A Novel Tissue-Engineered Corneal Stromal Equivalent Based on Amniotic Membrane and Keratocytes

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Corneal blindness due to injury or other diseases severely affect the quality of life of millions of people worldwide.1,2 Currently, corneal transplantation is considered to be an effective treatment for restoring vision. Gain et al.3 in their survey have estimated that 12.7 million people are waitlisted for corneal transplantation, while only 1 in 70 individuals have received a transplant owing to the shortage of donor tissue, necessitating the development of corneal equivalents. During the past decades, although artificial corneas like Boston KPro,4 AlphaCor,5 and the osteo-odontokeratoprothesis6 have been used in patients with previous graft rejections or other end-stage corneal diseases,7 tremendous efforts have been put in generating tissue-engineered corneal alternatives, which are based on biomaterials, living cells, or both. With the improvement of surgical techniques, corneal transplantation has achieved replacement of partial corneal tissue rather than the full thickness of the cornea. Accordingly, different studies have been conducted to develop transplantable bioengineered corneal tissue layers, such as corneal epithelium, endothelium, and stroma.8

The corneal stroma composes approximately 90% of the cornea; the complex hierarchical structure, high transparency, and mechanical strength make the development of bioengineered corneal stromal tissue a great challenge. To establish tissue-engineered corneal stroma, researchers have widely focused on a scaffold-based approach. Collagen hydrogels9,10 collagen sponges,11 collagen films,12 and magnetically aligned collagen13 have been used as scaffolds in various studies. Synthetic substrates such as polymers14 and gelatin hydrogel,15 or biomaterials such as decellularized porcine corneal stroma16 and silk fibroin,17,18 have also been studied to form stromal replacements.

For the cell-based approach, previous studies19,20 mainly have focused on the extracellular matrix secreted by long-term cultivation of CSCs to generate self-assembled corneal substitutes. The major obstacle for this approach is the
phenotypic instability of keratocytes in vitro. It has been demonstrated that keratocytes cultured in vitro can easily differentiate into fibroblasts that do not express the keratocyte-specific markers, such as keratocan, CD34, and CHST6 (corneal N-acetylglucosamine-6-O-sulfotransferase).21 resulting in production of abnormal extracellular matrix. Thus the preservation of keratocyte phenotype in vitro is critical in tissue-engineered cornea. Espana et al.22 have found that human keratocytes cultured on amniotic membrane (AM) stroma could maintain their phenotype and express keratocan by suppression of transforming growth factor-β.23 Our previous study has also proved that myofibroblasts could reverse to fibroblast phenotype when they are seeded on the stromal surface of AM,24 indicating AM helps maintain the quiescent phenotype of fibroblasts. Recently, we have developed an ultrathin amniotic membrane (UAM) through removing appreciable amount of loose matrix within the fibroblast and spongy layers; left with only a compact layer of matrix, it not only increased transparency, but also retained its strength and elasticity. We have applied this UAM successfully, constructed tissue-engineered corneal epithelium, and markedly improved the outcome of corneal epithelium transplantation surgery in rabbits.25

From the characteristics of AM, we raised the hypothesis that a combination of AM and keratocytes may develop into corneal stroma-like tissue in ex vivo culture conditions. To evaluate this hypothesis, we harvested corneal stromal cells (CSCs) from human and rabbit corneal tissues, seeded them on UAM to form a UAM-CSC construct. After cultivation for a set period, we stacked the constructs to develop a multilayer UAM-CSC laminate and tested its efficacy in rabbit lamellar corneal transplantation. Through this UAM-CSC combination strategy, we generated a stromal equivalent, which may have clinical application potential.

**Materials and Methods**

**Corneal Stromal Cell Culture**

Human CSCs were isolated from collagenase IV-digested human corneal stromal tissue obtained from the Eye Bank of Xiamen Eye Center (Xiamen, China), as previously described.22 CSCs were incubated with medium containing DMEM (Dulbecco’s modified Eagle’s medium)/MCDB-201 with 2% fetal bovine serum, 10 ng/mL epidermal growth factor (Peprotech, Rocky Hill, NJ, USA), 10 ng/mL platelet-derived growth factor (PDGF-BB; Peprotech), 5 mg/mL insulin (Sigma, St. Louis, MO, USA), 5 mg/mL transferrin (Sigma), 5 ng/mL selenous acid (Sigma), and 100 IU/mL penicillin and 100 µg/mL streptomycin (Life Technologies, Carlsbad, CA, USA). The medium was changed every 3 days. Rabbit CSC culture was performed with the same protocol.

