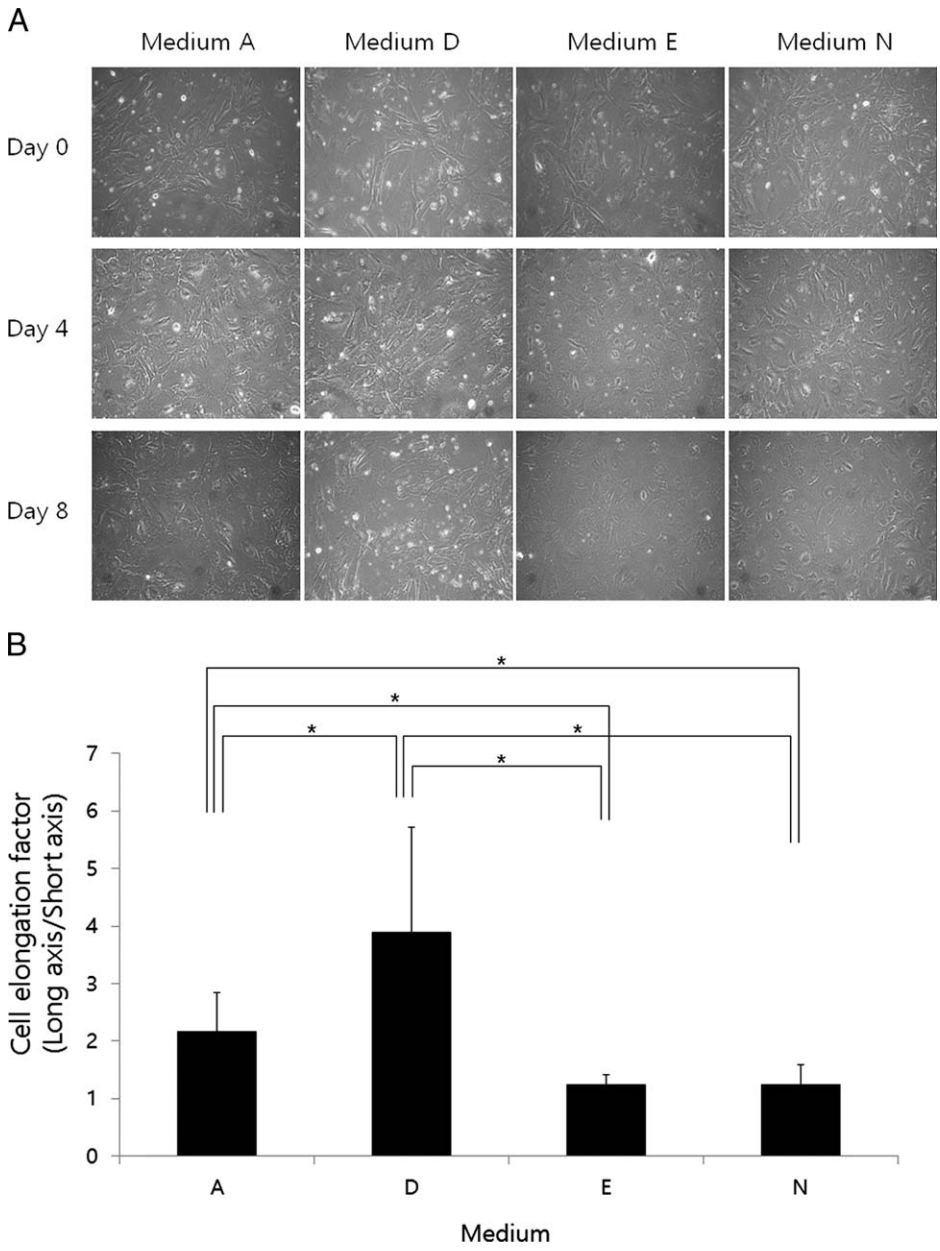

FIGURE 2. Morphology of the cells after 6 passages observed under phase-contrast inverted microscopy. (**A**) Cells in the different media had different shapes. The cells cultured in media A and D showed elongation of cytoplasmic processes, which is a fibroblast-like characteristic, whereas the cells cultured in media E and N were hexagonal. (**B**) The long-to-short-axis ratio was higher in medium D compared with the other media types ($P < 0.001$ for all, Mann-Whitney U test), and it was also higher in media A compared with media E and N ($P < 0.001$ for both). * Statistically significant by Mann-Whitney U test.

