



Full length article

Characterizing the impact of 2D and 3D culture conditions on the therapeutic effects of human mesenchymal stem cell secretome on corneal wound healing *in vitro* and *ex vivo*

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ABSTRACT

The therapeutic effects of secreted factors (secretome) produced by bone marrow-derived human mesenchymal stem cells (MSCs) were evaluated as a function of their growth in 2D culture conditions and on 3D electrospun fiber scaffolds.

Electrospun fiber scaffolds composed of polycaprolactone and gelatin were fabricated to provide a 3D microenvironment for MSCs, and their mechanical properties were optimized to be similar to corneal tissue. The secretome produced by the MSCs cultured on 3D fiber matrices versus 2D culture dishes were analyzed using a Luminex immunoassay, and the secretome of MSCs cultured on the 3D versus 2D substrates showed substantial compositional differences. Concentrations of factors such as HGF and ICAM-1 were increased over 5 times in 3D cultures compared to 2D cultures. *In vitro* proliferation and scratch-based wound healing assays were performed to compare the effects of the secretome on corneal fibroblast cells (CFCs) when delivered synchronously from co-cultured MSCs through a trans-well co-culture system versus asynchronously after harvesting the factors separately and adding them to the media. Cell viability of CFCs was sustained for 6 days when co-cultured with MSCs seeded on the fibers but decreased with time under other conditions. Scratch assays showed 95% closure at 48 h when CFCs were co-cultured with MSCs seeded on fibers, while the control group only exhibited 50% closure at 48 h. Electrospun fibers seeded with MSCs were then applied to a rabbit corneal organ culture system, and MSCs seeded on fibers promoted faster epithelialization and less scarring. Corneas were fixed and stained for alpha smooth muscle actin (α -SMA), and then analyzed by confocal microscopy. Immunostaining showed that expression of α -SMA was lower in corneas treated with MSCs seeded on fibers, suggesting suppression of myofibroblastic transformation.

MSCs cultured on electrospun fibers facilitate wound healing in CFCs and on explanted corneas through differential secretome profiles compared to MSCs cultured on 2D substrates. Future work is merited to further understand the nature and basis of these differences and their effects in animal models.

Statement of significance

Previous studies have shown that the secretome of bone marrow-derived mesenchymal stem cells (MSC) is promotes corneal wound healing by facilitating improved wound closure rates and reduction of scarring and neovascularization. The present research is significant because it provides evidence for the modula-

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tion of the secretome as a function of the MSC culture environment. This leads to differential expression of therapeutic factors secreted, which can impact corneal epithelial and stromal healing after severe injury.

In addition, this article shows that co-continuous delivery of the MSC secretome improves cell migration and proliferation over aliquoted delivery, and that MSCs grown on three-dimensional electrospun fiber constructs may provide a favorable microenvironment for cultured MSCs and as a carrier to deliver their secreted factors to the ocular surface.

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1. Introduction

The human mesenchymal stem cells (MSCs) secretome refers to the secreted cytokines, chemokines, growth factors, and extracellular matrix (ECM) molecules from MSCs, and it has generated great interest in wound healing due to their apparent therapeutic effects [1–5]. Although the MSC secretome is not fully characterized, the paracrine effects of MSCs on control of inflammation, tissue repair, regeneration, remodeling, and cellular recruitment have been supported by numerous studies [1–4]. For clinical applications, it is crucial to optimize the constituents of the secretome to increase efficiency. However, until recently, there have been only a few studies on the efficacy of secretome as a function of its composition.

Some studies have shown that the behavior of MSCs and their secreted factors vary in different cell culture environments, and the effects of the secretome on immunomodulatory properties and skin wound healing can be adjusted by modification of the fiber topography and alignment generated by electrospinning [6–8]. Scaffolds have been designed to differentiate stem cells into the desired lineages, and in particular, three-dimensional (3D) scaffolds have been extensively studied to provide ECM-like structures and environments [9,10]. These findings suggest that the therapeutic potential of MSCs and their secreted factors can be tuned for tissue-specific and target-specific effects as a function of culture conditions and cellular microenvironment.

In the field of ophthalmology, there have been various investigations into the use of the MSC secretome to promote corneal epithelial wound healing with promising results [11–14]. One study showed that continuous or multiple treatments of secretome suppress inflammation more than a single treatment during corneal wound healing [14]. The secretome can be incorporated through an eye drop which is a widely used therapeutic drug application method, but it requires frequent topical administration due to the volumetric turnover through the lacrimal system [15,16]. Therefore, it may be advantageous to develop a delivery system capable of continuously transmitting secreted factors without repeated application. Although the direct topical delivery of MSCs to the eye is one way to provide MSC secretome to corneal wound sites, MSCs delivered this way would need to engraft to the ocular surface or are likely to be relocated or washed away by tears. Immobilization of MSCs in or around the ocular surface in some fashion would provide a continuous, local supply of secreted factors that could treat a corneal wound.

In this study, we assessed and compared the effects of the MSC secretome several ways and in two different culture systems. Using *in vitro* corneal fibroblast cultures, we evaluated the effects of continuous versus discontinuous delivery on cell proliferation and migration by comparing a transwell-based co-culture system to simple inoculation of cell culture medium with harvested (cell-free) MSC secretome, as well as continuous delivery from MSCs cultured in 2D conditions versus a three-dimensional (3D) electrospun fiber scaffold. Using a rabbit corneal organ culture system, we compared continuous delivery of MSC secretome from cultured MSCs grown

in 2D on well plates versus those grown in the 3D electrospun fiber scaffold. The latter set of assays investigated the effects of continuous secretome delivery methods on both epithelial wound healing as well as formation of stromal haze after a severe alkaline burn injury.

Electrospun fiber membranes, which provide a 3D environment for MSC growth, were fabricated with polycaprolactone (PCL) and gelatin with adjusting mechanical property. The quantitative compositions of secretome from 2D and 3D environments were analyzed by Luminex multiplex protein assay. Cell viability and wound healing scratch assays were performed on corneal fibroblasts to evaluate the paracrine effects of secretome through co-culture of MSCs. The efficacy of corneal healing was then investigated using an organ culture model of chemical burn in rabbit corneas. The epithelial healing after injury was measured by fluorescein staining, and corneal haze was also evaluated. Stromal fibrotic reactions were evaluated by immunohistology through alpha smooth muscle actin (α -SMA) staining.

2. Materials and methods

2.1. Materials

Unless otherwise noted, all chemicals and solvents were of analytical grade and used as provided by the manufacturers.

Gelatin, polycaprolactone (PCL), trifluoroethanol (TFE), glutaraldehyde, thiazolyl blue tetrazolium bromide (MTT), sodium hydroxide, agar, phosphate-buffered saline (PBS), paraformaldehyde (PFA), fluorescein, and DAPI were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), antibiotic/antimycotic solution, Dulbecco's modified eagle medium (DMEM), Human 62-plex kits, DMEM/nutrient mixture F-12 (DMEM/F-12), penicillin G, streptomycin sulfate, insulin-transferrin-selenium (ITS), Dulbecco's phosphate-buffered saline (DPBS), Alexa Fluor 546-coupled goat anti-rabbit IgG, and Alexa Fluor 488-coupled goat anti-rabbit IgG were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Povidone-iodine solution was purchased from Dynarex (Orangeburg, NY, USA). Whatman filter paper was purchased from GE Healthcare Life Sciences (Pittsburgh, PA, USA). Mouse anti-alpha smooth muscle actin was purchased from Abcam (Cambridge, UK).