**Multilayer UAM Architecture Preparation**

Human placentas were obtained at the time of cesarean delivery from healthy donors in accordance with the tenets of the Declaration of Helsinki for research involving human subjects and received an approval from the institutional review board of Xiamen University-affiliated Chenggong Hospital (Xiamen, Fujian, China). Written informed consent was obtained from the donors. Placentas were preserved in −80°C within 1 week of processing. UAM was prepared according to our previous study. Briefly, the AM epithelial cells were dissociated with 0.02% EDTA and were removed with a cell scraper to produce denuded AM. Furthermore, the stromal side was digested by placing it on type IV collagenase-soaked sponge and incubated at 37°C for 75 minutes, to obtain the UAM.25 The monolayer UAM was then anchored with a specially designed acrylic culture ring-shaped insert measuring 1.5 cm in diameter, then human or rabbit CSCs were seeded on the UAM surface with a density of 5.0 × 10^4 cells/cm². When cells reached confluence after 3 days, the sides of the UAM with cells were face to face and stacked together, constituting a UAM sandwich, with cells between the interspace. To create three- or four-layer UAM constructs, three or four pieces of UAM with CSCs were superimposed with the cells between the interspace (Fig. 1). Then the sandwiches were assembled on the culture inserts and cultured for different durations to generate tissue-engineered corneal stromal tissue.

**Fluorescent Calcein AM Staining**

After culturing in KDM for 1 week, viability, morphology and confluence of CSCs seeded on the UAM substrate and culture dish (made from polystyrene) were evaluated by fluorescent calcein AM (Invitrogen) staining. In brief, the samples were rinsed with PBS solution three times (5 minutes each), followed by addition of 2 µg/mL calcein AM working solution and incubated at room temperature for 30 minutes, rinsed with PBS, then examined with a fluorescence microscope (Leica DM2500; Leica, Wetzlar, Germany).

**Hematoxylin-Eosin (H&E) Staining and Immunostaining**

UAM, UAM-CSCs, and corneal tissues after UAM or UAM-CSC laminate transplantation were fixed in 4% paraformaldehyde for 1 hour at room temperature, then embedded with OCT Compound (Tissue-Tek; SAKURA, Torrance, CA, USA). The frozen samples were cut into 6-µm-thick sections, then stained with the H&E Stain Kit (Auragene, Hunan, China) as per the manufacturer’s instructions and imaged with a light microscope. For immunostaining, tissue sections were incubated at 4°C overnight with anti-α-smooth muscle actin (anti-α-SMA) antibody (Abcam, Cambridge Science Park, UK). Negative controls were generated simultaneously by incubating sections with PBS without primary antibody. The next day, samples were incubated with Alexa Fluor 488-conjugated IgG (1:300) for 1 hour in the dark at room temperature, followed by PBS wash (three times), and covered by mounting medium (Vector, Burlingame, CA, USA) with 4’,6-diamidino-2-phenylindole (DAP) and examined with a fluorescence microscope (Leica DM2500).

**Gene Expression Analysis**

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to analyze gene expression of human keratocytes cultured on UAM and culture dish. Total RNA was extracted by using the Arcturus PicoPure RNA Isolation Kit (Thermo Fisher Scientiﬁc) according to the manufacturer’s instructions. The samples were treated with DNase I (Thermo Fisher) to eliminate genomic DNA. cDNA synthesis was performed using the SuperScript III First-Strand Synthesis System (Thermo Fisher) and quantitated with the Quantitect SYBR Green PCR (Qiagen). The expression of β-actin was used as an internal reference. The threshold cycle number (Ct) was used for comparison of the gene expression levels of the target genes (keratocan, CD34, and CHST6). The primers used are listed in Table 1.
Scientific, Waltham, MA, USA) and reverse transcribed to cDNA by RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific). qRT-PCR was performed with a StepOne plus Real-Time PCR detection system (Applied Biosystems, Darmstadt, Germany) using a SYBR Premix Ex Taq Kit (TAK-ARA, Shiga, Japan), according to the manufacturer’s instructions. For each experiment, template-minus controls were used to provide negative controls for subsequent PCR reactions. The amplification program included an initial denaturation at 95°C for 10 seconds, followed by denaturation at 95°C for 10 seconds, and annealing and extension at 60°C for 30 seconds, for 40 cycles. SYBR Green fluorescence was measured after each extension step, and the specificity of amplification was evaluated by melting curve analysis. The results of the relative qRT-PCR were analyzed by the comparative CT method and normalized to β-actin as an internal control. The primer sequence pairs used are listed in the Table.