minutes and frozen at −70°C until used for the measurement of COL8A2, Na^+-K^+ ATPase, OCT3/4, GFAP, nestin, β-catenin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. Aliquots of cell extracts containing 20 or 30 μg of protein were subjected to SDS-PAGE. Protein bands were then electrophoretically transferred by SDS-PAGE to a membrane (Immobilon-P; Millipore Corp., Bedford, MA, USA). After the membranes were incubated with 5% skim milk or gelatin in PBS for 1 hour, they were immersed in either goat anti-human COL8A2 antibody (Santa Cruz Biotechnology); mouse anti-human Na^+-K^+ ATPase antibody (Abcam, Cambridge, England); goat anti-human OCT3/4 antibody (Santa Cruz Biotechnology); mouse anti-human GFAP antibody (Santa Cruz Biotechnology); rabbit anti-human β-catenin antibody (Abcam); or rabbit anti-human GAPDH antibody (Abfrontier, Seoul, Korea) at 4°C overnight and alkaline phosphatase conjugated with a secondary antibody was added for 2 hours. The immunoreactive bands were detected using a chromogenic immunodetection kit (WesternBreeze; Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocols. Data were quantified with a video image analysis system. Protein bands were measured by densitometry. Each experiment was performed in triplicate.

Cell Viability With a Cell Counting Kit-8 (CCK-8) Assay

Cell viability was measured using a commercial CCK-8 assay kit (Dojindo, Kumamoto, Japan), according to the manufacturer's

