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Corneal regeneration by induced human buccal mucosa cultivated on an amniotic membrane following alkaline injury

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Various clinical disorders and injuries, such as chemical, thermal, or mechanical injuries, may lead to corneal loss that results in blindness. PURPOSE: The aims of this study were to differentiate human buccal mucosa (BMuc) into corneal epithelial-like cells, to fabricate engineered corneal tissue using buccal mucosal epithelial cells, and to reconstruct a damaged corneal epithelium in a nude rat model.
Methods: BMuc were subjected to 10 d of induction factors to investigate the potential of cells to differentiate into corneal lineages.
Results: Corneal stem cell markers β1-integrin, C/EBPδ, ABCG2, p63, and CK3 were upregulated in the gene expression analysis in induced BMuc, whereas CK3 and p63 showed significant protein expression in induced BMuc compared to the uninduced cells. BMuc were then left to reach 80% confluency after differential trypsinization. The cells were harvested and cultivated on a commercially available untreated air-dried amniotic membrane (AM) in a Transwell system in induction medium. The corneal constructs were fabricated and then implanted into damaged rat corneas for up to 8 weeks. A significant improvement was detected in the treatment group at 8 weeks post-implantation, as revealed by slit lamp biomicroscopy analysis. The structure and thickness of the corneal layer were also analyzed using histological staining and time-domain optical coherence tomography scans and were found to resemble a native corneal layer. The protein expression for CK3 and p63 were continuously detected throughout the corneal epithelial layer in the corneal construct.
Conclusions: In conclusion, human BMuc can be induced to express a corneal epithelial-like phenotype. The addition of BMuc improves corneal clarity, prevents vascularization, increases corneal thickness and stromal alignment, and appears to have no adverse effect on the host after implantation.

Corneal epithelial stem cells are located in the basal layer of the limbus [1], which is a transitional zone between the cornea and bulbar conjunctiva. Ocular surface injury or disease often leads to superficial scarring and vascularization of the cornea, persistent epithelial defects, and subepithelial scarring, which eventually lead to blindness. Surgical approaches to ocular surface diseases such as Stevens–Johnson syndrome (SJS), ocular cicatricial pemphigoid, and chemical injury include limbal transplantation [2-4] and amniotic membrane (AM) transplantation [5-8].

Autologous epithelial cells such as buccal, conjunctival, nasal, esophageal, rectal, and vaginal epithelia have a similar morphology to the corneal epithelium, which is derived from a stratified squamous structure [9]. The oral mucosa is recognized as an available source for biopsy because its epithelial cells and the cornea have similar morphological and cytochemical characteristics [10]; therefore, it could be considered as an alternative to allogenous limbal transplants. Buccal epithelial cells have some benefits as a cell source for the treatment of ocular surface disease, such as a lower stage of differentiation and a short cell turnover time. They require a shorter culture period and can be maintained in culture for long periods without undergoing keratinization [11]. The transplantation of cultivated buccal mucosal epithelial sheets offers a viable, suitable, and safe alternative in the reconstruction of a stable ocular surface necessitated by disease, burns, or chemical injuries [12-14]. Madhira et al. [9] have established cultures of oral mucosal epithelial cells on a human AM without the use of feeder cells, suggesting that oral epithelial cultures can be used for ocular surface reconstruction in patients with bilateral limbal stem cell deficiency. The long-term clinical results produced by

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Nakamura and coworkers [15] strongly support the conclusion that tissue-engineered cultivated oral mucosal epithelial sheets are useful in reconstructing the ocular surface of the scar phase of severe ocular surface disorders. Nishida et al. [16] reported that several patients consecutively treated with the oral mucosal approach showed remarkable improvements regarding the restoration of corneal transparency and postoperative visual acuity, and all corneal surfaces remained transparent during the follow-up period.

The main objective of this study was to differentiate human buccal mucosa (BMuc) into corneal epithelial lineages. The differentiated cells were characterized by assessing the upregulation of corneal stem cell and cornea-specific markers. The presence of p63 and CK3 was detected by performing immunocytochemical analysis in vitro. Next, the corneal constructs were implanted into a nude rat model of limbal stem cell deficiency as a proof of concept. The degree of vascularization and corneal haze were then assessed by slit lamp biomicroscopy, while the corneal thickness was evaluated 8 weeks after implantation via time-domain optical coherence tomography (OCT) scans. The animals were euthanized, and histological analysis was performed to detect the presence of CK3 and p63 in the corneal epithelial layer. All procedures for this study were approved by the Universiti Kebangsaan Malaysia Research and Ethics Committee with the approval code PP/FISIO/2009/RUSZYMAH/29-APRIL/261-MAY–2009-DECEMBER–2011.

METHODS

Buccal mucosa culturing process and formation of tissue-engineered corneal construct: BMuc was obtained from four non-cancerous patients who underwent maxillofacial surgery. This was performed by a qualified maxillofacial surgeon at the University Kebangsaan Malaysia Medical Centre. BMuc