### Western Blot Analysis
The protein from human CSCs on UAM and polystyrene was extracted in RIPA buffer (Sigma) with Halt protease and phosphatase inhibitor. Protein concentration was measured by BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein extracts (30 μg) were subjected to Western blot analysis by using anti-keratocan (Sigma), anti-lumican (Abcam), anti-collagen I (Proteintech, Chicago, IL, USA), anti-collagen V (Santa Cruz, Dallas, TX, USA), anti-α-SMA (Abcam), and anti-β-actin (Sigma) antibodies. Protein expression was visualized by using enhanced chemiluminescence reagent (Lulong, Inc., Xiamen, China) and imaged by using a transilluminator (ChemiDoc XRS; Bio-Rad, Hercules, CA, USA).

### Optical Transmittance of Multilayer UAMs
The UAM constructs cultured for different durations were placed on top of the printed letter A and the images were taken with a digital camera (Canon, Tokyo, Japan); the optical transmittance of multilayer UAMs was evaluated through macroscopic observation on the letters.

### Optical Coherence Tomography (OCT) Observation
Aoptovue OCT (Optovue, Fremont, CA, USA) was used to visualize cross sections and thickness of the samples. The samples were washed with PBS three times and were mounted in front of the preplaced mirror to obtain the images. The UAMs and engineered tissues were scanned by axial plane to obtain the 3D map.

### Table. Primer Sequence Pairs Used for Quantitative Real-time PCR

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<tr>
<th>Gene</th>
<th>Forward Primers</th>
<th>Reverse Complement Primers</th>
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<tr>
<td>β-Actin</td>
<td>5'-GCCCTGAAAGCTACCCAAGT-3'</td>
<td>5'-AGGAATCTTCTCTGACCATG-3'</td>
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<td>5'-AACTGGCTTACGCCTGATT-3'</td>
<td>5'-ATCTTTACTGGCTACACAGCC-3'</td>
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<td>Lumican</td>
<td>5'-GCCCTGAAAGCTACCCAAGT-3'</td>
<td>5'-TGGTCCAGAGGACCACAT-3'</td>
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<td>Collagen I</td>
<td>5'-GCGCTGTCCTCCGTGACAGAA-3'</td>
<td>5'-AGACGTCAGCCGATTTCT-3'</td>
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<tr>
<td>Collagen V</td>
<td>5'-TTGTCCTTTCTCCTGACAGAA-3'</td>
<td>5'-AGACGTCAGCCGATTTCT-3'</td>
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<td>CD34</td>
<td>5'-AGGAGAAAGCCGCTGGCGAAG-3'</td>
<td>5'-TGGTCTTCTCTGAAAACTCGG-3'</td>
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<td>5'-CATGGCGCCTCCATTCAAGGAC-3'</td>
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<td>CHST6</td>
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<td>PTGDS</td>
<td>5'-CAGTGCTGCGGACCCAGAC-3'</td>
<td>5'-CCAGTTGCTGCGGACCCAGAC-3'</td>
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<tr>
<td>Vimentin</td>
<td>5'-GAGCCAGCTTAAACCCAGCAA-3'</td>
<td>5'-AAGCTGCGGTCTGTTCTGGAAG-3'</td>
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<td>Fibronectin</td>
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<td>5'-CCAGGGTGAGCTGCTGGGAGA-3'</td>
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<td>α-SMA</td>
<td>5'-CTTGCTGAGGTTGGCCAGAT-3'</td>
<td>5'-TTCTTGGGCTTCTGATGCGA-3'</td>
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Ultrastructure Analysis