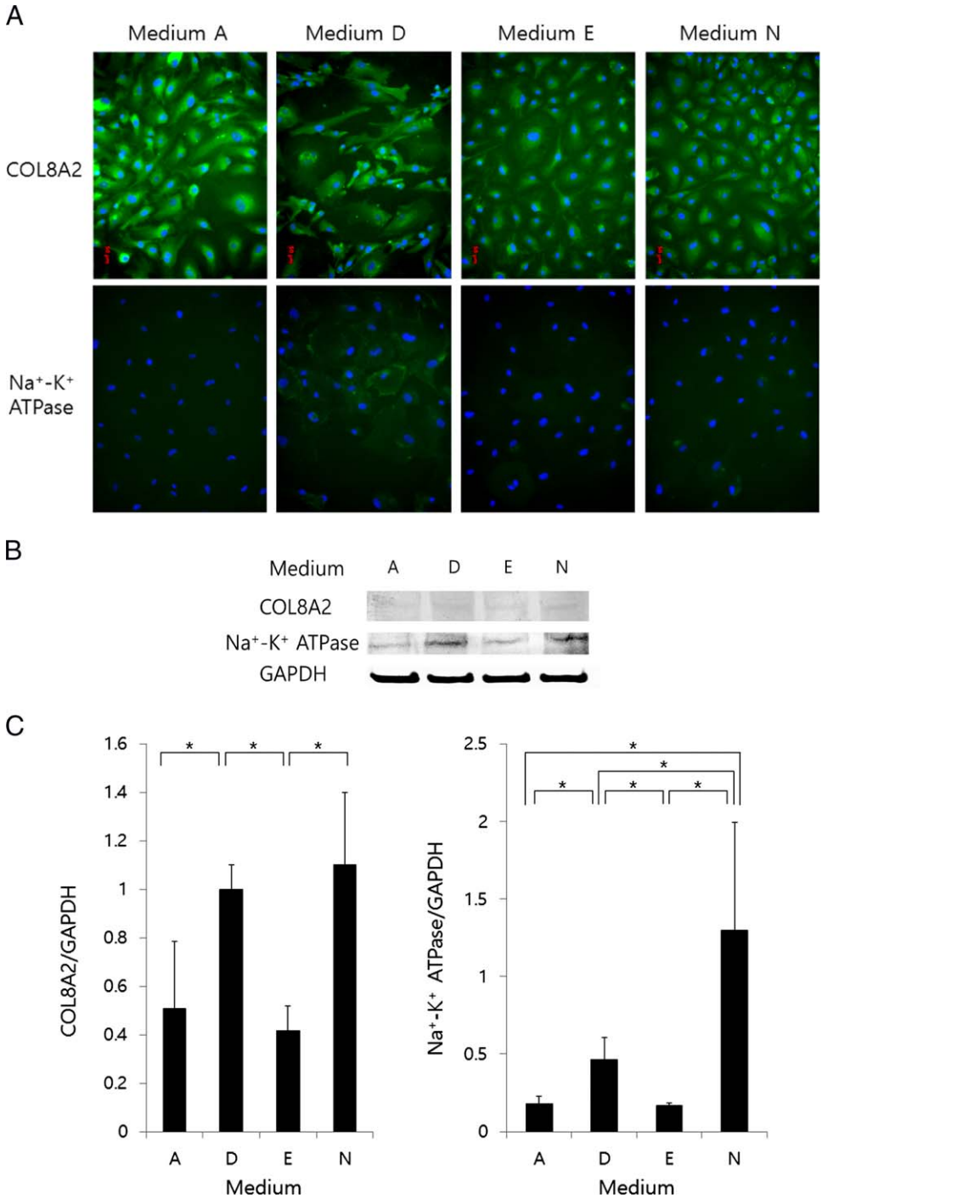

Figure 3. Expression of COL8A2 and Na^+-K^+ ATPase detected by immunofluorescence staining (**A**) and Western blotting (**B**, **C**). (**A**) Representative photos of cells stained with COL8A2 (*green*) and Na^+-K^+ ATPase (*green*). The cells in all four media types expressed COL8A2. The nuclei were stained with Hoechst 33342 (*blue*). (**B**) Representative band of COL8A2 and Na^+-K^+ ATPase expression levels by western blot analysis. (**C**) Expression levels of COL8A2 were higher in media D and N compared with E. Expression of Na^+-K^+ ATPase was higher in medium N compared with the other media types and higher in medium D compared with A and E. Graphic data shown are the means ± SD ($n = 3$). *$P < 0.05$ by Student's *t*-test.

protocol. Cells (1×10^3 cells per well) were placed in 96-well plates and incubated for 2 days in a humidified atmosphere containing 5% CO_2. Cells were cultured in medium A, medium D, medium E and medium N for 1, 4, or 7 days at 37°C in 5% CO_2. One hundred microliters of culture media without CCK-8 solution loading was used as the blank sample. The optical density was measured at 450 nm using an enzyme-linked immunosorbent assay reader (SpectraMax 384; Molecular Devices, Sunnyvale, CA, USA). The results are expressed as mean ± SD. Cell viability are expressed as the percentage of controls (medium A) after subtraction of the corresponding blanks.