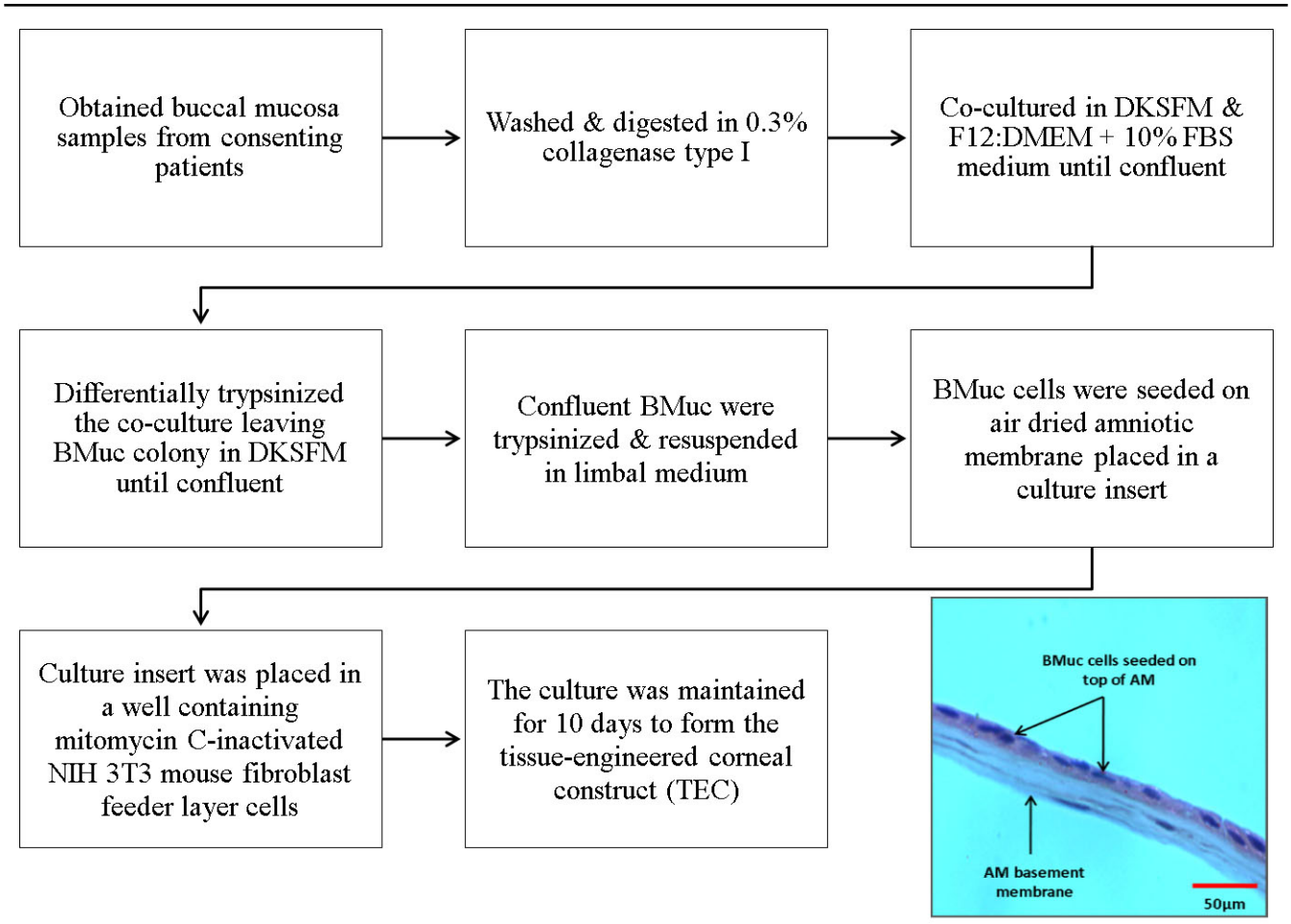

Figure 1. Schematic diagram of BMuc culturing process and the formation of tissue engineered corneal construct (TEC). Photo insert shows a TEC before implantation ((in vitro construct). Induced BMuc can be seen covering the epithelial side of the air-dried amniotic membrane (magnification: 400x).

Table 1. Marker genes and the sequences that were used in the real-time polymerase chain reaction analysis. Source: Modified from BLAST.

Marker gene	Product size (bp)	Sequence (5'-3')
β_1-integrin	104	F: AGTGAATGGGAACAACGAG
		R: CAATTCCAGCAACCACACCA
C/EBPδ	97	F: ACTTCAGCGCCTACATCGAC
		R: GCCTTGTGATTGCTGTTGAA
ABCG2	157	F: GCGACCTGCCAATTTCAAATG
		R: GACCCTGTTAATCCGTTCGTTT
p63	162	F: GAAACGTACAGGCAACAGCA
		R: GCTGCTGAGGGTTGATAAGC
CK3	106	F: GGATGTGGACAGTGCCTATATG
		R: AGATAGCTCAGCGTCGTAGAG
GAPDH	217	F: TCCCTGAGCTGAACGGGAAG
		R: GGAGGAGTGGGTGTCGTCGT

graft was harvested from the inner cheek or the lower lip during the biopsy of non-cancerous lesions with a size of 5 mm (length) ×5 mm (width) × 2 mm (depth). This graft was added to the existing excisional biopsy. The wound was approximated with 3/0 Vicryl sutures. Post-harvest healing was typically uneventful without any cosmetic or any other long-term defect [17].

The sample was transported to the laboratory and washed three times with phosphate buffered saline (PBS; Gibco Life Sciences, New York, NY). Collagenase type I (0.3%; Worthington Biochemical Corporation, Lakewood, NJ) was used to digest the tissue into single cells. The cells were resuspended in a mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F12, which contained 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine, and Defined Keratinocyte Serum-Free Medium (DKSFM; Gibco Life Sciences) and then plated in 6-well plates (Sigma Aldrich, St Louis, MO). The adherent cells were washed twice and cultured for 5–7 d until cell clones were formed. Co-cultured cells were differentially trypsinized to remove the fibroblasts, and epithelial growth was maintained until confluence. The confluent epithelial cells were grown on two separate plates containing either DKSFM or limbal medium. Uninduced cells were trypsinized and seeded on an AM (Cryocord Sdn. Bhd., Cyberjaya, Malaysia) in a culture insert (BD Biosciences, Woburn, MA). The culture insert was then placed in a well containing mitomycin C-inactivated NIH 3T3 mouse fibroblast feeder layer cells (ATCC, Cat. No: CRL-1658), and limbal medium was added to the culture to induce BMuc into corneal epithelial-like cells. This co-culturing technique is widely used to enhance the stratification of epithelial cells [18]. The culture was maintained for 10 d to form the tissue-engineered corneal construct (TEC). A schematic diagram of the BMuc culturing process and the formation of the TEC are shown in Figure 1.

Corneal epithelial cell isolation and culture: Redundant corneal rings were obtained from donor tissue during corneal transplant surgery. The culturing process was conducted using a previously described methodology [19]. Briefly, the samples obtained upon corneal transplant surgery were incubated with 0.05% trypsin ethylenediamine-tetra-acetic acid (TE; Gibco Life Sciences, New York, NY) for 20 min at 37 °C. Trypsin inhibitor (Gibco Life Sciences, New York, NY) was added to stop the TE reaction and the epithelial cells were then collected. The cell suspension was centrifuged, and the pellet cells resuspended in limbal medium were seeded on mitomycin C-inactivated NIH 3T3 mouse fibroblast feeder layer cells. The limbal medium consisted of DMEM with glutaMAX-1 mixed 3:1 with Ham's F12 medium, supplemented with 10% fetal bovine serum (Gibco Life Sciences), insulin 5 g/ml^{-1}, adenine 0.18 mM, hydrocortisone 0.4 g/l^{-1}, triiodothyronine 2×10^{-9} M, epidermal growth factor 10 ng/ml^{-1} (Sigma Aldrich), and cholera toxin 10^{-10} M (Merck, Sigma-Aldrich Inc.). The co-cultured cells were incubated at 37 °C in a 5% CO_2 incubator. The medium was replenished every 2 d. Corneal epithelial cells were subsequently used as reference in RNA and immunostaining analysis. An intact corneal ring was used for reference in immunohistochemical staining.