CSC-secreted extracellular matrix (ECM) and the underlying amnion morphology were imaged with scanning electron microscopy (SEM). Specimens were fixed in 2.5% glutaraldehyde for 2 hours; dehydrated by using an ethanol series at 4°C, followed by five tertbutyl alcohol washes, 10 minutes each, at room temperature; and crystallized in tertbutyl alcohol at 4°C overnight. The samples were freeze dried and imaged with SEM (JSM6390LV; JEOL, Tokyo, Japan). For the transmission electron microscopy (TEM) analysis, the specimens were fixed in 2.5% glutaraldehyde for 2 hours, dehydrated in an ethanol series at 4°C, followed by tertbutyl alcohol wash for five times. Then the samples were embedded in Epon, ultrathin sections (70 nm) were cut and stained with 3% uranyl acetate (Thermo Fisher Scientific) and lead citrate (Sigma). The sections were imaged at 80 kV by using TEM (JEM2100HC; JEOL).

Lamellar Keratoplasty

To evaluate the feasibility of the multilayer UAM lamination for surgery, the tissue-engineered stromal tissues were used to perform lamellar keratoplasty. Fourteen New Zealand white rabbits were included, and surgery was performed under intramuscular anesthesia with a mixture of xylazine hydrochloride (Shengda Animal Pharmaceutic, Jilin, China) and sodium pentobarbital (Boshida, Xiamen, China). The anterior stroma was dissected by trephination, approximately 150 μm in depth and 4.5 mm in diameter. The engineered UAM or UAM-CSC lamination stromal graft was excised with a 5-mm-diameter trephine and sutured onto the recipient rabbit cornea by using 10-0 nylon suture. At the end of the procedure, the eyelid was sutured to protect the transplanted tissues and the sutures were removed 1 week after the lamellar keratoplasty. Tobradex (Alcon, Fort Worth, TX, USA) and cyclosporine (NCPC, Shijiazhuang, China) eye drops were administered three times a day, and tobramycin eye ointment (Alcon) once a day. The rabbits were killed 4 weeks post surgery to evaluate the efficacy of transplantation. All animal experiments complied with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Statistical Analysis

All the experiments were performed at least three times unless specified. The statistical data are expressed as means ± standard deviation, which were analyzed by using Student’s t-test, and statistical significance was defined as $P < 0.05$.

RESULTS

Morphology and Phenotype of Corneal Stromal Cells Cultured on UAM

The SEM image showed that the fibrils of UAM surface were organized with a linear groove (Fig. 2A); higher magnification showed interspace between collagen fibers of the UAM (Fig. 2B). Calcein AM staining evaluated the viability and arrangement of keratocytes. CSCs were cultured in KDM for 1 week on UAM (C) and cell culture dish (D). mRNA expression of CD34, ALDH3A1, CHST6, and PTGDS (E), keratocan, lumican, collagen I, and collagen V (F), vimentin, fibronectin, and α-SMA (G) in keratocytes cultured on UAM surface or culture dish for 1 week. Western blot validated the expression of keratocyte-specific markers: keratocan, lumican, collagen I, collagen V, and fibrosis marker α-SMA in CSCs cultured on UAM and culture dish (H). Scale bar: 100 μm in (C) and (D). *P < 0.05, **P < 0.01.
were cultured on UAM as compared with culture dishes (Fig. 2E).

Keratocyte-specific proteoglycan keratocan was also highly expressed in CSCs cultured on UAM; another proteoglycan, lumican, and the ECM component collagen I showed no significant difference between cells cultured on UAM and culture dish, while collagen V was downregulated on UAM (Fig. 2F). Meanwhile, the expression of fibrotic markers fibronectin and α-SMA was lower on UAM than in cells grown in the culture dish (Fig. 2G). We further determined the expression of corneal stroma–specific proteoglycans and collagens in the CSCs by Western blot analysis. Similar to the qRT-PCR results, keratocan expression was higher in CSCs cultured on UAM, lumican and collagen I expression showed no significant difference between UAM and cell culture dish, while collagen V expression was higher in the cells grown on culture dish (Fig. 2H). Western blot analysis also confirmed that α-SMA expression was weakly detected in the cells grown on UAM, while it was highly expressed in CSCs cultured on cell culture dish (Fig. 2H).

We then performed SEM to observe the matrix produced by CSCs cultured on UAM and cell culture cover slides. The results showed dense, organized fibrils deposited on the UAM surface (Fig. 3A, B). CSCs cultured on cover slides produced mussy ECM (Fig. 3C, D).