2.2. Fabrication of gelatin/PCL electrospun fibers

An electrospinning solution was prepared by dissolving a mixture of gelatin and PCL (9:1 and 1:1 weight ratio) in TFE to form a 10% w/v solution. To electrospin PCL/gelatin fibers, a 10 kV positive voltage was applied to the solution via a needle, and a constant feeding rate of solution (0.5 mL/h) was provided by a syringe pump for 20 min (TL-01, Tong Li Tech, Shenzhen, China). The resulting PCL/gelatin fibers were crosslinked using glutaraldehyde vapor, which was carried out by placing fibers in a sealed desiccator containing 10 mL of 0.5% w/v aqueous glutaraldehyde solution at room temperature for 24 h. The residual glutaraldehyde was removed in vacuum at room temperature for 24 h.

2.3. Characterization of electrospun fibers

Mechanical uniaxial tensile testing was conducted using an Instron uniaxial testing instrument (Instron 8511, Canton, MA) to determine the maximum tensile strength, elongation at break, and Young's modulus. Rectangular shaped electrospun fiber membranes (6 cm × 3 cm) were subjected to a strain rate of 3 cm per min using a 5 N load cell until break ($n=3$). The thickness of each sample was measured, and force was divided by the thickness to obtain stress.

2.4. MSCs culture on electrospun fibers

Human bone marrow derived mesenchymal stem cells (MSCs; PT-2501) were purchased from Lonza, and the cells expressed CD29, CD44, CD105, CD166, CD90 and CD73, but did not express CD14, CD34, CD45, or CD19. The cells were used from passage three to five for all experiments. MSCs were cultured in culture medium (10% v/v FBS, and 1% v/v antibiotic/antimycotic solution in DMEM) and were incubated at 37 °C in 5% CO₂. Scaffolds were placed in 12-well plates and sterilized with UV light for 30 min. The cells (1.0×10^5 cells) were then seeded onto each scaffold in the 12-well plates followed by addition of 500 µL of serum-free culture medium (1% v/v antibiotic/antimycotic solution in DMEM) after 30 min. The MSC-seeded scaffolds were then moved into new 12-well plates 6 h after cell seeding to exclude the effect of the cells that adhered to the well plate.

For obtaining conditioned media from the 2D and 3D culture environments, the MSCs were cultured in 12-well plates with and without fibers in serum-free culture medium, and the culture media was collected after 24 h. The collected conditioned media were centrifuged at $500 \times g$ for 10 min, and the supernatants were analyzed by Luminex multiplex assay at the Human Immune Monitoring Center at Stanford University. Human 62-plex kits were used according to the manufacturer's recommendations. Plates were read using a Luminex 200 instrument with a lower bound of 50 beads per sample per cytokine.

2.5. Corneal fibroblast co-culture with MSCs

Primary corneal fibroblasts were isolated from rabbit corneas in the same manner as previous research in previously reported research [17]. Corneal fibroblasts were cultured in culture medium and were incubated at 37 °C in 5% CO₂. The cells (1.0×10^5 cells) were then seeded to each well of 12-well plates.

For evaluation of cell viability, MTT assay was performed. The culture media was removed after 24 h, and serum-free medium and the collected conditioned medium from the 3D culture environment was added to the well plates as a control group and the conditioned media group (CM-3D), respectively. Transwell inserts (PET, pore size: 3 µm, Falcon cell culture inserts) were placed in the wells and submersed in the media of the co-culture groups. For the MSC-2D group, MSCs were seeded on trans-well inserts and cultured in serum-free media for 24 h, and the MSC-seeded trans-well was assembled with corneal fibroblasts-cultured 12-well plates. For the MSC-3D group, the MSCs-cultured electrospun fiber mesh was transferred onto the transwell insert, and then the insert was placed into 12-well plates pre-seeded with corneal fibroblasts. The media was refreshed every two days. For MTT assays of corneal fibroblasts and MSCs, respectively, the transwell inserts were disassembled from well plates at day 2, 4, and 6. MTT solution (5 mg/mL) was added to the serum-free medium as 10% v/v. After 3 h incubation at 37 °C, the formazan crystals transformed from MTT were dissolved by DMSO. The absorbance was measured at 570 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

For the scratch wound healing assay, the corneal fibroblasts were cultured for 3 to 4 days until the cells formed a confluent monolayer. The cell monolayer was scraped in a straight line to create a scratch with a 200 µL pipette tip. The cell debris was gently washed with serum-free media. The control, CM-3D, MSC-2D, and MSC-3D groups were performed in the same manner as the cell viability assay. The images were taken at 24 and 48 h, and the quantification of scratch closure was evaluated by remaining open area via the wound healing measurement tool of ImageJ. The scratch closure was evaluated by dividing the remaining opened area by the initial scratch area.

2.6. Rabbit cornea organ culture

Fresh rabbit eyes were obtained from Vision Tech (Sunnyvale, TX, USA) and disinfected with 10% povidone-iodine solution. The chemical burn was generated to the corneal surface of the rabbit eyeball using sodium hydroxide solution. Whatman filter paper was punched with an 8 mm diameter biopsy punch (Miltex, Integra LifeSciences, Plainsboro, NJ), and the 8 mm circular filter papers were immersed in 1.0 M sodium hydroxide solution for 1 min. The sodium hydroxide-absorbed filter paper was placed on the cornea for 1 min, and great care and attention was taken to ensure time of exposure was standardized. Immediately the eyes were rinsed in flowing water for 5 min.

For the air/liquid organ culture system, the same method as a previous study was used [18] which has also been used extensively by other institutions [19–21]. Following wounding, the injured corneas were excised from the globes with a 1 mm scleral rim, grasping only scleral rims and not the clear cornea. Excised corneas were immediately carefully transferred onto individual preformed agar plugs to maintain normal culture and nutritional support. Agar plugs were made from 1:1 mixture of serum-free medium containing double strength antibiotic/antimycotic and 2% agar in distilled water. The agar plugs were made within polydimethylsiloxane (PDMS) molds. The base part and curing agent of Sylgard 184 (Dow Corning, Midland, MI, USA) were mixed as 10:1 weight ratio, and the 10 mL round bottom tubes were posted to the PDMS precuring mixture. The tubes were removed after PDMS curing, and the PDMS mold was autoclaved before use. Wounded corneas on the agar plugs were placed in a 12 well plate with 1 mL of complete serum-free culture medium, which was enough to bring the medium to the level of the scleral rim. The culture medium used was DMEM/F-12 containing 120 µg/mL penicillin G, 200 µg/mL streptomycin sulfate and ITS premix. Samples were incubated at 37 °C in 5% CO₂ in the air with once daily medium changes.

For the MSC group, MSCs (1.0×10^5 cells) were seeded on the wounded corneal tissue directly. For the MSCs-cultured electrospun fiber (MSC-fiber) group, the shape of the electrospun fiber membrane was refined as a donut shape with OD = 16 mm and ID = 8 mm. MSCs were seeded onto the electrospun fiber region carefully, and the MSCs-cultured electrospun fiber was transferred to the wounded corneal tissue 3 h after cell seeding. Corneal fluorescein staining was used to assess the epithelial integrity and barrier function. Fluorescein dye strips (Haag-Streit, Koeniz, Switzerland) were infused in saline (2 strips per 1 mL saline), and then the liquid mixture was applied to the corneas to determine the location and degree of the ocular surface staining under cobalt blue illumination. Photographs of fluorescein-stained corneas were taken with a smartphone ophthalmic imaging adapter (Paxos Scope, by Digisight Technologies, now Verana Health, San Francisco, CA, USA) equipped with a 15 × magnifying lens and a blue LED in the same manner as done in previous research [22].