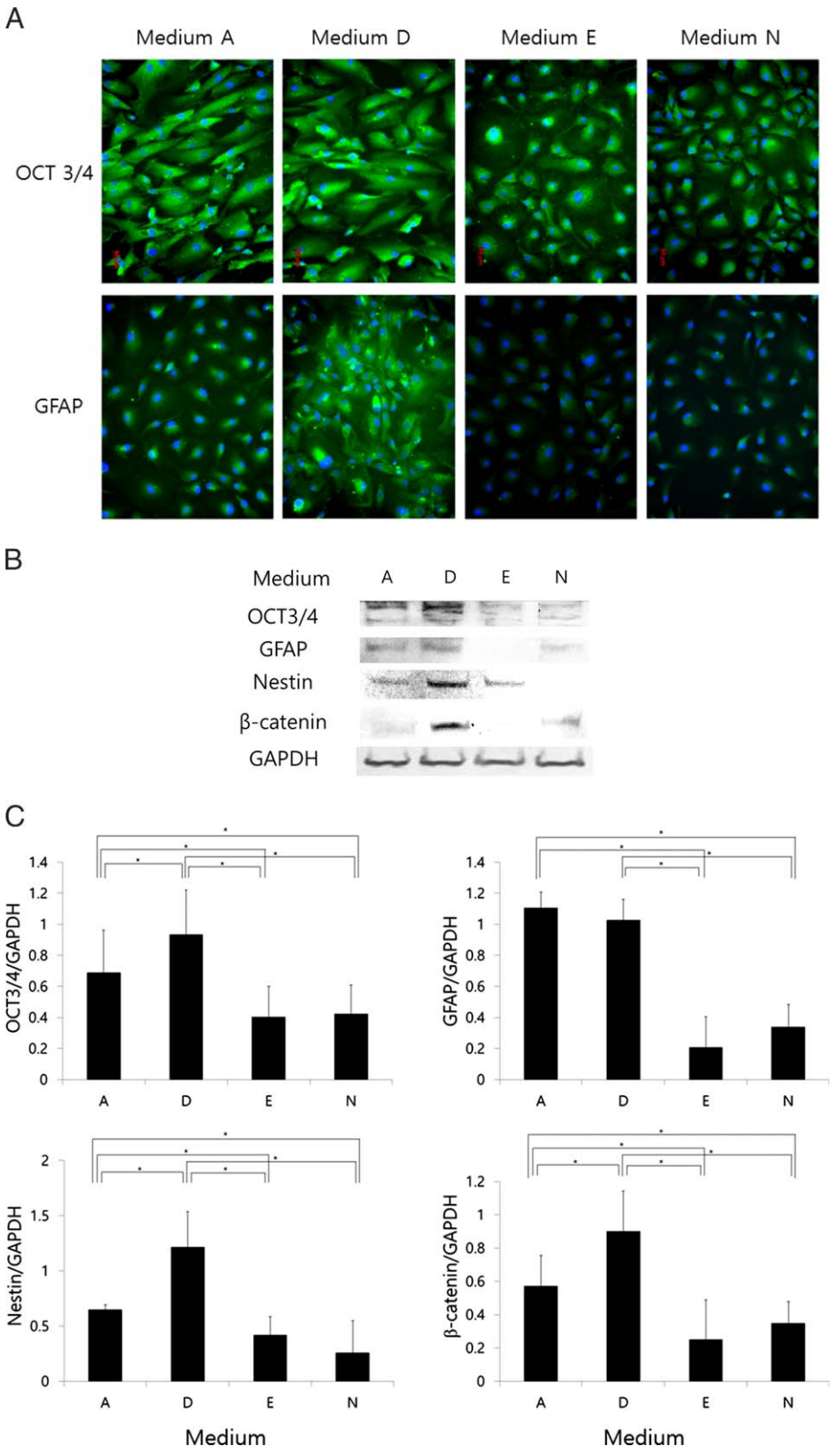

Figure 4. Expression of OCT3/4, GFAP, nestin, and β-catenin by immunofluorescence staining (**A**) and Western blotting (**B**, **C**). (**A**) Representative photos of cells stained with OCT3/4 (*green*) and GFAP (*green*). The nuclei were stained with Hoechst 33342 (*blue*). (**B**) Representative band of OCT3/4 and GFAP expression levels by western blotting. (**C**) OCT3/4 and GFAP expression levels were determined by Western blotting. OCT3/4 expression levels were higher in medium D compared to media A, E, and N and higher in medium A compared to media E and N. GFAP expression levels were higher in media A and D compared to media E and N. β-catenin and nestin expression was higher in the cells cultured in medium D compared to the other media and higher in medium A compared to media E and N. Graphic data shown are the means ± SD ($n = 3$). *$P < 0.05$ by Student's *t*-test.

Wound Healing Assay

Cell migration was assessed in a wound healing assay. The HCECs were cultured in 12-well plates coated with FNC coating mix (Athena Environmental Sciences, Inc.). Next, linear wounds were created by scraping confluent cell monolayers with the tips of sterile pipettes and the cells were then treated with medium A, D, E, or N. The detached cells were rinsed away with PBS and the wounded monolayers were replenished with the complete medium. Images were captured under an inverted light microscope. Cells were wounded and then treated with medium A, D, E, or N for 5 days. At 0, 1, 2, and 5 days, phase-contrast pictures of the wounds at three different locations were taken and then time to close the wound completely was calculated.