Figure 3. SEM images of the matrix produced by CSCs at low magnification (A, C) and high magnification (B, D) after culture for 8 weeks. Organized fibrils deposited on the UAM surface (A, B). CSCs cultured on cover slides produced mussy ECM (C, D).

**Characteristic of Engineered Corneal Stroma–like Tissue**

We then used UAM with CSCs to construct corneal stroma–like tissue. We assembled tissue constructs with two, three, and four layers of UAM, using the strategy showed in Figure 1. After assembly, the tissue constructs were further cultured for 8 weeks. By macroscopically observing the clarity of the letter A through the amniotic membrane, the optical transparency was estimated for single-layer UAM-CSCs (Fig. 4A) and stacked UAM-CSCs of two (Fig. 4C), three (Fig. 4E), and four (Fig. 4G) layers before culture. After culturing for 8 weeks, there was mild decrease of light transmittance in single-layer UAM (Fig. 4B) and in two-layer (Fig. 4D), three-layer (Fig. 4F), or four-layer UAM-CSCs (Fig. 4H). Generally, with the increase of UAM-CSC layers, the light transmittance decreased gradually.

We used Optovue OCT to scan the tissue constructs to investigate the matrix density and quantify the tissue thickness. The results showed that the entire UAM arrangement from single UAM without cells (Fig. 5A) to stacked UAM-CSCs of two (Fig. 5C), three (Fig. 5E), and four (Fig. 5G) layers, including native UAM, exhibited homogeneity, which showed that the CSC-secreted ECM may have been better integrated with native UAM and had similar density to UAM matrix. The density homogeneity was well documented by movie recording with Optovue OCT, as shown in Supplementary Figures S1 through S5. As a control, native human corneal tissue also showed homogenous density (Fig. 5I); however, the gray value of the image was different, maybe because of the difference in the collagen diameter between UAM-CSC construct and human cornea.

The OCT scan also showed thickness of the engineered tissue constructs. The monolayer UAM without cells was approximately 35 ± 6 μm in thickness (Fig. 5B) and the
monolayer UAM with CSCs cultured for 8 weeks was 58 ± 12 μm in thickness (data not shown), while the UAM-CSCs of two (Fig. 5D), three (Fig. 5F), and four (Fig. 5H) layers with cells cultured for 8 weeks was approximately 80 ± 10 μm, 198 ± 18 μm, and 250 ± 18 μm in thickness, respectively. As a control, the thickness of native human central corneal tissue was 500 ± 18 μm (Fig. 5J).

We then performed H&E staining to illustrate general tissue structure and cell distribution inside the constructs. The H&E staining of frozen cross sections showed acellular compact layer of UAM without seeding cells (Fig. 5K). In the two, three, and four layers of UAM tissue constructs (Figs. 5L–N, respectively), CSCs were evenly distributed among the matrix of tissue constructs, similar to those of native corneal stromal tissue.

To further investigate the detailed microstructure of the engineered corneal stroma–like tissue constructs, we performed TEM observation on native corneal tissue, acellular UAM, and multilayer UAM-CSCs. The cross-section images under different magnifications showed that the internal microstructure of human cornea (Figs. 6A–C) and the UAM (Figs. 6D–F) fibrils were organized into tightly spaced bands.
(lamellae), with uniform fibril in orthogonal direction, which was clearly demonstrated under high magnification (Figs. 6C, 6F). The CSCs after culture for 8 weeks exhibited a flattened appearance with long-range processes integrated into the developing ECM (Fig. 6G). When viewed at higher magnification, CSC-generated ECM between different cell layers showed stratified, multilayered lamellae with orthogonal fibril arrangement (Figs. 6H, 6I).

To determine the similarity of collagen fibril structure between native corneal tissue, UAM, and engineered corneal stroma–like tissues, we calculated fibril diameter and interfibrillar spacing by using high-magnification TEM images. The digital analysis showed that the average fibril diameter of human cornea, UAM, and CSC-secreted fibrils was $29 \pm 5.7 \text{ nm}$, $36.5 \pm 8.4 \text{ nm}$, and $33.8 \pm 6 \text{ nm}$, respectively. The distribution curve of fibril diameter in different groups showed that the fibril diameter of human cornea was smaller than that of UAM fibrils and CSC-secreted fibrils (Fig. 6J). The interfibrillar spacing of preserved human cornea, UAM, and CSC-secreted fibrils was $55.1 \pm 7.4 \text{ nm}$, $43.8 \pm 6.5 \text{ nm}$, and $45.0 \pm 5.8 \text{ nm}$, respectively. The distribution curve showed that the interfibrillar spacing of preserved human cornea was larger than that of UAM fibrils and CSC-secreted fibrils (Fig. 6K).

