

# Reproducible Derivation and Expansion of Corneal Mesenchymal Stromal Cells for Therapeutic Applications

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**Purpose:** A reproducible protocol for the production of corneal mesenchymal stem/stromal cells (cMSCs) is necessary for potential clinical applications. We aimed to describe successful generation and expansion of cMSCs using an explant method.

**Methods:** Corneoscleral rims of human cadaveric eyes were divided into four pieces and used as explants to allow outgrowth of cMSCs (passage 0, or P0). The cells were subcultured at a 1:10 ratio until passage 5 (P5). The characteristics as well as therapeutic effects of expanded cMSCs were evaluated both in vitro, using a scratch assay, and in vivo using epithelial debridement and chemical injury mouse models.

**Results:** All explants demonstrated outgrowth of cells by 7 days. Although the initial outgrowth included mixed mesenchymal and epithelial cells, by P1 only cMSCs remained. By subculturing each flask at a ratio of 1:10, the potential yield from each cornea was approximately 12 to 16 × 10<sup>10</sup> P5 cells. P5 cMSCs demonstrated the cell surface markers of MSCs. The secretome of P5 cMSCs induced faster closure of wounds in an in vitro scratch assay. Subconjunctival injection of P5 cMSCs in mouse models of mechanical corneal epithelial debridement or ethanol injury led to significantly faster wound healing and decreased inflammation, relative to control.

**Conclusions:** cMSCs can be reproducibly derived from human cadaveric corneas using an explant method and expanded with preservation of characteristics and corneal wound healing effects.

**Translational Relevance:** The results of our study showed that cMSCs produced using this scheme can be potentially used for clinical applications.

## Introduction

Mesenchymal stem/stromal cells (MSCs) are a population of cells defined by their ability to adhere to plastic, their expression of specific cell surface markers, and their ability to differentiate into multiple mesenchymal lineages (e.g., adipocytes, osteoblasts, chondroblasts) in vitro.<sup>1-3</sup> MSCs have potent immunomodulatory properties and are able to modulate both innate and adaptive immune

responses.<sup>4-7</sup> Due to their pluripotency, relative hypoimmunogenicity, and ability to modulate the immune response to tissue injury, MSCs have garnered significant interest in regenerative medicine.<sup>8-10</sup>

The antiinflammatory and healing effects of MSCs have recently been demonstrated in experimental models of ocular surface disease.<sup>11</sup> The beneficial effects of bone marrow-derived MSCs in corneal epithelial wounds and alkali burns have been shown in vitro and in vivo.<sup>12-16</sup> In this setting, MSCs are thought to improve corneal wound healing through

multiple mechanisms, including the suppression of inflammatory cytokines, as well as the upregulation of anti-inflammatory cytokines in the healing cornea.<sup>13,17</sup>

One important aspect of optimal wound healing in the cornea is the inhibition of neovascularization and fibrosis, as these processes affect corneal clarity. Recent studies have demonstrated the antifibrotic nature of corneal-derived MSCs (cMSCs).<sup>18</sup> Moreover, despite the pro-angiogenic nature of MSCs derived from other tissues, cMSCs may in fact be anti-angiogenic.<sup>19</sup> These findings, in addition to the relative heterogeneity of other MSCs,<sup>20,21</sup> make cMSCs particularly attractive for corneal wound-healing applications.

In most published reports, the isolation of cMSCs has been primarily through enzymatic digestion. In this method, tissue-digesting enzymes such as collagenases are used to digest the extracellular matrix of the limbal tissue and isolate a relatively small number of cMSCs, which can then be cultured and expanded *in vitro*.<sup>22,23</sup>

Another method for cMSC isolation is the explant method, which involves the use of intact explant tissue in culture media which leads to the release of cellular aggregates. Given the relatively low population of local cMSCs and the generally low yield of current cMSC isolation methods,<sup>24,25</sup> cell viability and reproducibility are of particular importance. Explant methods have been used in the past with variable yields of cMSCs.<sup>26</sup>

In this study, we investigated a reproducible protocol to isolate cMSCs from human cadaveric corneas using an explant method with a particular focus on optimizing an expansion method for clinical use. We further evaluated their wound healing and anti-inflammatory effects in both *in vitro* and *in vivo* corneal wound/injury models.