Upon conclusion of the experiment, corneas were cut in half and fixed in 4% PFA for 24 h at 4 °C, then processed through graded sucrose solutions. Corneas were then embedded in optimal critical

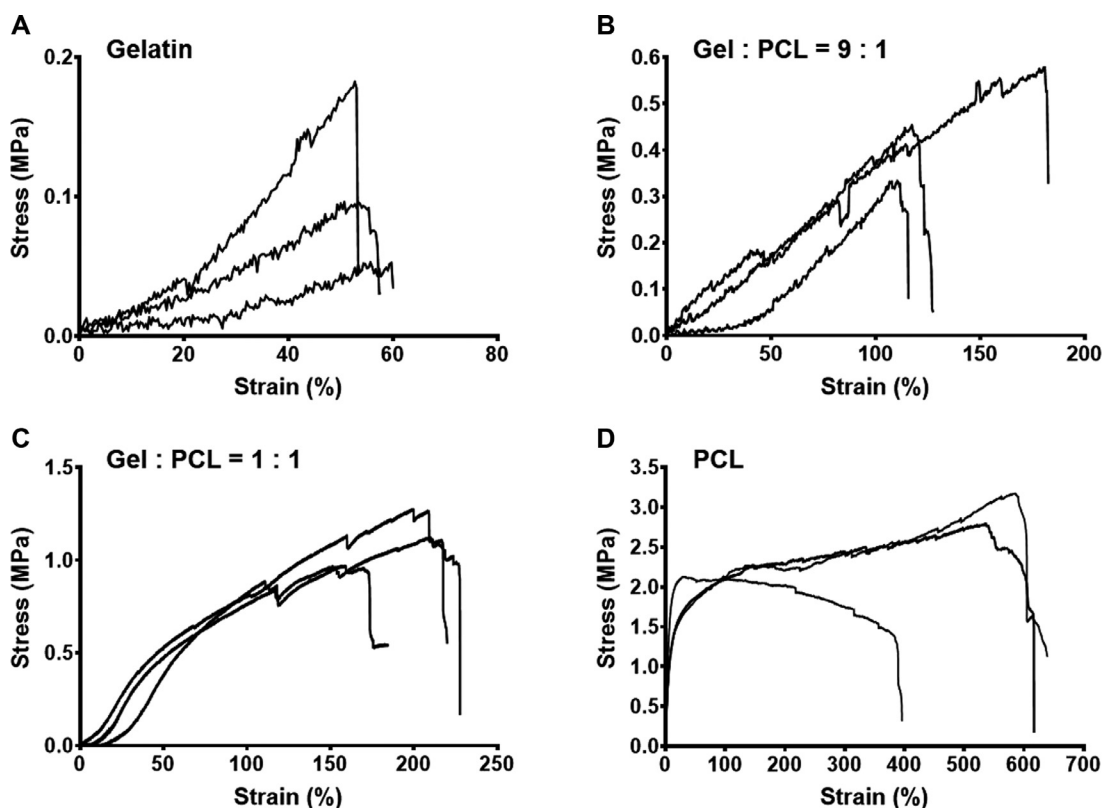


Fig. 1. Stress-strain curves of electrospun fibers consist of (A) gelatin, (B) gelatin: PCL = 9:1, (C) gelatin: PCL = 1:1, (D) PCL.

temperature (OCT) compound and 18-micron sections were taken with cryostat (Leica CM3050 S Research Cryostat). Sections were then stained with mouse anti- α smooth muscle actin primary antibody. The secondary antibodies were Alexa Fluor 546-coupled goat anti-rabbit IgG and Alexa Fluor 488-coupled goat anti-rabbit IgG. Cell nuclei were stained with DAPI. Negative controls were performed without primary antibodies. Images were taken at 10X and 20X with a confocal microscope. Alpha-smooth muscle actin positive areas were analyzed using ImageJ. The percentage area of alpha-smooth muscle actin was quantified for the stroma of each image in triplicate, and then normalized to the alpha-smooth muscle actin percentage area of the injury treatment arm.

2.7. Statistical analysis

All data are expressed as the mean $\pm$ standard deviation (SD). Each experiment was repeated at least 3 times unless otherwise indicated. Statistical evaluation was performed using a one-way ANOVA. A value of $p < 0.05$ was considered statistically significant. The statistical analysis was performed by using statistical software of GraphPad Prism 7.

3. Results

3.1. Fabrication of PCL/gelatin electrospun fiber

The mechanical properties of electrospun fibers were adjusted to provide appropriate cell environments that have similar Young's modulus of the native corneal tissue along with the easy handling of resultant scaffolds. The electrospun fibers, which are used for cell scaffolds, provide three-dimensional structure to the cells. These fibers which consist of gelatin and PCL were fabricated with different ratios of gelatin and PCL, and the mechanical properties

of each fiber were evaluated via a mechanical testing machine testing the varying compositions of fiber materials (Fig. 1 and Table 1). The gelatin-containing fibers were crosslinked via glutaraldehyde vapor to prevent the dissolution of gelatin into the aqueous solution. The stress-strain curve of gelatin electrospun fibers showed a slightly increasing slope with strain and had tensile strength values of 0.11 ± 0.05 MPa with Young's moduli of 0.3 ± 0.1 MPa (Fig. 1A). The tensile stress, elongation, and Young's modulus were increased with the addition of PCL. The stress-strain curve of PCL fiber was initially steep, increasing within 10% of strain, representing an elastic region, followed by a plastic region that exhibited relatively modest upward slopes until the failure point. Although the PCL fibers had tensile strength on the order of 2.7 ± 0.4 MPa with Young's moduli of 8 ± 2 MPa (Fig. 1D), the polymer material lacked biological support properties such as cell adhesion and proliferation. The combination of gelatin and PCL endows biological activity and while producing sufficient mechanical properties for surgical handling. The electrospun fibers, which consist of gelatin and PCL in a one to one ratio, were applied to *in vitro* and *ex vivo* experiments. The Young's modulus of the electrospun fibers of gelatin and PCL as 1:1 had 1.2 ± 0.1 MPa, which was close to the Young's modulus of human cornea (1.3 – 5.9 MPa) [23].

3.2. MSCs cultured on electrospun fibers

The compositions of MSC secretomes under various conditions were analyzed and compared. MSCs were cultured on well plates (2D) and electrospun fibers (3D) of gelatin and PCL as 1:1. The cell media was collected on day 2 after cell seeding on the substrates with media refreshing at day 1. The collected conditioned media were centrifuged, and the supernatants were analyzed by Luminex multiplex assay (Fig. 2).

Table 1
Mechanical properties of electrospun fibers.

	Tensile Stress (MPa)	Elongation (%)	Young's Modulus (MPa)
Gelatin	0.11 ± 0.05	56 ± 3	0.3 ± 0.1
Gelatin: PCL = 9: 1	0.5 ± 0.1	136 ± 32	0.40 ± 0.04
Gelatin: PCL = 1: 1	1.23 ± 0.07	211 ± 19	1.23 ± 0.07
PCL	2.7 ± 0.4	551 ± 110	8 ± 2
Human cornea [20]	–	–	1.3 – 5.9