Transendothelial Electrical Potential Difference (TEPD)

Endothelial barrier function was assessed by measurement of the TEPD. Cells were grown on membrane inserts of a transwell system in each medium for 7 days and TEPDs were measured using an epithelial voltohmeter (EVOM2; World Precision Instrument, Inc., Saratoga, FL, USA). The wells were then washed twice with serum-free medium and allowed to equilibrate for 1 hour before measurement.[18–20]

Statistical Analysis

Data were evaluated using the Mann-Whitney *U* test or Student's *t*-test. *P* values <0.05 were considered statistically significant.

Results

Culture of HCECs

Human corneal endothelial cells were cultured and observed in mosaic pattern at baseline passage (p0; Fig. 1).

Inverted Microscopy

Cell morphology was evaluated by phase-contrast inverted microscopy (Fig. 2A). The shapes of the HCECs changed over the passages. At passage six, HCECs showing fibroblast-like spindle shapes were used for evaluation of the effects of media type on the shape of the cells. Cells in media A, D, E, and N had different shapes. The cells cultured in media A and D showed elongation of cytoplasmic processes, which has been considered fibroblast-like, whereas the cells cultured in media E and N showed a mosaic pattern, similar to the p0 passage. The ratio of the long axis to short axis was higher in medium D compared with the other media ($P < 0.001$ for all, Mann-Whitney *U* test) and it was also higher in medium A compared with E and N ($P < 0.001$ for both, Mann-Whitney *U* test).

Expression of COL8A2 and Na^+-K^+ ATPase

The expression levels of COL8A2 and Na^+-K^+ ATPase are shown in Figures 3A through 3C. The cells in all four media types expressed COL8A2, which has been reported to be predominant in Descemet's membrane. Expression levels of COL8A2 were higher in media D and N compared with E ($P = 0.003$ for both, respectively, Student's *t*-test). Expression of Na^+-K^+ ATPase (green) was higher in medium N compared with A, D, and E ($P = 0.014$, 0.005, and < 0.001, respectively) and higher in medium D compared with A and E ($P = 0.001$ and 0.005, respectively).

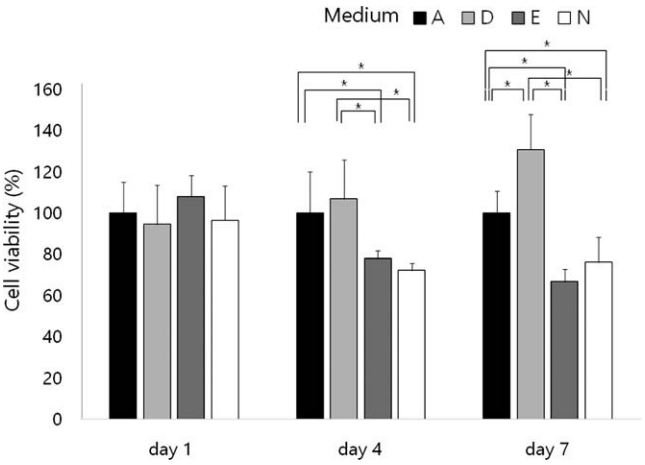

Figure 5. Cell viability depended on the medium used. Medium D gave the highest cell viability compared to the other media types at day four and day seven ($P < 0.001$ for all, Mann-Whitney *U* test). Medium A showed higher cell viability than both media E and N at day 7 ($P < 0.001$ for both). *Statistically significant by Mann-Whitney *U* test.

Expression of OCT3/4, GFAP, Nestin, and β-Catenin

The expression levels of OCT3/4, GFAP, nestin and β-catenin are shown in Figures 4A through 4C. Expression levels of OCT3/4 and GFAP were higher in media A and D compared with E and N. The nuclei were stained with Hoechst 33342 (blue). Expression levels of OCT3/4 were higher in medium D compared with A, E, and N ($P = 0.046$, 0.01, and 0.009, respectively) and higher in medium A compared with E and N ($P = 0.01$ and 0.009, respectively). Expression levels of GFAP were higher in media A and D compared with E and N ($P = 0.012$ and 0.003 in medium A, $P = 0.02$ and 0.011 in medium D). β-catenin and nestin expression was higher in the cells cultured in medium D compared with the other media, and higher in medium A compared with E and N ($P = 0.012$ and 0.006, respectively).