## Methods

### Harvesting Human Corneas

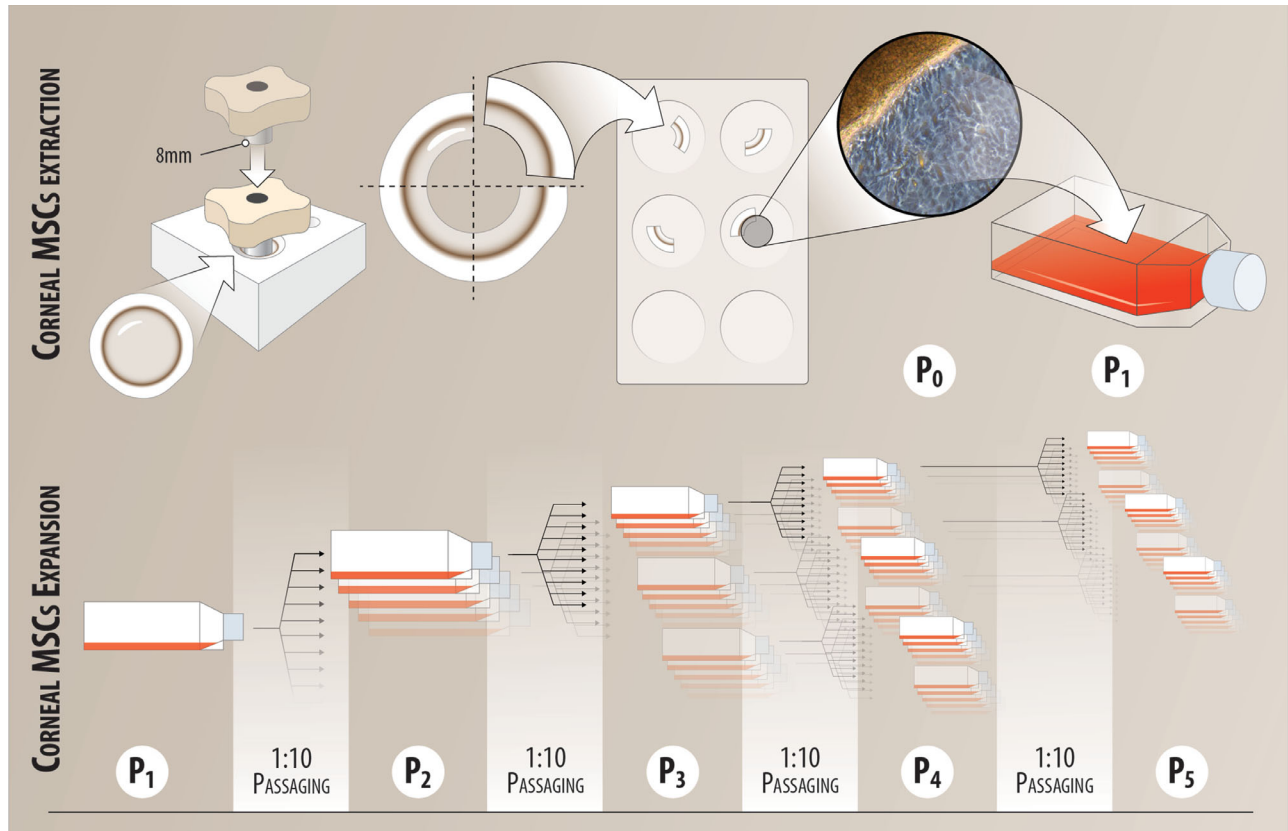
Human cornea tissue was generously provided by the Eversight eye bank (Ann Arbor, MI) from healthy cadaver eyes. All harvested corneas were stored in Optisol storage medium (Chiron Ophthalmics, Irvine, CA) at 4°C (average  $25.5 \pm 4.5$  days) until processing. The age of donors ranged from 50 to 65 years (32 donors, patients with a history of cancer and diabetes, were excluded). As this experiment did not include human subjects and the corneal tissues were obtained from cadaveric donors with no identifiable information, institutional review board approval was not required according to the University of Illinois at Chicago guidelines for the Protection of Research Subjects.

### Derivation of Human cMSCs Using Explant Method

All experiments were conducted under sterile conditions within biosafety cabinets and using autoclaved instruments. Human corneas were removed from storage medium and washed three times with sterile phosphate-buffered saline (PBS) containing 1% penicillin/streptomycin and 1% gentamicin. The Descemet's membrane and conjunctiva were removed, and the central corneas were trephined using an 8-mm trephine and discarded. As illustrated in [Figure 1](#), the remaining rings containing corneoscleral rim were cut into four, six, or eight pieces. Each piece was cultured, epithelial surface up, in one well of a six-well tissue culture plate. Two hundred microliters of media was put on top of each explant to prevent detachment of the explants from the bottom of the dish for the first few days until the explant attached. The media consisted of Minimum Essential Medium Alpha (MEM- $\alpha$ ) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, L-glutamine, and non-essential amino acid (NEAA) (Corning, Inc., Manassas, VA) incubated at 37°C in a humid atmosphere with 5% CO<sub>2</sub>. The culture media were replaced gently with fresh media every other day. Most of the pieces of tissues smaller than one-fourth had a higher likelihood of detachment from the plate following changing the media; therefore, the experiments were conducted using one-fourth-sized corneoscleral tissues. Following outgrowth of the cells, the explants were removed gently by using fine-tip iris forceps to prevent cell detachment. The outgrown cells (passage 0, or P0) from each one-fourth-sized piece were detached using 0.05% TrypLE (Thermo Fisher Scientific, Waltham, MA) and re-cultured in a T-175 flask (P1) with the same media and incubation. The media were changed every 2 to 3 days.