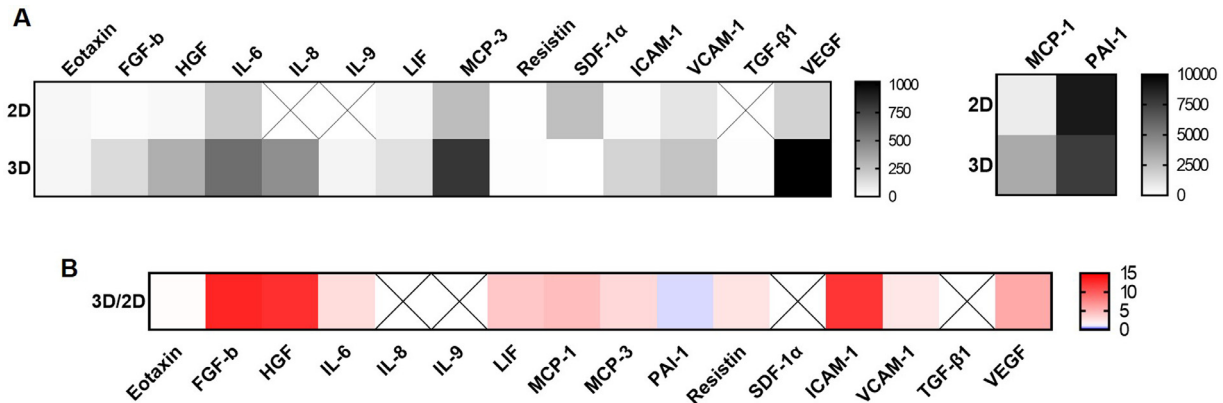


Fig. 2. (A) Luminex protein concentration analysis from MSCs cultured on electrospun fibers (3D) and well plates (2D). The scaled values represent the concentration of proteins (pg/mL). (B) Relative amounts of proteins in secretomes from MSCs cultured on 3D compared to 2D. The red and blue color represents the relative increase and decrease of secreted protein amounts, respectively. FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IL, interleukin; LIF, leukemia inhibitory factor; MCP, monocyte chemoattract protein; PAI, plasminogen activator inhibitor; ICAM, intracellular adhesion molecule; VCAM, vascular cell adhesion molecule; VEGF, vascular endothelial growth factor; TGF, transforming growth factor; SDF, stromal cell-derived factor (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

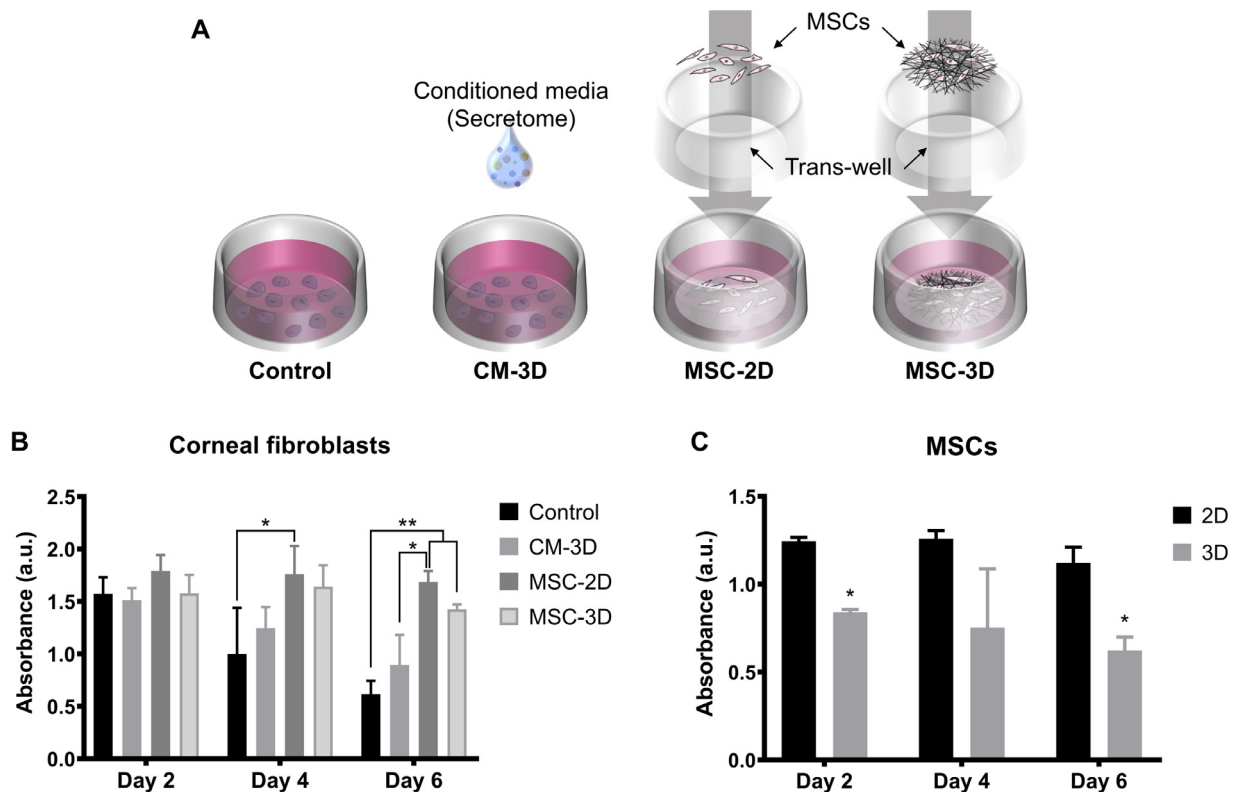


Fig. 3. (A) Schematics of the experimental groups. Corneal fibroblasts were cultured on well plates. The conditioned media (CM) obtained from MSCs cultured on electrospun fibers was added to the CM-3D group. Transwell inserts were utilized for the two co-culture groups, and the MSCs were cultured on the trans-well and electrospun fiber for MSC-2D and MSC-3D group, respectively. (B) Cell viability of corneal fibroblasts cultured at the bottom of the well. (C) Cell viability of MSCs that were co-cultured on 2D and 3D environments. The significant differences are represented by $0.01 < p < 0.05$ (*) and $p < 0.01$ (**).

A total of 16 proteins were analyzed in the secretomes from MSCs cultured on the well plate (2D) and electrospun fibers (3D) (Fig. 2A). MCP-1 and PAI-1 showed high expression with concentrations over 1 ng/mL, and the other 14 proteins were each expressed at concentrations under 1 ng/mL. IL-8, IL-9, and TGF- β 1 were not detected in the secretome from MSCs cultured in 2D, but they were detected in the secretome from MSCs cultured on 3D scaffolds. SDF-1 α was detected in the secretome from MSCs cultured in 2D, but it was not detected in the secretome from MSCs cultured on 3D scaffolds. The total amount of each protein in the secretome from 3D culture were divided by those in 2D culture for each protein, in order to evaluate the normalized change in secretome composition (Fig. 2B). The secretion of PAI-1 was reduced in MSCs cultured on electrospun fibers, while the secreted levels of Eotaxin, FGF-b, HGF, IL-6, LIF, MCP-1, MCP-3, resistin, ICAM-1, VCAM-1, and VEGF were promoted. Notably, the relative amounts of FGF-b, HGF, and ICAM-1 in secretome from 3D culture were increased over 5 times compared to 2D culture.

3.3. Corneal fibroblast co-culture with MSCs

The experimental groups were designed to evaluate the therapeutic effects of the MSC secretome under various MSC culture environments (Fig. 3A). The four conditions included corneal fibroblasts cultured in (1) serum-free media for 6 days with fresh media exchange every two days (control group), (2) conditioned media extracted from MSCs added to serum-free media (CM-3D) every two days, (3) co-culture of corneal fibroblasts with MSCs grown in 2D in an overlying transwell insert (MSC-2D), and (4) co-culture of corneal fibroblasts with MSCs grown on a 3D electrospun fiber membrane within an overlying transwell insert (MSC-3D). The MSCs were previously found to be unable to pass through the transwell insert, but the secreted factors from MSCs did penetrate and reach the corneal fibroblasts at the bottom of the well. Thus, the transwell system was found to be appropriate to evaluate the paracrine effect of MSCs.