Total RNA extraction and gene expression analysis: Total RNA from the cultured cells was isolated using TRI reagent

Table 2. Nude rat corneal scores after alkaline injury.

Score	0	1	2	3	4
Cornea haze/opacity	Transparent	Slight opacity with visible iris texture	Moderate opacity with unclear iris texture	Severe opacity; pupil can be seen vaguely	Very severe opacity; pupil cannot be seen
Neovascularization grade	No	2 mm within limbus	Around cornea ≤ 1/2 quadrant	Around cornea > 1/2 quadrant	Whole cornea

(Source: Jiang et al. 2010)

(Molecular Research Centre, Cincinnati, OH) according to the manufacturer's protocol and was subjected to gene expression analysis. Two-step reverse transcription PCR (RT-PCR; Invitrogen, Carlsbad, CA) was used to investigate the expression of β1-integrin, CEBPδ, ABCG2, p63, and CK3 as marker genes. Expression of the housekeeping gene, glycerylaldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization of gene expression data. The primer sequences are listed in Table 1.

Immunocytochemical analysis: Mouse monoclonal antibodies that were specific for CK3 were purchased from Chemicon International (Temecula, CA; Cat. No: CBL218, 1:50), and the p63 antibody was purchased from Abcam (Cambridge, UK; Cat. No: ab3239–500, 1:200). The antibodies cross react with both human and rat antigens. The procedure was conducted according to a published protocol [19]. Briefly, corneal epithelial cells, uninduced and induced BMuc were fixed with 4% ice-cold paraformaldehyde for 30 min, followed by washing three times with PBS. The plate was incubated with primary antibodies and left overnight at 4 °C. The cells were then incubated with Alexa Fluor 488 rabbit anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC; Chemicon International, Cat. No. AP160 F; 1:200) secondary antibody at 37 °C for 2 h. The cells were counterstained with 4', 6-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific, Waltham, MA) and mounted with anti-fading mounting medium (Biorad, Hercules, CA). The stained cells were observed using a Nikon $A1_R$ confocal microscopy system

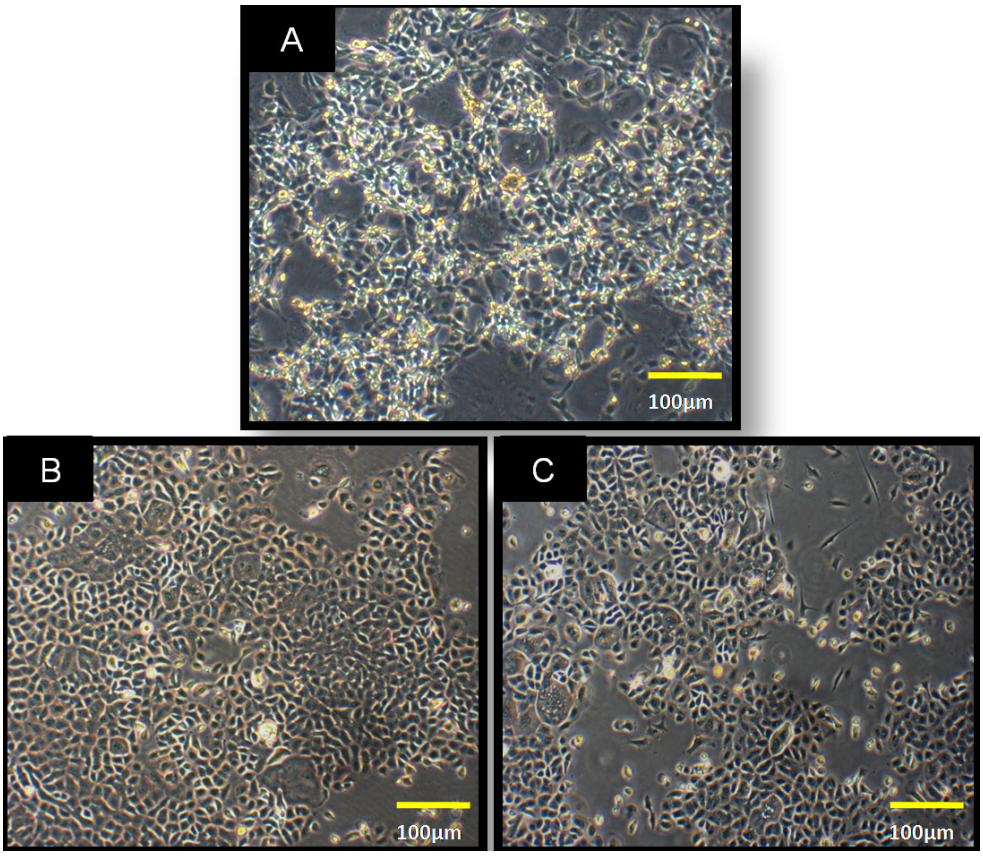

Figure 2. Morphology of the cells. **A**: Corneal epithelial cells from the native human corneal ring samples. **B**: Uninduced BMuc after 10 d of culture. **C**: Induced BMuc cells after 10 d of induction (magnification: 40x).

Table 3. Gene expression in corneal epithelial cells (positive control), uninduced BMuc and BMuc induced by limbal medium.