### Expansion of Derived cMSCs

As illustrated in [Figure 1](#), when P1 cells reached 80% to 90% of confluency, typically by 4 days, the cells were detached using TrypLE and counted. Then,  $3 \times 10^5$  cells were plated into 150-cm<sup>2</sup> dishes; subcultured (passage 2, or P2) at a ratio of 1:10 in T-175 flasks using MEM- $\alpha$  media supplemented with 10% FBS, 1% penicillin/streptomycin, L-glutamine, and NEAA; and incubated at 37°C in a humid atmosphere with 5% CO<sub>2</sub>. The media were changed every 2 to 3 days. Each passage of cells was subcultured with the same protocol and ratio until passage 5 (P5). Population doubling time (PDT) was calculated for MSCs using the



**Figure 1.** Illustration of derivation of cMSCs from human cadaveric corneas using the explant method. For the generation of cMSCs, the cadaveric human corneas were trephined using an 8-mm trephine, and the central part was discarded. The remaining corneoscleral rim was cut into four pieces, and each piece was cultivated epithelial side up in one well of a six-well tissue culture plate. After around 7 days, a mixture of epithelial and mesenchymal cells outgrowth was evident and designated as passage 0 (P<sub>0</sub>). P<sub>0</sub> cells were subcultured in a T-175 flask (P<sub>1</sub>). The confluent P<sub>1</sub> cMSCs were then subcultured at a ratio of 1:10 in T-175 flasks (P<sub>2</sub>). The passaging process was continued with the same ratio (1:10) until P<sub>5</sub>.

following formula:  $PDT = CT/PD$ , where CT is culture time, and PD is population doubling.<sup>27</sup>

## Flow Cytometry

The expression of positive and negative MSC cell surface markers was evaluated using flow cytometry. P<sub>5</sub> cMSCs at 80% to 90% confluency were detached using TrypLE. The collected cMSCs ( $5 \times 10^5$  cells per sample) were incubated for 1 hour in the dark at room temperature with Cell Staining Buffer (BioLegend, San Diego, CA) and cell surface antibody diluent: human anti-CD90, 1:100 (BioLegend); human anti-CD73, 1:100 (BD Pharmingen, San Diego, CA); human anti-CD45, 1:100 (BioLegend); and human anti-HLA-DR/MHC II, 1:100 (BioLegend). Then, the cells were washed three times in cold PBS using centrifugation at  $350 \times g$  for 10 minutes at 4°C. The final cell pellet was re-suspended in cold PBS containing 10% fetal calf

serum and analyzed using a CytoFLEX Flow Cytometer (Beckman Coulter, Inc., Brea, CA) within 2 hours. Data were analyzed using FlowJo software (FlowJo LLC, Ashland, OR).

## Obtaining cMSC Secretome

To obtain cMSC secretome, cultured cMSCs (collected from P<sub>5</sub> cells) at 90% to 100% confluency were washed three times with pre-warmed PBS and incubated in a T-175 flask with FBS-free, phenol red-free MEM- $\alpha$  (containing L-glutamine, NEAA, and 1% penicillin/streptomycin, 20 ml) for 48 hours. The supernatant was collected as the conditioned media (CM) containing cMSC secretome. To eliminate any cell contamination, the collected CM were centrifuged at  $500 \times g$  for 15 minutes at 4°C, and the supernatant was used for further experimentation.

## In Vitro Effects of cMSC Secretome on Wound Healing (Scratch Assay)

To evaluate the wound-healing effects of collected secretome from cMSCs, the CM were tested in an in vitro wound-healing (scratch) assay as explained before.<sup>28</sup> In brief, human corneal limbal epithelial cells (HCLEs) were cultured in a six-well plate using keratinocyte serum-free medium (KSFM; Invitrogen, Grand Island, NY). Following 100% confluency of HCLEs, a plus-shaped wound (perpendicular vertical and horizontal scratch lines) was created using a sterile 200- $\mu$ L pipette tip. Each well was then washed three times with pre-warmed PBS to remove the detached cells, followed by administration of media treatments. Three out of six wells were treated with a mixture of 1 ml KSFM and 2 ml of freshly derived CM from P5 cMSCs (two wells). Three remaining wells were treated with a mixture of 1 ml KSFM and 2 ml of FBS-free, phenol red-free MEM- $\alpha$  (containing L-glutamine, NEAA, and 1% penicillin/streptomycin) as control. The proliferation/migration of HCLEs and closure of the created wounds were evaluated every 3 hours for 24 hours using an inverted microscope with digital camera (Carl Zeiss, Oberkochen, Germany). The captured photographs ( $n = 6$  wells per group) were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, <https://imagej.nih.gov/ij/>).

## In Vivo Effects of Expanded cMSCs on Corneal Epithelial Wound Healing

All animal experiments in this study were conducted in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The animal protocols were approved by the Animal Care Committee at the University of Illinois at Chicago.