Co-culture of MSCs showed a positive effect on the maintenance of viability of corneal fibroblasts (Fig. 3B). The viability of corneal fibroblasts under serum-free conditions were decreased with time. Conditioned media slightly reduced cell viability, but this reduction was not statistically significant. In the MSC co-cultured systems (MSC-2D and MSC-3D), the corneal fibroblasts maintained viability for 6 days, and the results showed statistically significant differences compared to the control group at day 6.

The viability of MSCs were measured in the transwell environment as well as separately, as were the corneal fibroblasts, to verify the effects of culture condition on the cellular activities, and there were no decreases with time for each group (Fig. 3C). There was an initial difference between 2D and 3D conditions, derived from the difference in initial cell number. Although the number of MSCs in the 3D condition was lower than 2D, there was no significant difference in the paracrine effects on corneal fibroblasts. The viability results illustrate that the paracrine effects of continuously delivered MSCs secretome improved the viability of not only corneal fibroblasts but also the MSCs themselves as compared to the serum-free environment.

A scratch-based wound healing assay was performed to evaluate the secretome's effects on corneal fibroblasts migration as well (Fig. 4). After generation of the scratch, serum-free media and MSCs conditioned media were added to the well plates for the control and CM-3D group, respectively. Transwell inserts were utilized for the MSC-2D and MSC-3D groups, and the MSCs were cultured on transwell and electrospun fibers, as mentioned above. The lateral movement of the scratch border was assessed by the wound healing measurement tool provided by ImageJ software, and internal "open" areas devoid of cells were measured simultaneously. At

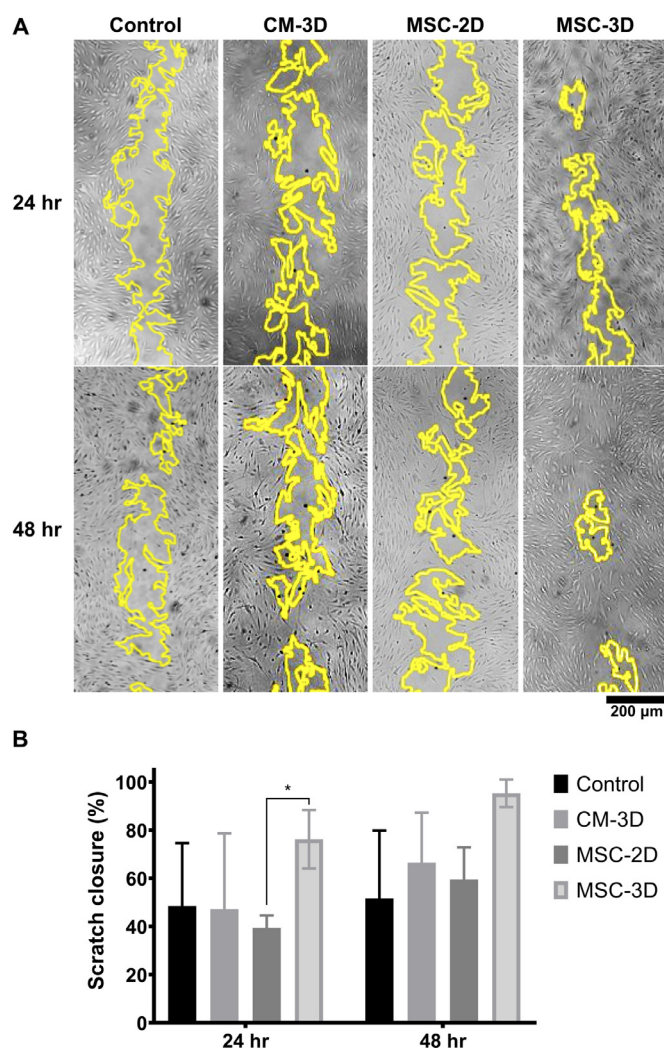


Fig. 4. Wound healing scratch assay of corneal fibroblasts as a function of MSC secretome treatment conditions (A) Representative images taken at 24 and 48 h after applying MSCs conditioned media (CM-3D) and MSCs co-culture (MSC-2D and MSC-3D). The yellow lines, generated by the wound healing measurement tool of ImageJ, represent the wound borders. (B) Quantification of scratch closure using the wound healing measurement tool of ImageJ. The scratch closure was evaluated by dividing the open area by initial scratch area. The significant differences are represented by $0.01 < p < 0.05$ (*).

24 h, the scratch closures of control, CM-3D, MSC-2D, and MSC-3D group were found to be 48 ± 21 , 47 ± 26 , 40 ± 4 , and $76 \pm 10\%$. The co-culture of MSCs on the electrospun fiber promoted faster wound closure of corneal fibroblasts, although there were significant differences compared to MSC-2D. At 48 h, the scratch closures of control, CM-3D, MSC-2D, and MSC-3D group showed 52 ± 23 , 67 ± 17 , 60 ± 11 , and $95 \pm 5\%$. The MSC-3D group showed faster closure rate than the other groups.

3.4. Rabbit cornea organ culture

Rabbit organ culture was performed to further study the effects of MSCs on corneal epithelial wound healing and stromal scarring when applied topically only versus on an applied electrospun fiber membrane. In an organ culture setup, an air-liquid interface is created on the corneal surface, with nutrients provided to the ocular surface through either diffusion from an underlying agar plug on the posterior side of the cornea, or through capillary wicking from a moat of culture media that the corneal-scleral rim is partially submersed within along its periphery (Figs. 5 and 6). When MSCs

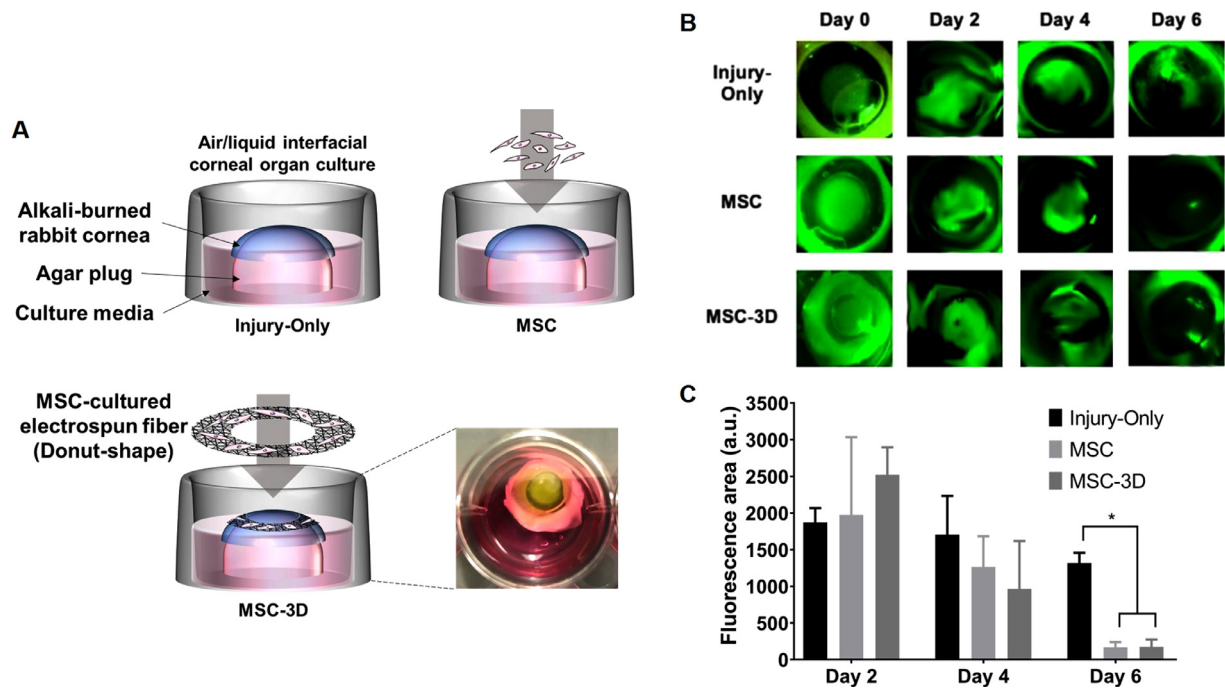


Fig. 5. (A) Schematics of air/liquid interface created by corneal organ culture. The corneas of all groups were cultured in serum-free media. MSCs and MSCs-cultured on electrospun fibers were applied onto the damaged cornea, respectively. The inset photo shows the corneal organ culture after electrospun fiber application with fluorescein staining. (B) Blue LED light photos of representative rabbit corneas in the 3 treatment arms following alkali burn, at days 0, 2, 4, and 6. The corneas were stained with fluorescein and images taken under blue LED light to highlight wound size. (C) Quantification of fluorescence area which represents uncovered area by epithelial cells. Fluorescence area was captured by ImageJ after staining of the corneas with sodium fluorescein.