Gene of interest	Corneal epithelial Cells	Uninduced BMuc	Induced BMuc	P value (Uninduced vs Induced)
β1-integrin	$4.11 \times 10^{-1} \pm 3.42 \times 10^{-2}$	$4.50 \times 10^{-5} \pm 3.50 \times 10^{-6}$	$3.23 \times 10^{-1} \pm 1.01 \times 10^{-1}$	p=0.01 *
C/EBPδ	$1.86 \times 10^{-2} \pm 2.55 \times 10^{-3}$	$2.30 \times 10^{-6} \pm 4.60 \times 10^{-7}$	$1.61 \times 10^{-2} \pm 6.29 \times 10^{-3}$	p=0.02 #
ABCG2	$2.64 \times 10^{-3} \pm 1.05 \times 10^{-3}$	$6.90 \times 10^{-7} \pm 4.20 \times 10^{-7}$	$1.16 \times 10^{-3} \pm 9.00 \times 10^{-4}$	p=0.03 Ł
p63	$1.54 \times 10^{-2} \pm 6.87 \times 10^{-3}$	$1.39 \times 10^{-8} \pm 3.70 \times 10^{-9}$	$9.77 \times 10^{-6} \pm 4.48 \times 10^{-6}$	p=0.99
CK3	$1.41 \times 10^{-3} \pm 7.42 \times 10^{-4}$	$1.24 \times 10^{-8} \pm 4.32 \times 10^{-9}$	$1.26 \times 10^{-6} \pm 6.31 \times 10^{-7}$	p=0.98

* Induced BMSCs compared to uninduced BMSCs for β1-integrin ($p<0.05$) # Induced BMSCs compared to uninduced BMSCs for C/EBPδ($p<0.05$) Ł Induced BMSCs compared to uninduced BMSCs for ABCG2 ($p<0.05$)

(Nikon, Tokyo, Japan), and the images were processed using NIS-Elements Viewer 3.20 image analysis software (Nikon, Japan).

Induction of damage to nude rat corneas: Nude rats were used in this study as a proof of concept. The rat strain was Crl: NIH-Foxn1^{rnu} and was purchased from Charles River (Taiwan). A corneal defect was created in 16 nude rats. Briefly, the nude rats were anesthetized using a mixture of

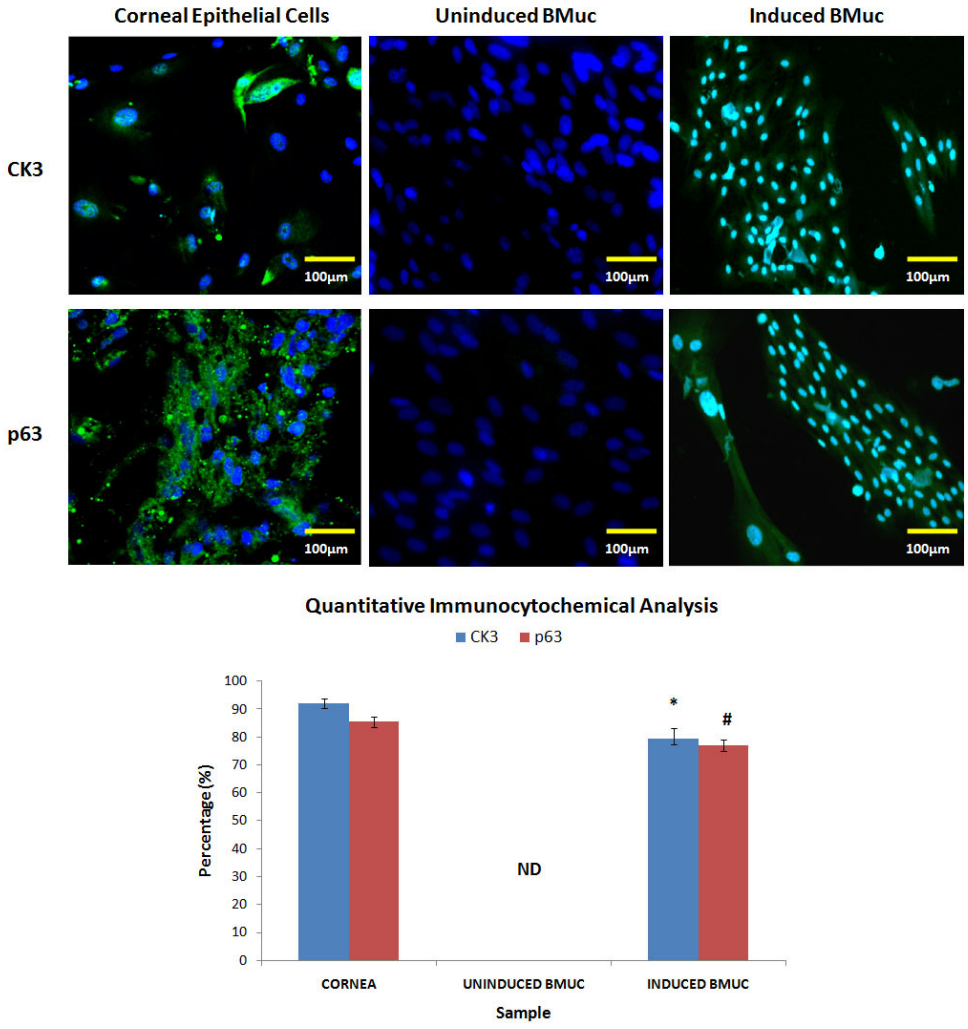

Figure 3. Immunocytochemical analysis using CK3 and p63 antibodies in corneal epithelial cells from native human corneal ring samples; uninduced BMuc and induced BMuc after 10 d of induction (magnification: 100x). The blue color represents the 4', 6-diamidino-2-phenylindole (DAPI) stain in cell nuclei, and the green color represents the fluorescein isothiocyanate (FITC) stain, either in nuclei or cytoplasm. The percentage of positive stains in three different groups was also measured. *Induced BMuc compared to uninduced BMuc for CK3 ($p<0.05$). #Induced BMuc compared to uninduced BMuc for p63 ($p<0.05$). ND: not detected.