The effect of expanded P5 cMSCs on corneal epithelial wound healing was evaluated using a 2-mm corneal epithelial debridement wound model in mice. Twenty male C57BL/6J mice (4–5 months-old) were anesthetized with intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg). Anesthetized mice were positioned under a surgical microscope, one drop of 0.5% proparacaine was applied to the eye, and a 2-mm area was demarcated using a 2-mm trephine. The corneal epithelial layer in the demarcated area was removed gently using an AlgerBrush II (The Alger Companies, Lago Vista, TX). After taking a baseline photograph of the fluorescein-stained eye using a Nikon (Tokyo, Japan) FS-2 slit-lamp biomicroscope, P5 cMSCs were injected subconjunctivally using a Hamilton (Reno, NV) syringe ( $n = 10$ , one

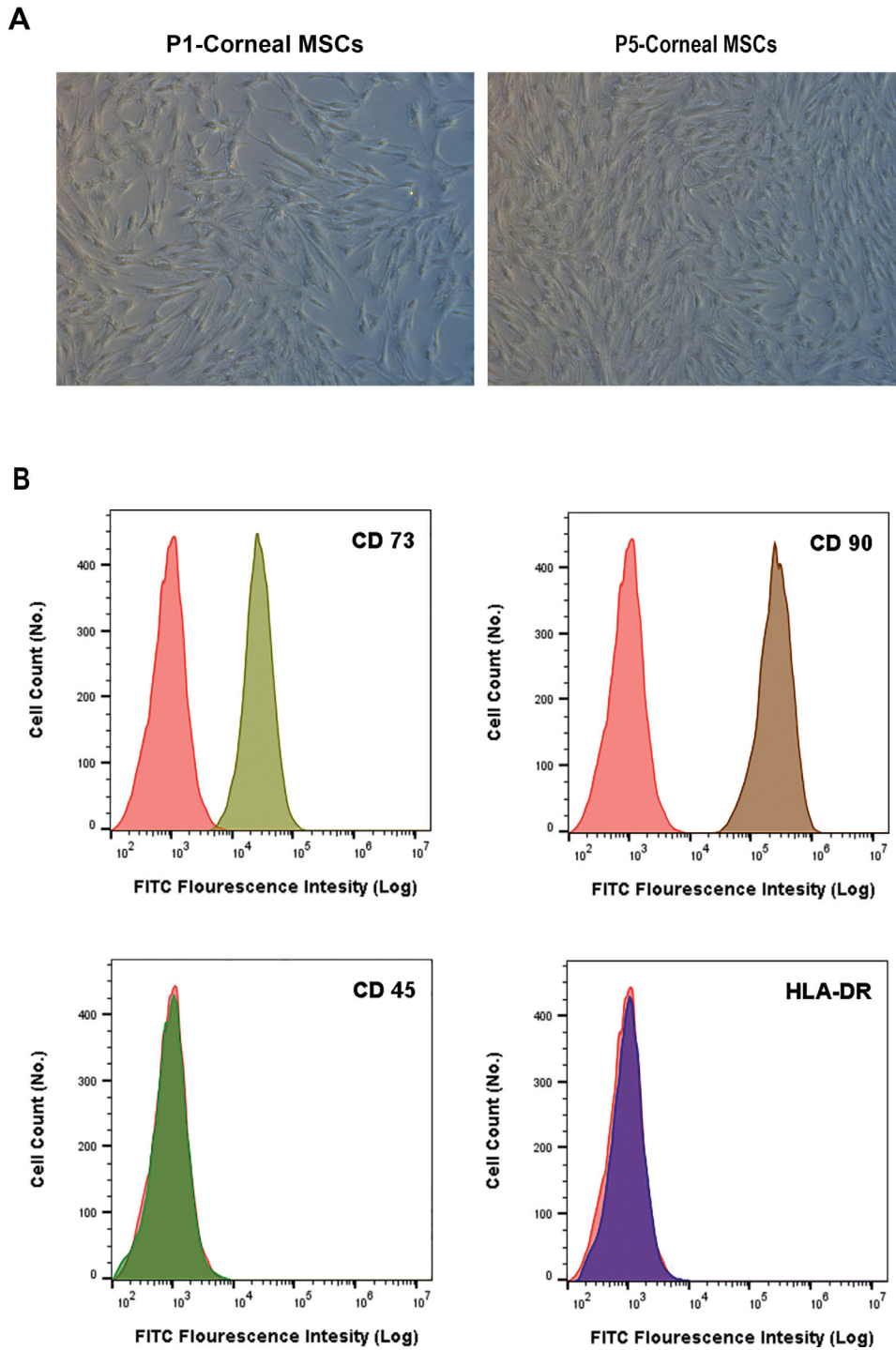
eye per mouse; two injections per eye at superior nasal and inferior temporal near the limbus;  $1 \times 10^5$  cells in 10  $\mu$ L MEM- $\alpha$  media per injection). MEM- $\alpha$  was injected with the same pattern in the control group ( $n = 10$ , one eye per mouse). The treated eyes were evaluated after 18 hours and photographed. The wounded area in captured photographs was measured using ImageJ software, and the percentage of wound closure compared to baseline was calculated.