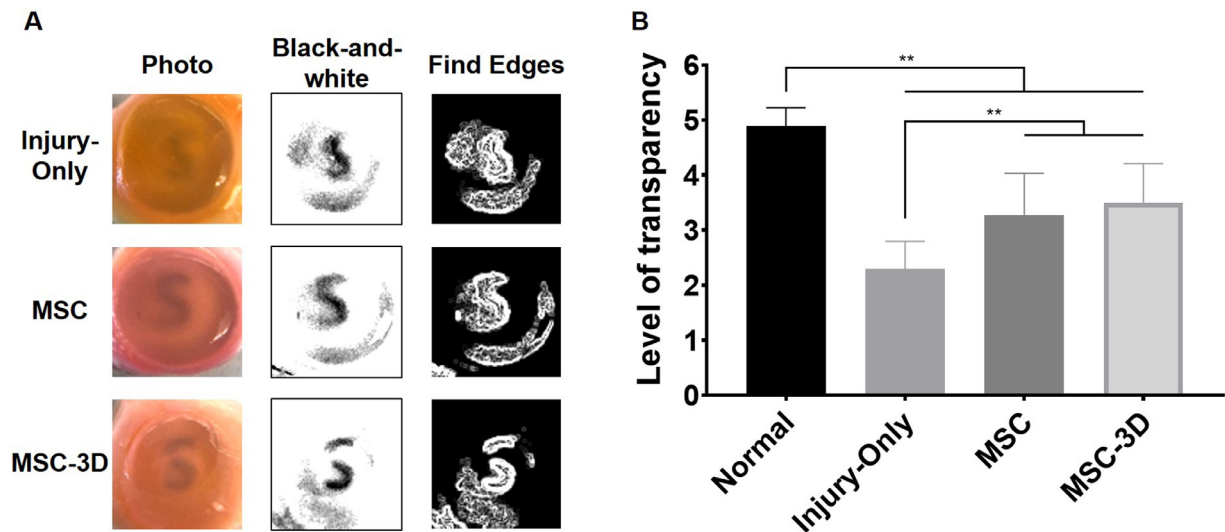


Fig. 6. (A) Representative visual comparison and digital image analysis of letter photos that represent printed letters laid below the corneas to evaluating scarring. The images were adjusted by ImageJ through the same process including black-and-white with contrast and brightness regulation. (B) Manual haze grading of organ cultured corneas as observed by three independent observers blinded to the application. The scale was from 0 to 5, with 5 representing un-injured/untreated corneas and 0 representing complete haze that occludes visualization of the underlying letter.

are applied topically, the cells likely settle into the culture well surfaces in contact with the culture media. Thus, any secreted factors from the MSCs would actively condition the media and nourish the cornea through a capillary wicking effect. For the electrospun fiber approach, we created an annular ring containing the cultured MSCs and this ring was laid directly on the corneal surface at the corneal limbus. Thus, secreted factors in this arrangement were produced in the immediate vicinity of the corneal epithelium. There were 3 rabbit corneas per treatment group. Over the course of 7 days, daily photographs were taken of each alkali-burn-wounded cornea under white light and blue LED light with fluorescein dye applied

to the cornea to observe wound size. The corneas treated with the MSCs and the MSC-seeded fibers had similar rates of wound closure, and by day 6, all corneal wounds in these 2 groups had healed (Fig. 5). In the injury-only group, one cornea healed at day 6, while wounds remained in the other 2 injured corneas at the end of the experiment.

Corneal opacification was measured qualitatively at the conclusion of the experiment by placement of printed letters on a sheet of paper placed below the corneal tissue culture dish. At 7 days after injury, corneal haze of some degree was observed in all eyes (Fig. 6A). Representative images of the MSC and MSC-3D group

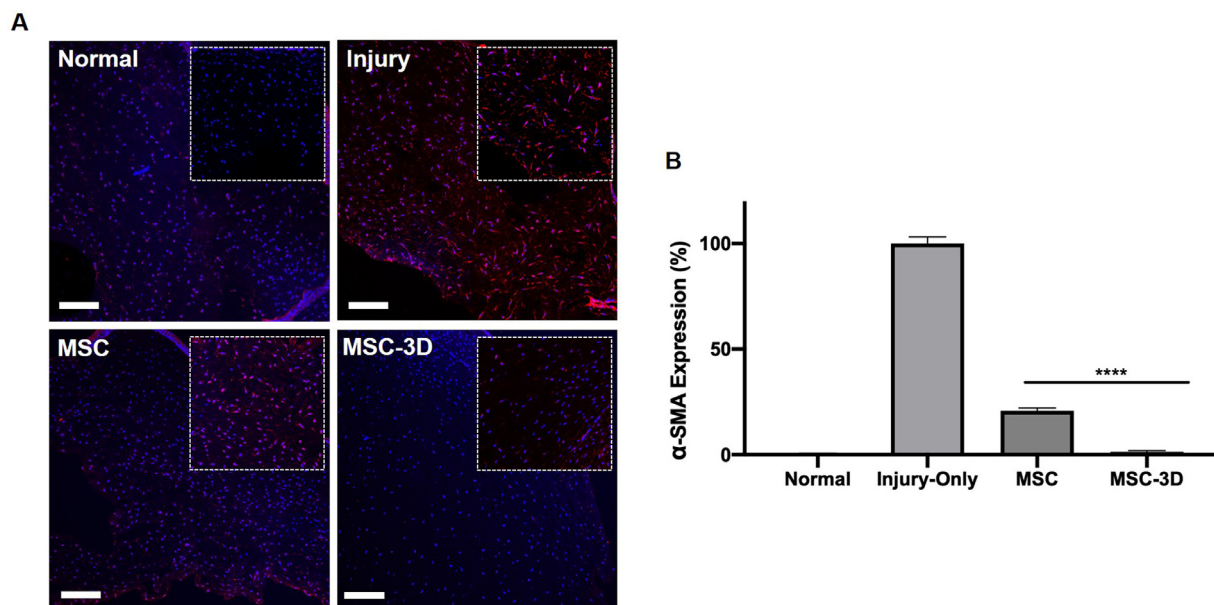


Fig. 7. (A) Fluorescence confocal microscopy images of rabbit corneas in the normal, injury-only, MSC, and MSC-3D treatment groups at completion of organ culture experiment. Tissue was stained for α -SMA (red) and DAPI (blue). 10X with 20X inset, bar = 100 μ m. (B) Quantification of α -SMA staining for each treatment group normalized to the injury group, using Image J analysis (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

showed that stromal haze formation was relatively reduced compared to the injury-only group. Corneal haze was graded manually by authors KC, KN, and DM, and were as follows for normal, injury-only, MSC, and MSC-3D groups: 4.9 ± 0.3 , 2.3 ± 0.5 , 3.3 ± 0.7 , and 3.5 ± 0.7 , respectively. Scarring was apparent in all 3 groups, but it was most significant in the injury-only (no treatment) group and least significant in the MSC followed by the MSC-3D group, although difference between the MSC groups was not statistically significant. The MSC and MSC-3D groups showed similar rates of corneal epithelial wound healing.