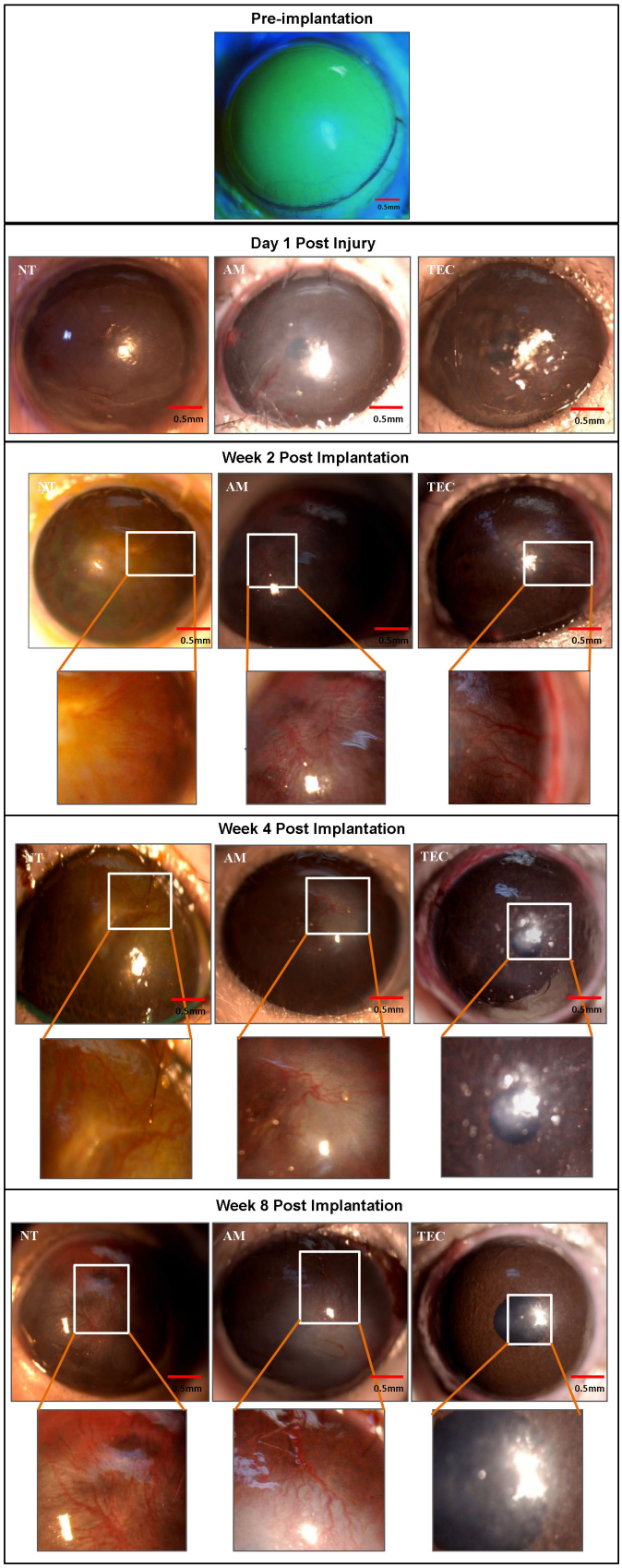

Figure 4. Slit lamp examination of nude rat eyes showing the total epithelial defect of the corneal surface on Day 1 after corneal injury. A representative photo of corneal defect pre-implantation showed positive fluorescein staining (green). The absence of the green color in the rest of the photos shows that full coverage of the corneal epithelium has been achieved by Week 2. Microscopic evaluation is also shown at 2 weeks, 4 weeks, and 8 weeks after implantation.

ketamine, xylazine, and zoletile at 0.01 ml/kg via intramuscular injection. Corneal damage was created by applying a 1N (mol l^{-1}) of NaOH-soaked disc paper to the right eye of each nude rat, resulting in corneal epithelial stem cell deficiency. This protocol for generating corneal defects in animal models has been previously validated [20,21]. Dexamethasone-neomycin (Maxidex) was applied to the defective eye three times per day for up to 5 weeks in all groups.

Treatment of corneal defect: A 360° conjunctival peritomy was carried out, and scar tissue and vascularization were removed from the damaged site under anesthesia in all nude rats. The nude rats were then divided into three groups—a TEC group where the TEC was sutured onto the corneal surface using 8/0 Vicryl sutures with the cells facing up (n=4); an AM group where only an AM (without cells) was sutured onto the corneal surface (n=6); and an NT group where the defect was left empty (n=6). The implantation was performed 7 d after injury. Tarsorrhaphy was performed to protect the implanted site in cases of corneal exposure. The implanted constructs were maintained in vivo for 8 weeks, followed by histological and immunohistochemical analyses.

Histological and immunohistochemical analyses: Histological and immunohistochemical analyses were carried out as previously described [22]. Briefly, samples were fixed with formalin, and paraffin embedded samples were sectioned. Hematoxylin and eosin (H&E) staining was used in the histological analysis. The purple stain represents the nuclei, whereas the cytoplasm and connective tissue were stained pink or red. The sections were also subjected to immunohistochemical staining using primary and secondary antibodies for immunocytochemical analysis. Briefly, the tissue was treated with antigen retrieval for 20 min and was incubated with 10% goat serum at 37 °C. Next, the tissue was incubated with primary antibodies and left overnight at 4 °C. The sections were counterstained with DAPI to visualize the nucleus. The stained tissue was observed using a Nikon $A1_R$ confocal microscopy system (Nikon), and the images were processed using NIS-Elements Viewer 3.20 image analysis software (Nikon).

Ocular surface evaluation: The corneal surfaces of live rats were investigated for clarity and neovascularization using a slit lamp and were documented after chemical injury (pre-implantation) at Week 2, Week 4, and Week 8