## In Vivo Effects of Expanded cMSCs on Corneal Inflammation

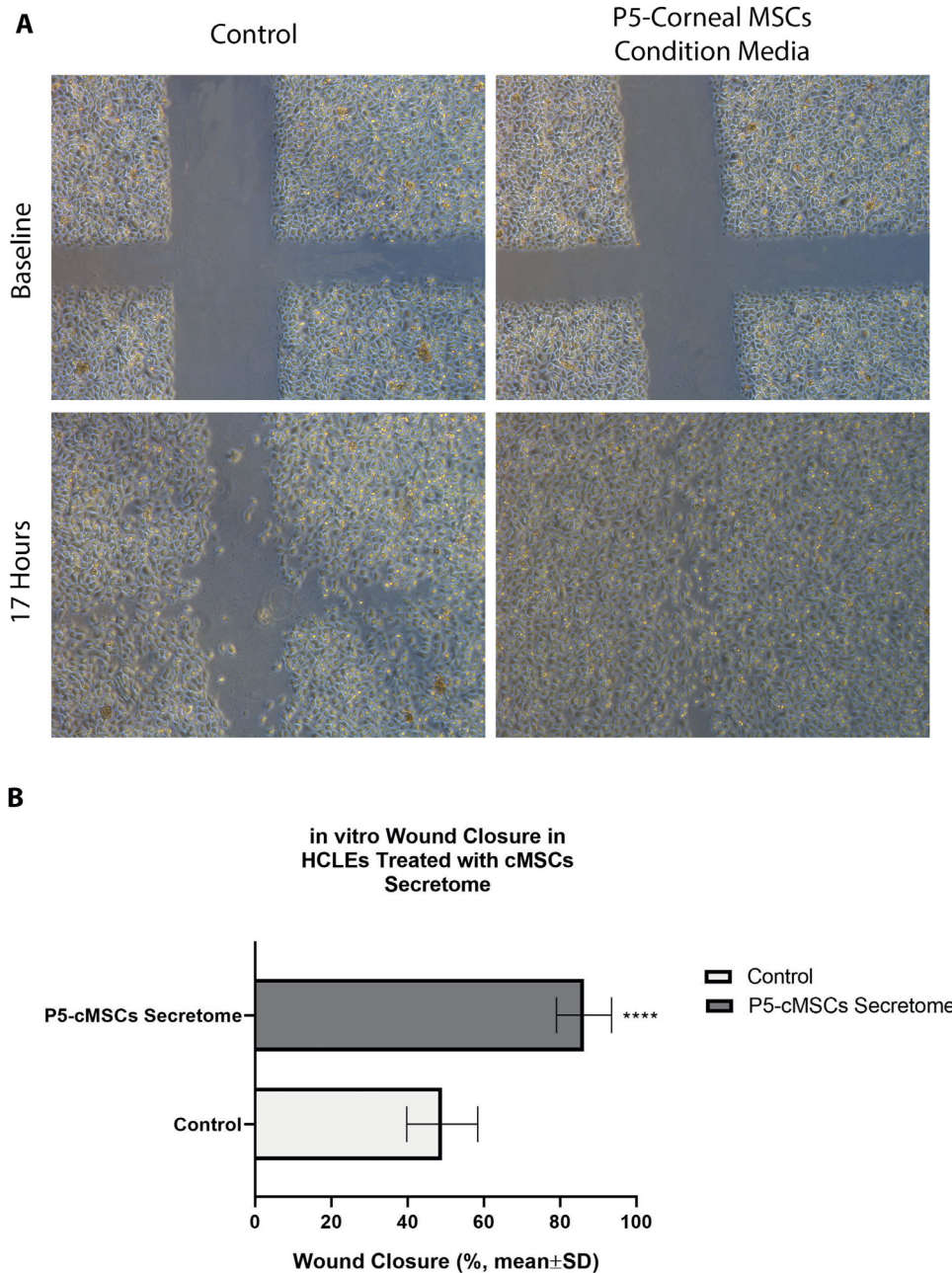
To evaluate the effects of expanded cMSCs on corneal inflammation, an ethanol injury model was used. Forty male C57BL/6J mice (5 months old) were anesthetized, and each was positioned under a surgical microscope. One drop of 0.5% proparacaine was applied to the ocular surface. To induce inflammatory corneal injury, a 2-mm-diameter filter paper pre-soaked in 100% ethanol was placed on the cornea surface for 90 seconds followed by washing with sterile PBS. P5 cMSCs were injected subconjunctivally using a Hamilton syringe ( $n = 20$  eyes, one eye per mouse; two injections per eye at superior nasal and inferior temporal in two opposite sites near the limbus,  $1 \times 10^5$  cells in 10  $\mu$ L MEM- $\alpha$  media per each injection). MEM- $\alpha$  was injected with the same pattern in the control group ( $n = 10$ , one eye per mouse). The treated eyes were photographed every 4 days. The photographs at day 12 were selected for comparison. Corneal haziness was used as a proxy for degree of inflammation, and six blinded experts graded each photo according to a standardized grading system (Supplementary Figure S1). A consensus of four or more out of six was considered as the accepted grade for each particular eye.