Cell Viability

Cell viability differed depending on the media (Fig. 5). Medium D resulted in the highest cell viability compared with the other media types at days 4 and 7 ($P < 0.001$ for all, Mann-Whitney *U* test). Medium A resulted in higher cell viability compared with E and N at day 7 ($P < 0.001$ for both).

Wound Healing

The wound closure rate differed depending on the media used for culture (Figs. 6A, 6B). The wound closure rate was higher in media A and D compared with the other media types ($P < 0.001$ for all). Medium N showed delayed wound closure compared with the other media types ($P < 0.001$ for all).

TEPD

Transendothelial electrical potential difference differed according to the medium used for culture (Fig. 7); TEPD was higher in medium N compared with media A, D, and E ($P = 0.016$, 0.026, and 0.016, respectively). The TEPD in medium E was higher than that in media A and D ($P = 0.008$ and 0.016, respectively).

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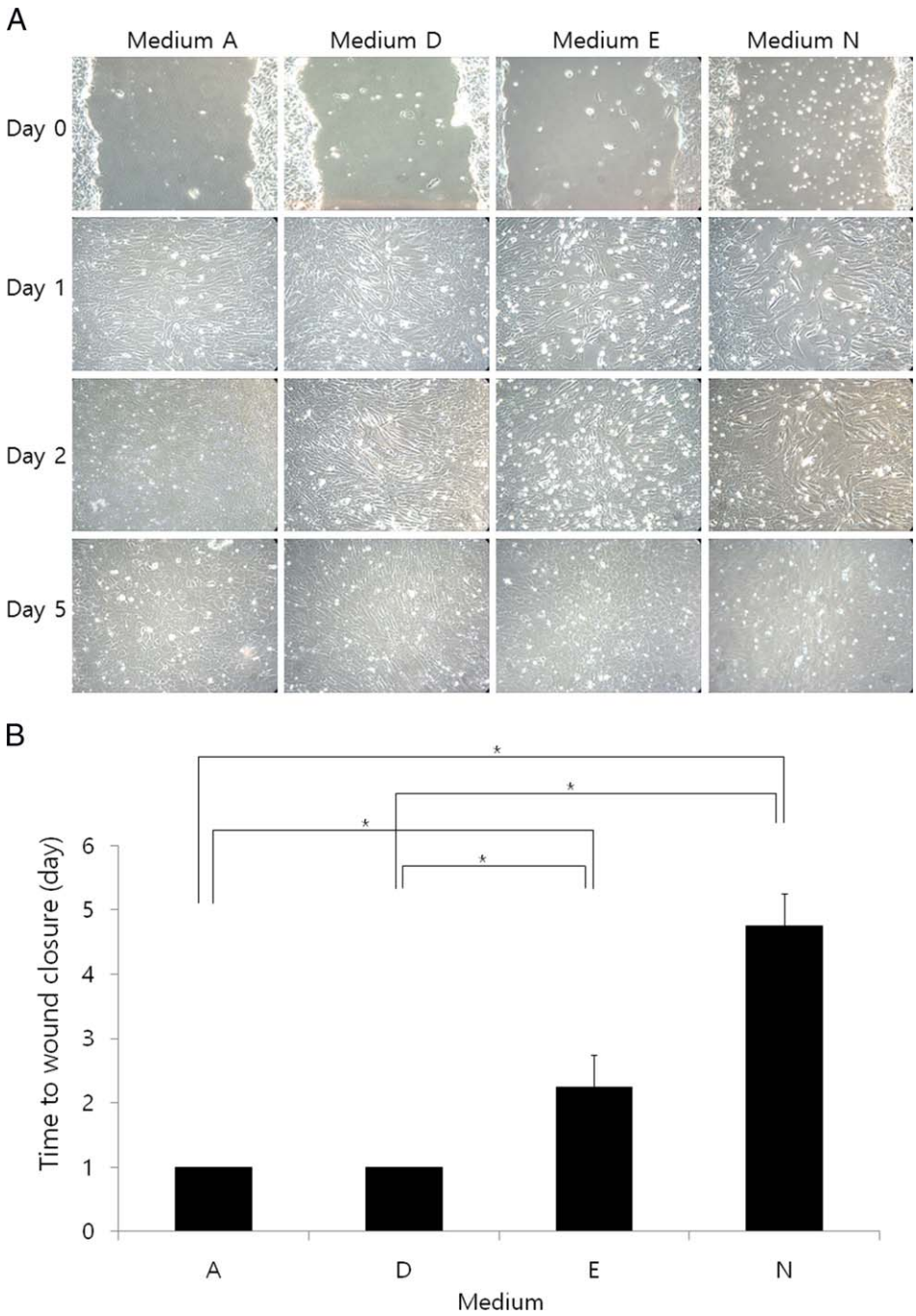