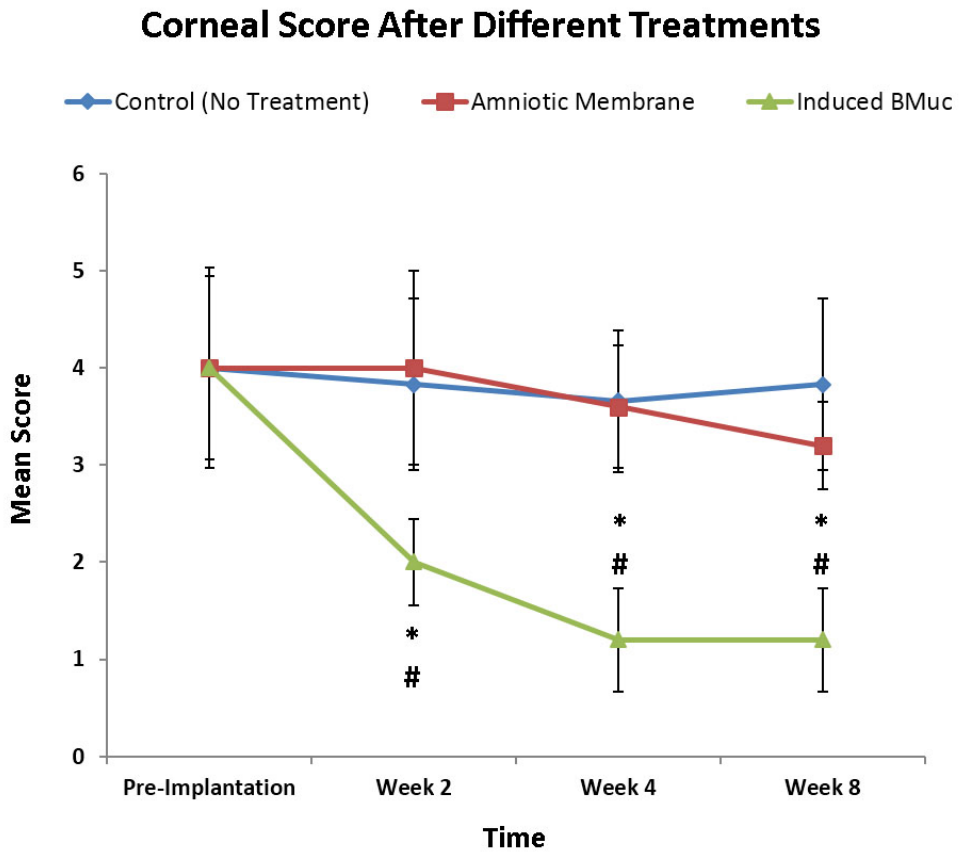

Figure 5. Corneal scoring after different treatments at pre-implantation and at Week 2, Week 4, and Week 8 after implantation based on the corneal grading in Table 2. * TEC compared to NT at Week 2, Week 4, and Week 8 ($p<0.05$). # TEC compared to AM at Week 2, Week 4, and Week 8 ($p<0.05$).

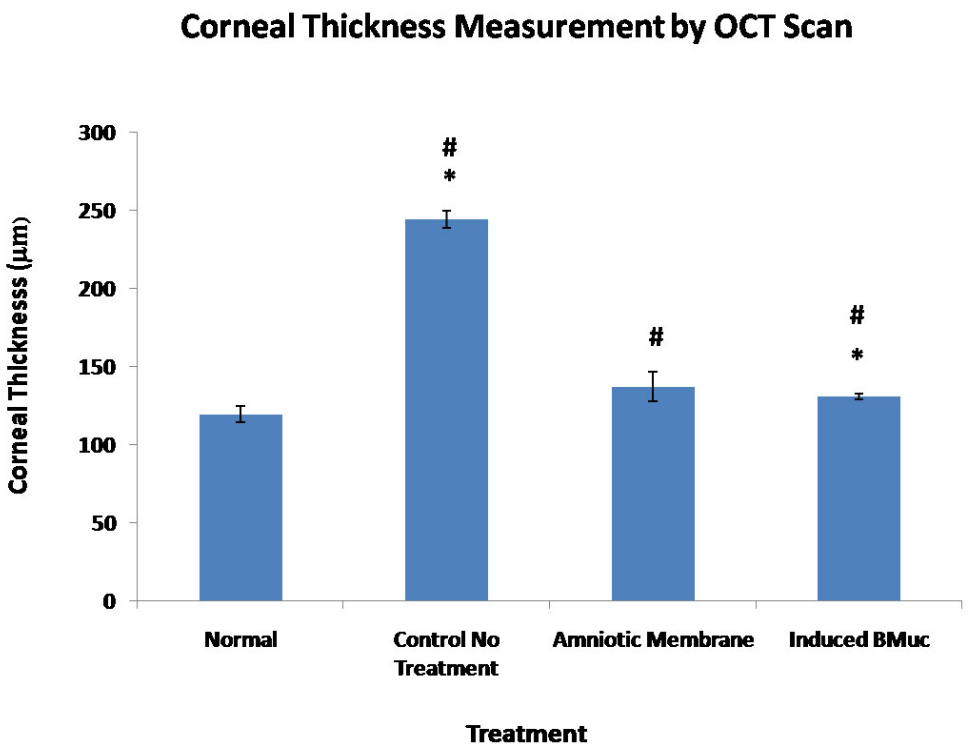

Figure 6. Corneal thickness measurement by optical coherence tomography (OCT) scan at 8 weeks post implantation.* Normal cornea compared to NT and TEC ($p<0.05$). # NT compared to AM and TEC ($p<0.05$).

post-implantation. The nude rat corneas were scored based on the criteria in Table 2. Corneal thickness was measured at the end of the experiment using OCT scans. The central region of the anterior segment of the corneas was measured in triplicate.

Statistical analysis: All the data obtained from corneal surface evaluations were processed using the Statistical Package for the Social Sciences (SPSS Version 18.0) software and analyzed using one-way ANOVAs. A value of $p<0.05$ was regarded as statistically significant.

RESULTS

Morphology of corneal epithelial cell cultures: Corneal epithelial cells were cultured until 80% confluence. The corneal epithelial cell morphology showed a compact polygonal shape with small and dense colonies (Figure 2A).

Cell morphology of uninduced and induced BMuc: Uninduced BMuc cells were large, compact, dense, and polygonal in shape. Upon induction, no change in the morphology of the induced BMuc was noted (Figure 2B,C).

Gene expression analysis: Gene expression analysis using qualitative RT-PCR showed that corneal cells expressed higher levels of all the corneal markers compared to uninduced and induced BMuc. Induced BMuc showed upregulation of β-integrin ($3.23 \times 10^{-1} \pm 1.01 \times 10^{-1}$), C/EBPδ ($1.61 \times 10^{-2} \pm 6.29 \times 10^{-3}$), ABCG2 ($1.16 \times 10^{-3} \pm 9.00 \times 10^{-4}$), p63 ($9.77 \times 10^{-6} \pm 4.48 \times 10^{-6}$), and CK3 ($1.26 \times 10^{-6} \pm 6.31 \times 10^{-7}$) compared to uninduced BMuc: β-integrin ($4.50 \times 10^{-5} \pm 3.50 \times 10^{-6}$; p=0.01), C/EBPδ ($2.30 \times 10^{-6} \pm 4.60 \times 10^{-7}$; p=0.02), ABCG2 ($6.90 \times 10^{-7} \pm 4.20 \times 10^{-7}$; p=0.03), p63 ($1.39 \times 10^{-8} \pm 3.70 \times 10^{-9}$; p=0.99), and CK3 ($1.24 \times 10^{-8} \pm 4.32 \times 10^{-9}$; p=0.98; Table 3). The expression levels of p63 and CK3 were not significantly different between uninduced BMuc and induced BMuc.

Immunocytochemical analysis: Immunocytochemical analysis demonstrated positive protein expression of CK3 (92.15% ± 1.57) and p63 (85.54% ± 1.91) in corneal epithelial cells and induced BMuc (79.61% ± 3.43 and 77.09% ± 2.12, respectively; p=0.01). These two proteins were not expressed in the uninduced BMuc (Figure 3).