**Lamellar Keratoplasty With Engineered Corneal Stroma–like Tissue**

To determine the efficacy of engineered corneal stroma–like tissue on corneal reconstruction, we assembled the stroma-like tissues, we calculated fibril diameter and interfibrillar spacing by using high-magnification TEM images. The digital analysis showed that the average fibril diameter of human cornea, UAM, and CSC-secreted fibrils was $29 \pm 5.7 \text{ nm}$, $36.5 \pm 8.4 \text{ nm}$, and $33.8 \pm 6 \text{ nm}$, respectively. The distribution curve of fibril diameter in different groups showed that the fibril diameter of human cornea was smaller than that of UAM fibrils and CSC-secreted fibrils (Fig. 6J). The interfibrillar spacing of preserved human cornea, UAM, and CSC-secreted fibrils was $55.1 \pm 7.4 \text{ nm}$, $43.8 \pm 6.5 \text{ nm}$, and $45.0 \pm 5.8 \text{ nm}$, respectively. The distribution curve showed that the interfibrillar spacing of preserved human cornea was larger than that of UAM fibrils and CSC-secreted fibrils (Fig. 6K).

Transplanted in the recipient rabbit cornea by using lamellar keratoplasty technique (Figs. 7A, 7B), with multilayer UAMs without cells as control (Figs. 7C, 7D). No postoperative complications including conjunctival congestion or corneal neovascularization were recorded during the follow-up period. One week after transplantation, fluorescence staining showed partial epithelialization of the graft in the UAM-CSC group (Fig. 7F), and no epithelialization in the UAM group (Fig. 7H). Four weeks post surgery, slit-lamp microscopy images showed complete integration of the graft and recipient corneal tissue in both the UAM-CSC group (Fig. 7I) and the UAM group (Fig. 7K).

Fluorescence staining on the ocular surface showed complete epithelialization of the graft in both the UAM-CSC group (Fig. 7J) and the UAM group (Fig. 7L). H&E staining of the rabbit corneas 4 weeks post surgery showed that CSCs were evident in the matrix of engineered corneal stroma–like tissue UAM-CSCs (Figs. 7M, 7N). In contrast, in the UAMs along the graft, few rabbit native keratocytes migrated into the amniotic matrix near the implant bed (Figs. 7O, 7P). The thickness of UAM-CSC laminate (Fig. 7M) at 4 weeks after surgery was obviously higher than that of the UAM laminate (Fig. 7O).

We further performed TEM on the transplanted cornea and found there was an obvious desmosomal attachment formed between the epithelial cells on the surface of the UAM-CSC construct (Fig. 8A). Populated keratocytes could be seen among the extracellular matrix, with procollagen deposited around the cells (Fig. 8B). Under high magnification, the procollagen fibrils were loose but still characterized by packed, orthogonal architecture with uniform diameter of $32.5 \pm 6.6 \text{ nm}$ (Figs. 8C, 8D). The corneal button, at 4 weeks post transplantation with UAMs, showed epithelial cells on the graft surface (Fig. 8E); few rabbit keratocytes migrating into the
amniotic matrix near the implant bed (Fig. 8F); and aligned collagen fibers of amnion (Figs. 8G, 8H). The normal rabbit cornea as a control was characterized by desmosomal attachments between the epithelial cells (Fig. 8I), dendritic keratocytes among the ECM (Fig. 8J), and well-organized collagen fibers (Figs. 8K, 8L).

We further performed immunofluorescence staining of α-SMA on the corneas after UAM-CSC and UAM laminate transplantation at 4 weeks. There was no α-SMA expression in the transplanted laminates or recipient corneal tissues in both the UAM group (Fig. 9A) and the UAM-CSC group (Fig. 9B), while limbal blood vessels from both groups showed positive staining as control (Figs. 9C, 9D).