## Statistical Analysis

The results are presented as mean  $\pm$  standard deviation or median (95% confidence interval [CI]). The statistical analyses were performed using GraphPad Prism 8 for Windows (GraphPad Software, San Diego, CA; [www.graphpad.com](http://www.graphpad.com)). Student's *t*-test and one-way analysis of variance were used to analyze mean difference in continuous, normal data (scratch assay results and in vivo 2-mm corneal epithelial wound model results), and the Mann–Whitney *U* test was used to evaluate the difference in distribution of ordinal data (haziness grades in corneal inflammatory injury model).  $P < 0.05$  was considered statistically significant.



**Figure 2.** The characteristics of cMSCs following expansion. (A) Representative images show that the spindle shape morphology of cMSCs did not change following expansion from P1 to P5. (B) Flow cytometric analyses of P5 cMSCs show that the cells expressed the defining cell surface markers, with more than 95% positive for CD73 and CD90 and nearly 100% negative for CD45 and HLA-DR ( $n = 3$ ).



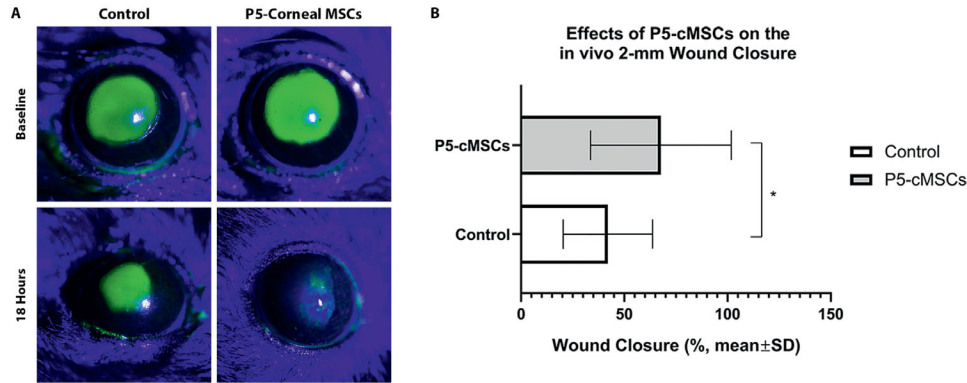
**Figure 3.** The effects of P5 cMSCs-derived secretome on in vitro proliferation and migration of HCLE cells in scratch-wound assay. (A) Representative images show faster closure of scratch wounds in HCLE cultures treated with P5 cMSCs conditioned media compared to control. (B) Quantitative comparison of wound closure showed that on average  $86.3 \pm 7.2\%$  of the wounded areas were repopulated after 18 hours after treatment with P5 cMSCs secretome. On the other hand, the wounded area was repopulated by  $49.1 \pm 9.3\%$  in controls ( $n = 6$  per group; \*\*\*\* $P < 0.0001$  compared to control).

## Results

### Characteristics of Explant-Derived cMSCs

Human corneoscleral rims cut into four pieces were found to be the preferred size for obtaining cMSCs, as the smaller size explants had a higher likelihood of

detachment from the plate. All of the one-fourth-sized explants gave rise to cell outgrowth by 7 days (Fig. 1). Although the initial outgrowth included a mixture of both epithelial and mesenchymal cells (P0), by the end of P1 only cMSCs remained, as other cell types (e.g., epithelial cells) cannot be passaged in the MSC media. The cMSCs maintained their normal spindle morphol-



**Figure 4.** The effects of expanded P5 cMSCs on corneal epithelial wound healing in 2-mm epithelial wounds in rodent models. (A) Representative images of wound closure in mouse 2-mm epithelial wounds injected subconjunctivally with P5 cMSCs. (B) That resulted in  $67.7 \pm 34.1\%$  closure after 18 hours, whereas the wound closure percentage was  $42.2 \pm 21.6\%$  in control injected eyes ( $n = 10$  eyes per group;  $*P < 0.05$ ).

ogy (Fig. 2A). By the end of each passage (P1–P5), the total cMSCs yield from each one-fourth-sized explant was approximately  $3$  to  $4 \times 10^6$  in each flask (80%–90% confluency in a T-175 flask). Each flask was subcultured 1:10 at each cycle of passaging (Fig. 1). The potential yield of expanding cMSCs from each cornea was approximately  $12$  to  $16 \times 10^{11}$  P5 cells. PDT was evaluated to define proliferation rate. The mean proliferation potential for cMSCs at P1 through P5 was  $32 \pm 3$  hours, which remained almost constant up to P10. Moreover, the actual age of culture for in vitro aging is determined by the number of cell population doubling.<sup>29</sup> The number of cell population doubling was calculated approximately 3 times per each passage and was 16 to 17 times from P0 until P5 at a 1:10 ratio. By flow cytometry, more than 95% of the cells were positive for cell surface markers CD73 and CD90 and negative (less than 5%) for CD45 and HLA-DR at P5 (Fig. 2B).

### Expanded cMSCs Promote Epithelial Wound Healing in Vitro

The secretomes (conditioned media) of P5 cMSCs were applied to a scratch assay to evaluate their in vitro wound healing effects. After 18 hours, wounds treated with P5 cMSC secretome were closed by  $86.3 \pm 7.2\%$ , and the control wounds were closed by  $49.1 \pm 9.3\%$  ( $P < 0.0001$ ;  $n = 6$  per group) (Fig. 3).