Immunofluorescence confocal imaging showed strong expression of α -SMA (red) in the injury-only cryosections, but not in the normal, MSC, or MSC-3D treated corneas' cryosections (Fig. 7). Quantification of α -SMA staining was performed by Image J, which showed stained percentage area to be 0.49%, 20.8%, and 1.3% for the normal, MSC-2D, and MSC-3D groups, respectively, as compared to the injury group (100%). The decreased α -SMA expression of the MSC and MSC-3D groups as compared to the injury group was statistically significant with $p < 0.0001$.

4. Discussion

In this study, we investigated how culture conditions could influence the secretome of MSCs and, in turn, how this affects corneal wound healing. A previous research study showed that the topography of a cell scaffold is a key factor that modulates the paracrine function of cells, and that electrospun fiber scaffolds provide an environment in which cells produce more effective wound healing factors than two-dimensional cell culture plates do [7]. Another research study paid attention to time-dependent healing effects, showing that transplantation of MSCs achieves a better result than once and three-times application of MSC conditioned media [14]. Here, we studied the sustained secretion of factors from MSCs grown on three-dimensional electrospun fiber-based scaffolds versus conventional 2D tissue culture conditions, which we surmised would provide differential effects on corneal wound healing. We chose to compare these conditions because delivery of MSCs to the ocular surface could be accomplished with either a 2D or 3D delivery system, where MSCs are provided either as

a monolayer or encapsulated and grown within a matrix, respectively. In particular, electrospun fibers are generally easily handled with surgical instruments and their mechanical properties enable them to be glued or even sutured in place. Providing a scaffold that could be held in place on the ocular surface could have advantages over topical application of MSCs, where cells would generally be rapidly washed out by tear film turnover and/or loss through the lacrimal system or overflow over the eyelid margins, since a typical eyedrop has far more volume (~40 microliters) than the ocular surface (~7 microliters).

We used gelatin/polycaprolactone (PCL) composites for the electrospun fiber scaffolds to provide 3D microenvironments for the MSCs in this study. We chose electrospun fiber meshes because they provide both a biochemically and mechanically biomimetic environment for the MSCs, and also allow for free diffusion of secreted factors out of the scaffolds. Gelatin, which is a hydrolyzed form of collagen, was used to mimic the collagenous matrix of corneal stroma while PCL, a well-characterized, degradable polymer, was used to provide additional structural support. In a previous study, author HJL fabricated gelatin/PCL electrospun fibers and followed the procedure with crosslinking of the gelatin component [24]. The ratio of gelatin and PCL in this study was adjusted to obtain the Young's modulus of human cornea, and the 1:1 ratio was close to the range of published values (Table 1). We found that the higher the PCL content, the greater the mechanical strength, but the resultant reduction in gelatin led to a decrease in bioactivity when seeded with MSCs. The ratio of gelatin and PCL was fixed at 1:1 as a result, which we found provided the right balance between mechanical strength and bioactivity.

A commercial cell line of bone-marrow derived MSCs were cultured on fabricated electrospun fiber and well plates, and the secreted factors from the cells showed differences in composition depending on various cell culture environments tested. About 60 different growth factors, cytokines, and chemokines were analyzed by the Luminex protein assay, and 16 factors were within the detectable range (Fig. 2). Overall, the concentrations of detectable proteins were increased for MSCs cultured on 3D environments except for two secreted factors (PAI-1, and SDF-1 α). PAI-1 is known as a promoter of corneal wound healing through the regulation

of corneal epithelial cell migration and adhesion [25]. SDF-1 α is known to enhance stem cell proliferation, chemotaxis and migration, so the factor may play a role in accelerated corneal epithelialization [26]. In secretome derived from MSCs grown on electrospun substrates, the amounts of three particular proteins (FGF-b, HGF, and ICAM-1) were noticeably elevated. FGF-b enhances cell proliferation *in vitro* and corneal wound healing *in vivo* [27–29], while HGF promotes lamellar differentiation of corneal epithelial cells *in vitro* and restoration of corneal transparency *in vivo* [30–32]. ICAM-1 has a role in efficient corneal wound healing through epithelial recruitment [33]. The increase of these factors in the secretome from the MSC-3D condition could explain the enhanced wound healing effects seen in the *in vitro* migration assay results as well as the organ culture experiments. We believe that the effects of various secretomes depend on the interplay between the factors and not just the factors' direct effects themselves. In this study, we focused on fabricating a construct that allows for sustained secretome generation from MSCs cultured on electrospun fibers and tissue culture plates rather than on isolating and systematically studying combinations of specific recombinant versions of factors themselves.

The effects of the secretome on corneal fibroblast viability were remarkable in the co-culture groups (MSC-2D and MSC-3D)—where secreted factors are continually supplied directly from the MSCs to the fibroblasts—compared to providing exogenous secretome in the form of aliquots of conditioned media (CM-3D) in the absence of MSCs (Fig. 3B). The corneal fibroblasts that were cultured in serum-free media (control) showed decreased cell viability with time. The CM-3D group maintained slightly higher viability compared to the control group, but there were no significant differences observed over 6 days. On the other hand, the fibroblasts treated in the two MSCs co-culture groups maintained viability, and there were statistically significant differences compared to the control group at day 6. The result of the corneal fibroblast viability assays suggest that co-culture environments may be more effective than static aliquots of harvested, cell-free secretome at enhancing wound healing. Since the secretome in a co-culture environment is dynamic and likely changing over time, it is difficult to compare the quantitative and compositional differences of the secretomes obtained by co-culture and single culture of MSCs. The Luminex assay we used only shows “snapshots” in time of the specific secretome profile at the moment it was sampled. We have several hypotheses for this result. The first is that a sustained supply of secretome is more effective than supplying a static inventory of factors as single aliquots because factors that are consumed are constantly replenished in the co-culture case. A second hypothesis is that the amounts and compositions are actually fluctuating over time due to cell-cell crosstalk between the fibroblasts and the MSCs. Although there was no direct cell-cell contact between MSCs and corneal fibroblasts, the cells are likely communicating via mutual paracrine effects (as cells do *in vivo*) since the transwell insert membranes are permeable to proteins. This intercellular crosstalk is outside the scope of this study and merits further investigation. A third hypothesis is that other factors not captured by the Luminex assay may be at play and are exerting their influence in the co-culture scenarios.

The cell viability of the co-cultured MSCs themselves were also evaluated (Fig. 3C), and the MSCs grown on the electrospun fibers showed significantly lower absorbance than the MSCs grown directly on the transwell inserts. The same number of cells were seeded on the electrospun fibers, but some cells were adhered to the bottom of well plate where the fibers were placed, and then the electrospun fibers were moved onto the transwell. From the cell viability results of MSCs on 2D and 3D (Fig. 3C), the fraction of MSCs adherent to the electrospun fiber when cells were seeded could be estimated. The ratio between 2D and 3D was maintained

for 6 days without a considerable change, and the ratio of cell numbers of 3D/2D was about 0.6 – 0.7. This represents 60 – 70% of MSCs adherent on the electrospun fiber, with the rest of the cells adherent to the well plate underneath the electrospun fiber. The total number of MSCs on the electrospun fiber scaffolds was lower than in the 2D environment, but there was no significant difference in the effects on the viability of corneal fibroblasts.