Slit lamp biomicroscopy evaluation: Fluorescein stain was applied to the outer surface of damaged corneas. The stained areas in the damaged sites were indicated by a green color when a blue light was shone onto the eyes, which apparently indicated signs of inflammation on the nude rat ocular surface. Both the NT and AM groups showed considerable vascularization that remained after 2 weeks and 4 weeks post-implantation (Grade 4), whereas in the TEC group the vascularization was significantly reduced from Grade 3 to Grade

2. At week 8 post-implantation, no significant difference was detected while total healing with little vascularization was apparent in the TEC group, which was graded at Grade 1 (Figure 4 and Figure 5).

Corneal thickness measurement: Figure 5 shows the corneal thickness measurements obtained using OCT scans at 8 weeks post-implantation. TEC generated thin corneas measuring 130.97±2.03 µm (p=0.15); in the NT group, the thickness was 244.50±5.63 µm (p=0.01) and in the AM group the thickness was 137.44±9.46 µm. Normal corneas has a mean thickness of 119.66±5.04 µm. There was no significant difference in corneal thickness between TEC, AM and normal corneas (p=0.37). The non-treated groups generated thick corneas approximately double the thickness of a normal cornea (p<0.05; Figure 6).

Histological analysis: Induced BMuc were cultivated on an AM before implantation, covering the epithelial side of the air-dried AM (Figure 1). The corneal epithelial and stromal layers were assessed and compared with those of normal rat corneas. The NT group showed a thin epithelial layer supported by a loose stromal layer with the absence of blood vessels. In the AM group, irregular epithelial layers with goblet-like cells were observed with an unaligned stromal layer. In the TEC group, epithelium consisting of four to five irregular layers of cells with a compact and aligned stromal layer was noted, resembling a normal rat cornea (Figure 7).

Immunohistological analysis: Immunohistological analysis revealed positive CK3 and p63 staining in the corneal epithelial layers of normal rats and in the TEC and AM groups. The expression was observed in nearly the entire epithelial layer. However, neither of these markers were expressed in the NT group (Figure 8).

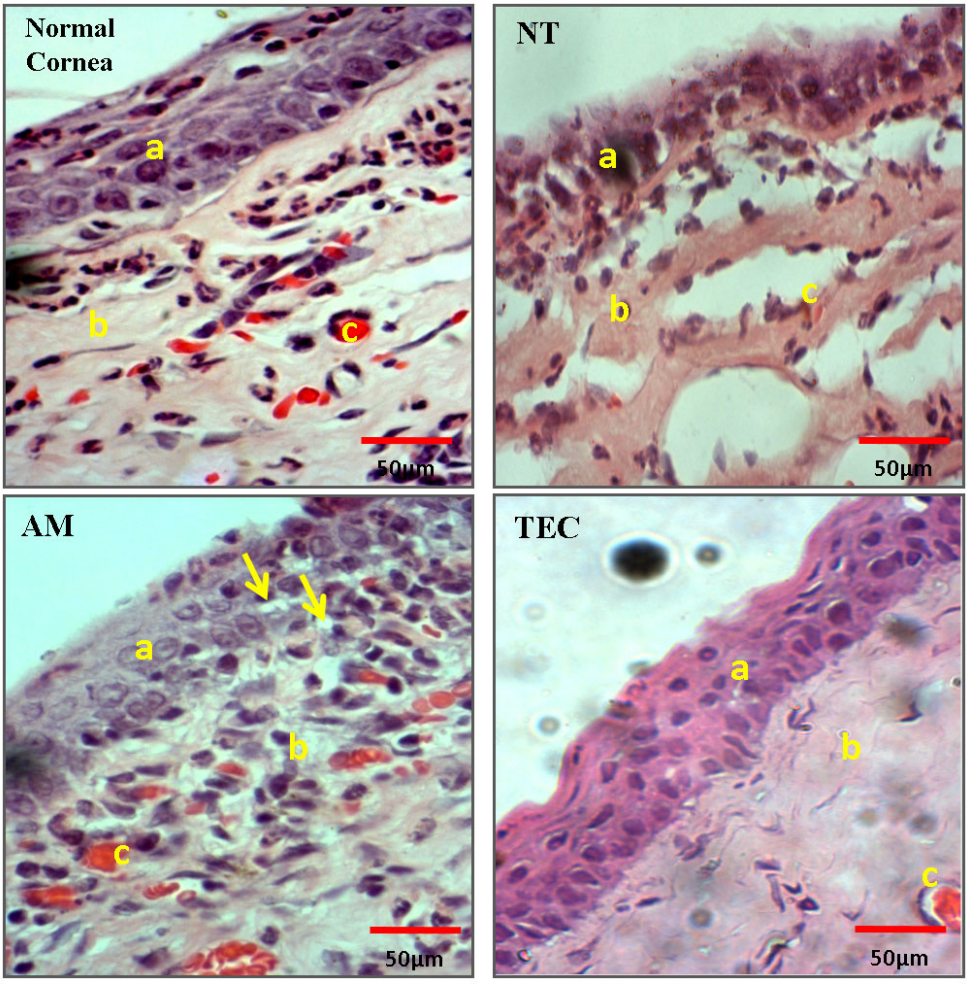

Figure 7. Histological analysis using hematoxylin and eosin-stained normal corneal layer, NT, AM, and TEC. a: epithelial layer. b: stromal layer. c: blood vessel. Yellow arrow: goblet cells (magnification: 400x).