DISCUSSION

AM is widely used in the treatment of ocular surface diseases owing to its biological properties of stimulating epithelial cell proliferation,\textsuperscript{26,27} antifibrosis, anti-inflammation, and antimicrobial features;\textsuperscript{28} it is also commonly used in corneal epithelial tissue engineering as cell carrier.\textsuperscript{29-30} In the current study, we used a stepwise strategy to combine keratocytes and multilayer UAM to generate corneal stroma–like tissue and successfully applied it in rabbit corneal lamellar transplantation. UAM used in our study included a compact layer of AM, which surmounts the disadvantages of AM, such as low transparency and sagginess. The highly organized collagen fibers make the UAM an outstanding carrier for keratocytes by promoting cell-cell, cell-matrix, and matrix-matrix communication to maintain the keratocyte phenotype, and further to promote synthesis of ECM in vitro.

We found that keratocytes cultured on UAM could maintain their phenotype and express specific markers such as keratocan, CD34, ALDH3, CHST6, and PTGDS. Fibronectin and α-SMA, the fibrotic markers, were decreased in keratocytes cultured on UAM compared with cell culture dish. Previous studies have proved full-thickness AM is effective in reducing scar formation during ocular surface reconstruction,\textsuperscript{31} and soluble AM stromal extracts can prevent myofibroblast differentiation.\textsuperscript{24} Our current results further proved that UAM retained the characteristic of antifibrosis on keratocytes during ex vivo tissue engineering, thus preserving their normal phenotype, which is critical in corneal stromal tissue engineering in terms of matrix synthesis. Collagen I gene expression was slightly higher in UAM, while collagen V expression was higher in CSCs cultured on dishes. Since collagen I is the major matrix component of corneal stroma, our results indicated that collagen synthesis of keratocytes cultured on UAM was similar to that of in vivo collagen components.

In this study, we found that newly synthesized collagen fibers laid down by keratocytes cultured on UAM were well organized, while collagen fibers produced by CSCs cultured on cover slides were arranged in random direction. Recently, researchers\textsuperscript{17,18} have processed material surfaces with a uniform groove for corneal tissue engineering, which guide cell growth and collagen deposition. The native structure of AM used in our study showed similar unique features. SEM results showed that the topography of the UAM surface was characterized by linear grooves between aligned collagen fibers.
fibrils that aided as a cell-guiding and ECM deposition substrate. Thus on the UAM surface, CSCs oriented along the in situ fiber orientation and CSC-secreted collagen fibers were aligned in parallel manner to form lamellae. Additionally, a previous study has demonstrated that the primary corneal stroma in chick corneal development can act as a template to guide the deposition of the secondary stroma secreted by the stromal cells. The native UAM matrix made up of fibril lamellae with tightly packed, highly organized collagen fibrils in a regular orthogonal array may provide a template for the generation of the CSC-secreted ECM. Moreover, the keratocytes sandwiched between the lamellae construct a 3D system with multilayer UAMs, providing a biomimetic architecture to guide the cornea-specific ECM formation.

After 8 weeks’ culture of multilayer UAM-CSC laminate, we trephined the laminate and found that the multilayer UAMs had intact mechanical strength, while different layers of the UAMs easily separated in the control group without CSCs seeded, indicating matrix produced by CSCs promoted adhesion of UAMs. At low magnification, the CSC-secreted ECM was arranged into tightly spaced bands approximately 1- to 2-μm thick, like the human corneal laminares, with uniform fibril in orthogonal direction. The homogeneous, thin-diameter collagen fibrils generated by CSCs were regularly packed with an ordered hierarchical organization. The CSC-secreted ECM between two layers was approximately 30 ± 10 μm. The CSCs were sandwiched between the multilayered UAM to form a high-density cellular system; thus, layers can be in close proximity to promote cell-cell, cell-matrix, matrix-matrix communication as remodeling progresses. On the other hand, the strong antifibrosis microenvironment provided by the 3D-confined architecture favors the tissue-engineered cornea formation. Multilayered UAM-CSC laminates resemble the corneal stroma lamination. The four-layer UAM reached a thickness of 250 ± 18 μm, half the thickness of human cornea, making its use possible for lamellar keratoplasty.