### In Vivo Wound Healing and Anti-Inflammatory Effects of cMSCs

To evaluate the in vivo effects of expanded cMSCs, the cells were injected subconjunctivally in murine eyes

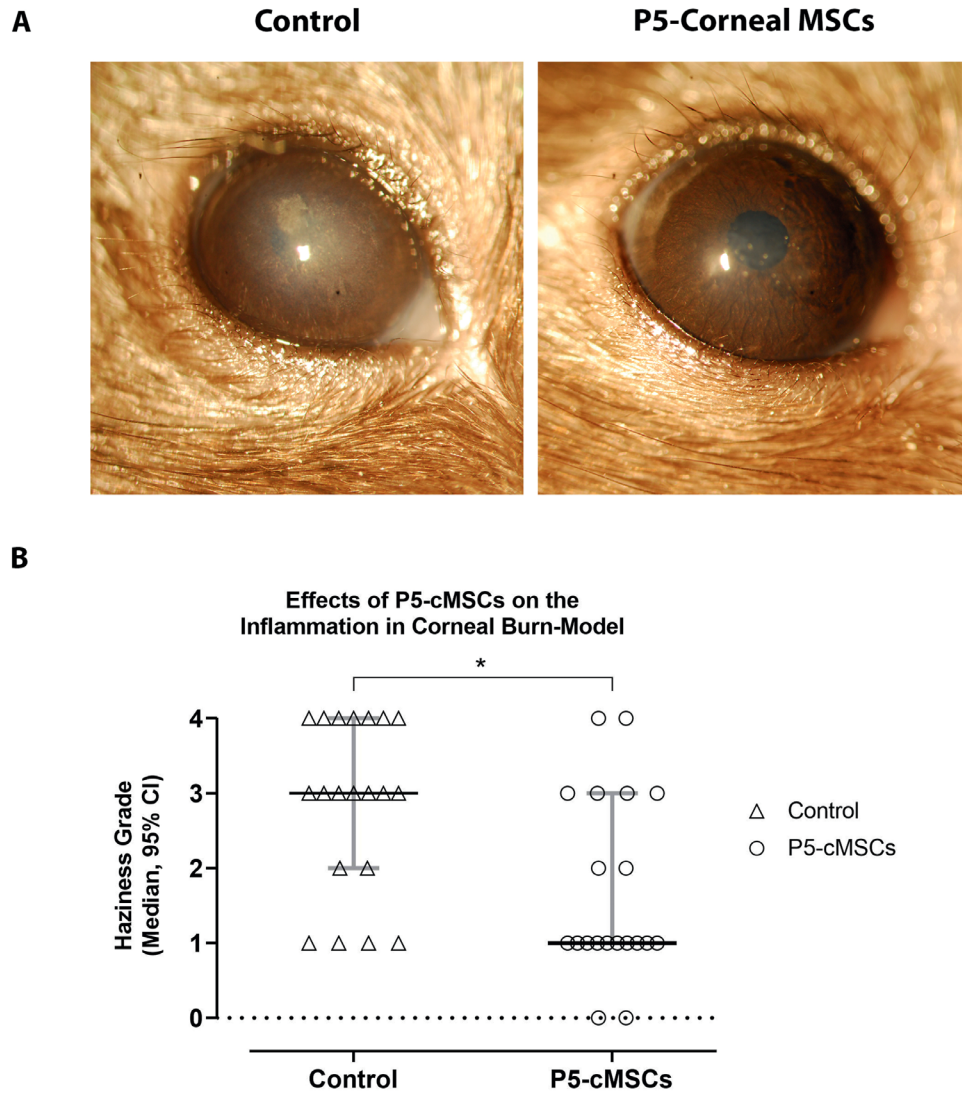
following corneal epithelial debridement or corneal ethanol injury. At 18 hours after the 2-mm epithelial wounds, the healed (re-epithelialized) area was significantly higher in mice injected with P5 cMSCs compared to controls ( $67.7 \pm 34.1\%$  vs.  $42.2 \pm 21.6\%$ , respectively;  $P < 0.05$ ) (Fig. 4).

Likewise, corneal haziness after subconjunctival injection of P5 cMSCs was significantly reduced after 12 days compared to controls as evaluated by a haziness grading system: median haziness grade 1 (95% CI, 1–3) versus median haziness grade 3 (95% CI, 2–4), respectively ( $P < 0.05$ ) (Fig. 5).