FIGURE 6. Wound healing and cell migration assays. (**A**) Wound-healing assays demonstrated that media A and D enhanced wound closure and cell migration. (**B**) The time to wound closure was shorter in media A and D compared to the other media types ($P < 0.001$ for all). Medium N showed delayed wound closure compared to the other media types ($P < 0.001$ for all). *Statistically significant by Mann-Whitney U test.

DISCUSSION

Cultured HCECs have been suggested to be useful for in vivo transplantation in corneal endothelial diseases[9]; thus, it is important in regenerative medicine to develop suitable HCEC culture media.[10] The culture medium regulates cell phenotypes, including survival, differentiation, transdifferentiation, migration, and proliferation.[10] In this study, we investigated the differences in cellular phenotype in response to culture in different types of media. We found that the shapes of HCECs differed depending on the medium used. Media A and D caused the HCECs to become elongated and fibroblast-like compared with the other media types used. Conversely, HCECs cultured in media E and N reverted to a polygonal shape, similar to cells in vivo. Cell shape is related to various cell functions, such as the communication with other cells, regulation of cell movement,[17] and collagen I expression.[21] Polygonal cells can fit together and cover surfaces with an apparent tendency to minimize surface-free energy.[22] It has been reported that more elongated cells express higher levels of collagen type I than less stretched cells, even when the cell coverage area is the same.[21] Nevertheless, COL8A2

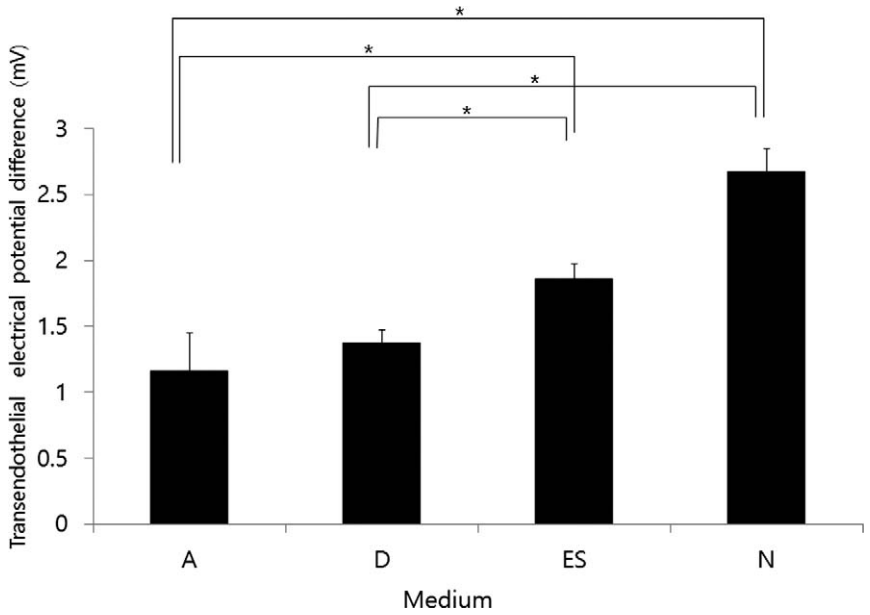

FIGURE 7. Transendothelial electrical potential difference (TEPD) differed according to the medium used. Transendothelial electrical potential difference was higher in medium N compared to media A, D, and E ($P = 0.016$, 0.026, and 0.016, respectively). The TEPD in medium E was higher compared to that in media A and D ($P = 0.008$ and 0.016, respectively). *Statistically significant by Mann-Whitney U test.