To evaluate the feasibility of the multilayer UAM lamination, we performed lamellar keratoplasty using this tissue-engineered stromal substitution. During the surgery, we found the UAM laminates were sufficiently resilient to be stably sutured in the recipient cornea. However, the multilayer UAMs without cells—each layer would be easily separated during the surgery—further proved that the matrix secreted by the CSCs provided strong adhesion at the layer interfaces to ensure the mechanical toughness and integrity of the implant. Four weeks post surgery, the implant UAM-CSC laminates were seamlessly

**Figure 8.** TEM of corneal button at 4 weeks post transplantation with UAM-CSCs. Epithelial cells on the surface of graft tissue showed obvious desmosomal attachments (A). Populated keratocytes were distributed in the graft tissue (B), and the surrounding collagen fibers showed packed, orthogonal architecture (C, D). The corneal button at 4 weeks post transplantation with UAM showed epithelial cells on the graft surface (E), rabbit keratocytes migrating into the amniotic matrix near the implant bed (F), and aligned collagen fibers of amnion (G, H). Normal rabbit cornea as a control showing desmosomal attachments between the epithelial cells (I), dendritic keratocytes among the ECM (J), and organized collagen fibers (K, L). *White arrowheads* refer to desmosomal attachment between epithelial cells.
and stably integrated with the native corneal tissues without any rejection. Significantly, the excised corneal button displayed a normal corneal architecture on histologic examination. The rabbit epithelial cells grew over the implants, CSCs distributed among the ECM, and the implant were closely attached to the implant bed. In the UAM control group, few stromal cells from rabbit cornea migrated into the amniotic matrix near the implant bed, and the graft was not closely attached to the epithelium and the graft bed.

Thus our results indicated that multilayer amnion could provide an ideal scaffold and favorable milieu for keratocytes to flourish, together with an in vivo environment that promotes generation of collagen fibers that mimic corneal stroma. So these cells participate in laying down ECM at these sites, effectively rebuilding stromal tissue, which is important for corneal repair and regeneration.

Said et al.33 have transplanted multilayer amnion in humans and report that the CSCs migrate into amnion, with most cells showing α-SMA positivity and a few cells with CD34 positivity, demonstrating that the stromal cells are metabolically active among amniotic stroma even after 1 year of follow-up in vivo.33 In our study results, there was no α-SMA expression in the transplanted laminates or recipient corneal tissues in both the UAM group and the UAM-CSC group. These results indicated that although the transparency of the anterior stroma did not reach the level of transparency of the native cornea, there was no scar formation or active myofibroblast differentiation in the corneal stroma after UAM or UAM-CSC laminate transplantation.

The natural environment of CSCs is three dimensional, and their behavior in a 3D environment is of more relevance to both the cellular biology features and tissue engineering.54 The multilayer UAM construct acted as a 3D scaffold for keratocytes and enabled in vivo cell behavior studies during the corneal stroma–like tissue development. In the future, our model can be used to elucidate keratocyte performance, which may also aid in better understanding the process of corneal stroma development. In conclusion, our study established a novel 3D biomimetic corneal model as corneal substitute in vitro, which functioned to promote ECM generation and remodeling after transplant in vivo, providing a new avenue for basic research and therapeutic potential.

Acknowledgments

Supported by the National Key R&D Program of China (No. 2018YFA0107301, No. 2018YFA0107304), the National Natural Science Foundation of China (NSFC; No. 81770894, No. 81470602, and No. 81330022), the Fundamental Research Funds for the Central Universities of China (No. 20121101049 and No. 20720150171), the Natural Science Foundation of Fujian Province, Fujian, China (No. 2015J01355). The funders had no role in study design, data collection and analysis, decision on publishing, or preparation of the manuscript.

Disclosure: X. Che, None; H. Wu, None; C. Jia, None; H. Sun, None; S. Ou, None; J. Wang, None; M.V. Jeyalatha, None; X. He, None; J. Yu, None; C. Zuo, None; Z. Liu, None; W. Li, None

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FIGURE 9. Immunofluorescence staining of α-SMA on the corneas after UAM-CSC and UAM laminate transplantation for 4 weeks. There was no α-SMA expression in the transplanted laminates or recipient corneal tissues in both the UAM group (A) and the UAM-CSC group (B). The limbal region showed positive α-SMA expression in both the UAM group (C) and the UAM-CSC group (D), surrounding the blood vessels in the limbal stroma. Scale bar: 100 μm.


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