A scratch assay was performed using the same groups and conditions as the cell viability experiments (Fig. 4). The MSC-3D group was more effective in accelerating corneal fibroblast wound closure than the MSC-2D group, although both groups showed similar corneal fibroblast viability. The control group showed about 50% closure at 24 h, and the level was maintained until 48 h. The other three secretome treatment groups, CM-3D, MSC-2D and MSC-3D, showed continuous closure until 48 h. The groups showed about 20% increased degree of closure between 24 and 48 h, while there was less than 5% increase in the control group. The secreted factors from the MSC-3D group produced the most rapid corneal fibroblast migration, and this group showed 76 ± 10 and $95 \pm 5\%$ closure at 24 and 48 h, respectively. The improved wound closure effects of the MSC-3D group as compared to the CM-3D group are again likely due to the dynamic environment whereby MSCs continuously supply vital secretome factors as needed in response to cues from the neighboring fibroblasts. This result showed the secretome derived from MSCs in 3D culture is more effective than that derived from 2D culture at stimulating corneal fibroblast migration.

The rabbit cornea organ culture experiments were conducted because it enabled us to further investigate wound closure, scarring, and cellular expression as a result of MSC co-culture in an environment that mimics the ocular surface. Corneal organ culture creates an air-liquid interface and nutrient sources that emulate the tear film (cell culture media encircling a cornea-scleral rim) as well as the aqueous humor (agar plug posterior to the cornea) (Fig. 5A). When MSCs are applied topically they tend to slide and grow in the surrounding cell culture well, and their nutrients can intermingle with the media that reaches the cornea via capillary action and tissue absorption. Laying an electrospun fiber on the corneal surface provides information about whether physical proximity of the MSCs to the wound site makes any difference in the action of the factors secreted. Three groups were observed which all sustained a chemical corneal injury: one group was untreated, one group received MSCs directly as an “eyedrop”, and one group received the MSCs cultured on an annular electrospun fiber mesh (Fig. 5). The fiber mesh was fabricated in the donut shape as shown in order to not interfere with the epithelial defect directly, but to provide a nearby, physically stationary, but real-time source of MSC secreted factors. Photographs taken every two days of fluorescein staining patterns showed that the MSC and MSC-3D groups exhibited faster wound closure rates than the injury-only groups (Fig. 5B and C). All MSC and MSC-3D corneas had almost healed by day 6. There was no significant difference between MSC and MSC-3D on epithelial layer closure in corneal organ culture. The organ culture environment, containing multiple cell types and cell-matrix interactions, is more complicated compared to the *in vitro* cell culture environment, so further studies to confine the effect of migration difference *in vitro* cell migration is required, such as fractionating the secretome into growth factor combinations.

Scarring was also evaluated via the rabbit cornea organ culture experiments, and while haze was evident in all treated groups, the transparency index determined by pixel analysis demonstrates a difference between the groups. Similar to wound closure results of alkali-burned rabbit corneas, the MSC and MSC-3D groups exhibited less corneal opacification than the injury-only group, with decreased scarring when the MSCs were applied to the wounded corneas as evident by the clarity of printed letters below the corneas (Fig. 6). The representative photographic images of the

injury-only group showed the most blurring of the 'S' letters under the corneas, compared to the uninjured and untreated "normal" corneas at Day 7 in culture. The MSC and MSC-3D groups provided better clarity and visualization of the 'S' letter shape through the injured and treated corneal tissue, and while the MSC-3D group corneas were generally clearer, the differences were not statistically significant. The *ex vivo* corneal organ culture results showed that continuous delivery of secreted factors from co-cultured MSCs were superior to no treatment controls, but that 2D versus 3D MSC culture conditions were not substantially different in their effects on the rate of epithelial healing and degree of haze formation.

Histologically, there were some additional differences noted. Immunofluorescence imaging of the cryosections showed that the MSC-3D group exhibited an apparent difference in the expression of α -SMA compared to the injury-only and MSC-2D conditions (Fig. 7), similar to that of the normal condition. α -SMA is the most common marker used to detect myofibroblast morphology and indicative of increased myofibroblastic transformation [34,35]. As expected of cells within a corneal wound, the injury group showed increased α -SMA expression. The MSC-2D group did as well, but to a much lesser degree (Fig. 7B). The MSC-3D group had the least α -SMA expression, which suggests possible effects on the formation of subepithelial and stromal scarring long-term, although this was not as apparent clinically in the organ culture results. This data is consistent with the corneal haze findings, showing highest levels of α -SMA expression and corneal haze in the injury group, and to a lesser degree in both treatment groups, with MSC-3D conditions superior to MSC-2D. It is not clear why the MSC-2D group may have led to relatively higher α -SMA expression in these cases; one thought is that the physical location of the seeded MSCs at the bottom of the culture well was too far from the corneal wound site itself to exert as optimal of an effect. The MSCs grown on the annular electrospun fiber mesh were well-positioned to provide very localized and constant paracrine effects. The immunohistochemical results point toward this delivery vehicle being a potentially more favorable microenvironment for the MSCs and a therapeutically more effective secretome. A physically adjacent and biochemically more optimal secretome could potentially lead to a local decrease in TGF- β 1, which can lead to apoptosis of myofibroblasts [36], and a resultant decrease in α -SMA and reduction in myofibroblastic activity in general.

There are a number of limitations inherent to this study. First, *in vitro* scratch assays and organ culture assays only provide information on stromal and/or epithelial aspects of the corneal wound healing response to injury, without any indication of the vascular or inflammatory (immunogenic) effects. In particular, the angiogenic and inflammatory response to alkaline burns of the ocular surface is profound. The authors appreciate this limitation, especially in the context of our recently published results on the *in vivo* response to alkaline burns and the therapeutic effects of topically applied, cell-free MSC secreted factors [37]. Nevertheless, we feel that the *ex vivo* assays used in this study provide useful information on the epithelial and stromal response to injury and treatment, and can guide future work *in vivo*. A second limitation is the fact that it is difficult to simulate an *in vivo* "co-culture" situation where living MSCs secrete their factors on/within the ocular surface milieu. The circular sheet and annular, electrospun scaffolds used in our assays provide a generalized concept of a vehicle with sufficient mechanical and handling properties that can be placed and/or secured onto the ocular surface or perhaps implanted subconjunctivally. The same applies to the 2D condition; we did not feel the need to culture the MSCs on a separate 2D substrate and instead relied on tissue culture polystyrene plates. There may be differences in the MSC biological response as a function of fiber and culture plate materials and material properties that were outside the scope of this study and thus not accounted for.

5. Conclusions

We presented the effects of secretome produced under different MSC culture conditions on corneal wound healing. Secretome composition was analyzed using a multiplex assay, and we found the composition to be significantly different between those obtained from 2D and 3D microenvironments. The effects of these different compositions were evaluated by *in vitro* cell and organ culture experiments. The viability and migration of corneal fibroblasts were measured using an MSC co-culture system compared against harvested, cell-free secretome treatments, and the secretome that was produced from the MSCs cultured on electrospun fibers yielded increased cell viability and rates of migration. Although there was no statistically significant differences between the secretomes derived from 2D and 3D environments on epithelial wound closure and stromal haze formation in rabbit corneal organ culture in terms of clinical evaluation, there were some notable immunohistological differences, including decreased α -SMA expression with continuous secretome delivery in the MSC-3D condition. Further work is merited to evaluate the delivery of MSCs on and within 2D and 3D scaffolds to the ocular surface *in vivo*.

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Conflict of interest

The following authors have no financial disclosures: Kaylene Carter, Kyung-Sun Na, and Ignacio Jesus Blanco. Hyun Jong Lee, Gabriella Fernandes-Cunha, Ali Djalilian, and David Myung are co-inventors on a patent application.

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