## Discussion

The therapeutic potential of cMSCs has been demonstrated in both in vitro and in vivo laboratory studies,<sup>19,30</sup> and cMSCs could provide a novel source of MSCs for corneal regenerative treatments. For clinical applications, however, protocols for cMSC isolation and expansion will have to be scalable while maintaining high reproducibility. Efficiency in terms of cellular yield has numerous obvious benefits, including decreased demand for corneal donor tissue, less batch-to-batch variability, and greater availability to patients. Process scalability will become important when going from preclinical to clinical trials and ultimately to clinical practice. Finally, reproducibility at the cellular level is of particular importance in the clinical application of cell-based therapies. Although standards for cell-based therapies are still in development, quality control will become an important consideration.<sup>31,32</sup>

With these considerations in mind, in this study we aimed to demonstrate a reproducible protocol for



**Figure 5.** The effects of expanded P5 cMSCs on corneal stromal inflammation following ethanol-induced burn injury in mouse model. (A) Representative images show less haziness in the corneas treated with P5 cMSCs compared to control. (B) The distribution of haziness grades was significantly different between eyes treated with P5 cMSCs: median haziness grade 1 (95% CI, 1–3) for treated eyes and median haziness grade 3 (95% CI, 2–4) for control eyes ( $n = 20$  eyes per group;  $*P < 0.05$ ).

the derivation and expansion cMSCs with a relatively high yield. With this protocol, a single corneal donor would theoretically provide at least  $1 \times 10^{12}$  cells at P5. These cells maintained consistent morphology into P5 and adhered to the current standards regarding the classification of MSCs established by the International Society for Cellular Therapy.<sup>1</sup> The explant method for deriving and expanding cMSCs produced viable cells at a yield similar in magnitude to various published yields for adipose tissue, umbilical cords, and Wharton's jelly with high reproducibility.<sup>33–35</sup> When the described protocol is translated to clinical applications, a single one-quarter-sized corneoscleral rim specimen would be expected to produce 3 to  $4 \times 10^7$

cells (Fig. 1) with a total yield of approximately  $1 \times 10^8$  P2 cells per cornea which can be used as the master cell bank. Each aliquot of cells from the master cell bank (P2) can be expanded 1:10 for a total of  $1 \times 10^9$  working cell bank (P3) cells. Each aliquot of a working cell bank can then be thawed when desired, passaged to P5 (1:100 expansion), and administered to patients. Importantly, this process would permit the use of some aliquots for quality control measures and to ensure homogeneity and allow for sustainable long-term regeneration of aliquots for patient use.

Population doubling is typically used to track cellular age in vitro because different cell culture techniques can cause variation in the passage number. Previous



studies have reported senescence at over 40 population doublings from the initial MSCs passage<sup>36,37</sup>; however, in other studies, senescence has been reported at between 15 and 30 passages.<sup>38,39</sup> Our results showed that the number of cell population doubling was 16 to 17 times from P0 until P5 at a 1:10 expansion ratio, which is similar to previous reports.<sup>29,39</sup> Our result showed that the mean population doubling time for cMSCs at P1 to P5 was  $32 \pm 3$  hours, which is comparable to the reported population doubling time of 40 hours for bone marrow MSCs and 24 hours for umbilical cord MSCs.<sup>40</sup> In addition to cultivation conditions, the specific media and different origins of MSCs may affect the population doubling time.<sup>41</sup>

The generation of cMSCs must also consistently preserve the therapeutic effects in order to translate clinically. Prior studies on human cMSCs have demonstrated antifibrotic properties with reduced corneal scarring after injury, along with regeneration of injured stromal tissue.<sup>18</sup> We have also described direct antiangiogenic properties of human cMSCs that have resulted in decreased corneal neovascularization in both in vitro and in vivo studies. These antiangiogenic properties were found to be conferred in part by the secretion of pigment epithelium-derived factor and soluble fms-like tyrosine kinase-1 and low secretion of vascular endothelial growth factor A.<sup>19</sup> cMSCs also induce secretion of antiinflammatory factors by the macrophages and modulate their phenotype to an inflammation-resolving phenotype,<sup>30</sup> in addition to inhibiting T-cell proliferation.<sup>42</sup> Other studies have shown that cMSCs can induce increased cytokeratin 12-positive corneal epithelial cells and decreased neovascularization, conjunctivalization, and K8-positive conjunctival goblet cells.<sup>43-45</sup> In this study, subconjunctival cMSC injection resulted in improved epithelial wound healing and reduced corneal haze in mouse models, corroborating prior results.

In addition to process parameters such as yield, reproducibility, and preservation of efficacy, other challenges related to the clinical translation of MSC therapies are postprocessing logistics such as temperamental handling and storage conditions, as well as biologic challenges such as immunological rejection and poor cellular retention at the site of injury.<sup>46</sup> Given that much of the purported benefit of MSCs may be attributed to the secretome,<sup>47</sup> the use of the secretome rather than the cells may mitigate some of these issues. Secretome would require less restrictive storage and transportation conditions, have higher shelf stability, and have more consistent bioavailability at target tissues,<sup>48</sup> thus representing an attractive therapeutic option. Our in vitro experiments demonstrated enhanced corneal wound

healing with factors secreted by cMSCs, consistent with previous studies.<sup>49</sup> Moreover, the secretome wound-healing properties were maintained through P5 without diminished effect. Future studies might compare the wound-healing efficacy of cMSCs to that of the secretome.

## Conclusions

The study reports the reproducible isolation and expansion of explant-derived cMSCs and confirmed their in vitro and in vivo therapeutic benefits. These studies may serve as a foundation for the development of protocols for clinical production of cMSCs and clinical applications of secretome-based therapies.

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