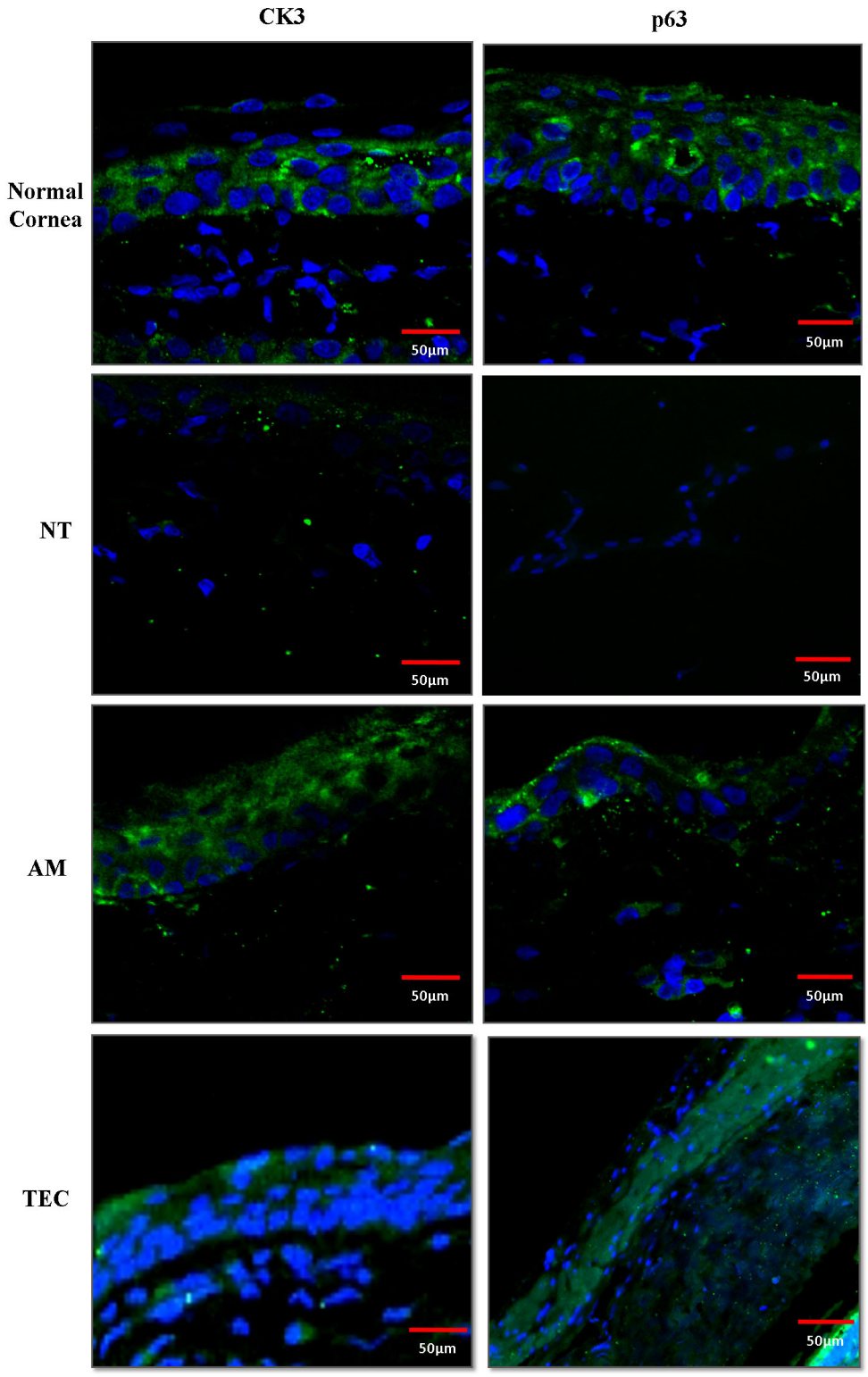

Figure 8. Normal cornea, AM, and TEC stained positive for CK3 and p63. No expression of either antibody was detected in NT (magnification: 400x).

DISCUSSION

In this study, we have shown that AM and TEC are effective in facilitating corneal epithelial repair. This is proven by the restoration of corneal thickness as seen in OCT assessment and good stromal alignment in histological evaluation in both groups. It is noteworthy that both epithelial and stromal layers in TEC appeared to be more compact compared to those of AM. In terms of corneal clarity and the absence of vascularization, TEC scored significantly better compared to AM. This difference was noted as early as Week 2 post-implantation. This suggests that the rate of healing and remodeling of the cornea may be faster in TEC.

In our previous study, TEC formed using induced bone marrow mesenchymal stem cells (BMSCs) was tested in damaged nude rat corneas [19]. It was shown that TEC formed using induced BMSCs significantly reduced vascularization and improved corneal clarity when compared to treatment with an AM alone or to no treatment. In this study, we explored the feasibility of inducing BMuc for the same purpose. The rationale is that BMuc are easily available and are inherently epithelial in nature. We have shown that BMuc can be successfully induced into corneal epithelial cells in vitro, as demonstrated by the positive gene and protein expression of specific markers of the corneal epithelial cells after induction.

The in vitro results showed significant upregulation of β-integrin, C/EBPδ, and ABCG2 in BMuc after induction. β-integrin, C/EBPδ, and ABCG2 are corneal stem cell markers that are involved in regulating epithelial cell differentiation [23], the cell cycle, and self-renewal in human limbal stem cells [24] and that protect corneal epithelial progenitor cells against oxidative stress induced by toxins and hypoxia [25]. Immunocytochemical analysis indicated that CK3 and p63 were highly expressed in induced BMuc but were absent in uninduced BMuc, confirming the results reported by Kolli et al. [26] and Madhira et al. [9]. We concluded that limbal medium is suitable for inducing a corneal epithelial-like phenotype [19].

In the slit lamp biomicroscopy evaluation, the TEC group showed remarkable improvement in terms of corneal clarity and non-vascularization compared to the AM and NT groups. Judging from the improvement of corneal score over time, we predict that both AM and TEC will be able to achieve total clarity and non-vascularization at a later time but not NT.

In our previous study, the corneal thickness of the TEC prepared using induced BMSCs was significantly higher compared to that of the control group [19]. Corneal thickness using TEC prepared with induced BMSCs (276.50 µm) seemed to be thicker compared to TEC prepared with induced BMuc (130.97 µm). The initial thickness of the rat corneas was the same in both studies. In both studies, the corneal thickness of the TEC group was significantly higher than that of the AM and NT groups. However, in terms of epithelial thickness, both TEC-treated groups appeared to have four to five epithelial layers that stained positive for p63 and CK3. This suggests that the increased corneal thickness seen in the groups treated with TEC prepared with induced BMSCs is due to the thickening of the stromal layer. However, both groups maintained a compact and aligned stroma that in the AM and NT groups appeared loose or irregular. In both studies, treatment using AM seeded with cells resulted in significant improvement in terms of corneal clarity and non-vascularization compared to AM alone.

Treatment with AM, which is still the gold standard, provided a reasonable restoration of the cornea. The addition of epithelial-like cells on AM improved corneal clarity, prevented vascularization, and increased corneal thickness and stromal alignment. The translation of these improvements into corneal function is yet to be proven. The role of induced BMSCs or BMuc may be more relevant in cases of limbal stem cell deficiency. One limitation of the study is that functional parameters such as visual acuity testing and ocular surface regularity [27] were not evaluated.

In conclusion, human BMuc can be induced to express a corneal epithelial-like phenotype. The addition of BMuc improved corneal clarity, prevented vascularization, and increased corneal thickness and stromal alignment and showed no adverse effect on the host after implantation.

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