expression was not affected by the different media, or different cell shapes observed, as COL8A2 was expressed in all four media types. It has been reported that COL8A2 is abundant in Descemet's membrane, which is a basement membrane for HCECs in vivo; COL8A2 has been described as a marker of HCECs.[23] However, Na^+-K^+ ATPase expression was higher in medium D compared with the other media types. Sodium-potassium adenosine triphosphatase is the largest protein complex in the family of P-type cation pumps, and its minimum functional unit is a heterodimer of α- and β-subunits.[24] It is expressed in the basolateral membrane of corneal endothelial cells, and is primarily responsible for the pump functions of the corneal endothelium.[25] Sodium-potassium adenosine triphosphatase is activated by phosphorylation of alpha(1)-subunit and translocation of the α1-subunit and translocation of the α1 and β1 subunits to the basolateral membrane via the extracellular-signal-regulated kinase 1/2 pathway.[26] Sodium-potassium adenosine triphosphatase and COL8A2 have been described as differentiation markers of HCECs.[23] Medium D induced Na^+-K^+ ATPase expression, whereas COL8A2 expression did not differ between the media types. These results suggest that there may be different pathways that increase Na^+-K^+ ATPase and COL8A2 expression. It has been reported that the α1 isoform of the Na+/K+ ATPase is upregulated in dedifferentiated progenitor cells.[27] Further study is necessary to investigate the mechanism of increased Na^+-K^+ ATPase and COL8A2 expression.

Proliferation rates were different depending on the medium used. Medium D provided the higher proliferation rates compared with the other media types at days 4 and 7. Medium A showed a higher proliferation rate compared with media E and N at day 7. Expressions of OCT3/4, GFAP, nestin, and β-catenin were all associated with proliferation rate. These expressions were higher in medium D compared with the other media, and higher in medium A compared with E and N. Expressions of OCT3/4 and GFAP have been reported to be abundant in embryonic stem cells and neural stem cells.[28] These molecules have been suggested to be associated with cell proliferation and regenerative capacity after damage.[10,29,30] Nestin has been reported to be a neural stem cells marker, and it is required for the proper self-renewal of neural stem cells.[10] Wound closure rates were higher in both media A and D compared with the other media types. These results also can be correlated with the higher OCT3/4, GFAP, nestin, and β-catenin expressions levels seen in medium D.

In this study, medium N increased TEPD compared with the other media types. Transendothelial electrical potential difference is generated by the ionic transport and is a quantitative indicator of corneal endothelial function.[20] Transendothelial electrical potential difference is a very sensitive index of endothelial transport. It is a manifestation of the activity of the endothelial fluid pump which maintains the cornea at the level of hydration required for transparency.[31] The maximal values of transendothelial potential difference has been reported at 1.3 ± 0.1 mV.[18]

Media A and D have previously been used for HCEC culture.[5,8,9] These media included FBS, which contains a variety of growth factors as well as major proteins such as globular protein and BSA.[32] Platelet-derived growth factor, transforming growth factor, fibroblast growth factor, EGF, and insulin-like growth factor in FBS have all been reported to induce cell transdifferentiation.[33–36] Unlike media A and D, media E and N were serum-free. Medium E has been used previously for embryonic stem cell culture,[15] and medium N has been used for neural stem cell culture.[16] Thus, media E and N may be able to provide a more similar environment to that found in vivo because HCECs in vivo are not in contact with serum, but instead face the aqueous humor produced by the ciliary body.

In this study, the cells at passage 6 were used. Primary mammalian cells reach replicative exhaustion after several passages in vitro, a process called replicative senescence.[37,38] In general, the primary cells at passages 4 through 6 have been used for evaluating the cell shape and physiological activity.[14,39] It has been reported that HCECs at passages 3 and 7 have a similar phenotype.[40] However, HCECs were reported to become irregular in shape and eventually were found to be large and flat (typical of senescent cells) after six passages.[41] Thus, cells at passage 6 were considered to be the

most appropriate to evaluate the changes of cell shape and physiological activity depending on the media.[41,42] In addition, although the cells were cultured in the common media in the early passages and it could already channel the cells down a development path, the changes of cell environment including nutrients, growth factors and transcription factors can affect and even reverse the cell phenotypes.[43,44]

In conclusion, medium D was superior to the other media types with regard to the expression of stem cell–associated proteins and proliferation. However, after cell passage, medium N was more appropriate than the other three media types to restore cell shape and function.

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