# Kinetics and Function of Mesenchymal Stem Cells in Corneal Injury

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**Purpose.** Bone marrow–derived mesenchymal stem cells (MSCs) hold great promise for wound healing and tissue regeneration. In the present study, we investigated the impact of corneal injury on the homeostasis of endogenous MSCs, and the potential of MSCs to home to injured tissue and promote corneal repair.

**Methods.** Corneal injury in mice was induced by thermal cauterization. Circulating MSCs were quantified by flow cytometric analysis. Ex vivo expanded red Q-dot–labeled or GFP+ bone marrow–derived MSCs were intravenously injected after injury and detected using epifluorescence microscopy. Corneal fluorescein staining was performed to evaluate epithelial regeneration.

**Results.** Following the induction of corneal injury in mice, a 2-fold increase in the frequency of circulating endogenous MSCs was observed within 48 hours of injury, which was accompanied by increased levels of the stem cell chemoattractants, substance P and SDF-1, in both the injured cornea and blood. Systemically administered MSCs homed to the injured cornea, but not to the normal cornea, and showed long-term survival. In addition, in the setting of corneal injury, MSC administration showed significant and rapid corneal epithelial regeneration.

**Conclusions.** These findings provide novel evidence that corneal injury causes significant mobilization of endogenous MSCs into blood, and that MSCs home specifically to the injured cornea and promote regeneration, highlighting the therapeutic implications of MSC-mediated tissue repair in corneal injury. (*Invest Ophthalmol Vis Sci.* 2012;53:3638–3644) DOI:10.1167/iovs.11-9311

The limbal stem cell (LSC) compartment is the critical source of renewal for the corneal epithelium. Partial or total LSC deficiency is a major cause of corneal transparency loss and ocular morbidity. In addition to trauma and burn injuries, intrinsic factors that lead to LSC deficiency include dysfunction or reduction in the numbers of LSCs due to autoimmune diseases, multiple surgeries, or diabetes.[1] Current therapeutic options for LSCs currently include autologous limbal grafts for unilateral lesions and limbal allografts for extensive or bilateral lesions.[2] However, these strategies are associated with potential adverse effects, including the induction of LSC deficiency in the healthy eye following autologous procurement, and the risks of immunosuppressive therapy post allograft.[3] As a consequence, other strategies, including the potential usage of bone marrow mesenchymal stem cells (MSCs) in corneal injuries, are gaining prominence.[4]

MSCs are self-renewing, nonhematopoietic stromal stem cells comparable to embryonic stem cells in terms of their multipotency and proliferative and differentiation potential.[5] Due to their multilineage differentiation potential and immunomodulatory properties, MSCs present a promising tool for cell therapy, and are currently being tested in Food and Drug Administration–approved clinical trials for myocardial infarction, stroke, meniscus injury, limb ischemia, graft-versus-host disease, and autoimmune disorders (http://clinicaltrial.gov). They have been extensively tested and proven effective in preclinical studies for these disorders.[6] In addition, MSCs have a unique property of homing to the inflamed/injury sites. A significant tissue injury, such as myocardial infarction, stroke, or renal injury, causes the release of cyto-chemokines and other factors, which stimulate bone marrow to mobilize MSCs into the blood and direct their trafficking to the site of injury to promote tissue regeneration/repair.[7,8]

The application of MSCs, or conditioned media derived from MSC cultures, to the injured cornea, has been demonstrated to improve corneal wound healing.[9–12] The impact of corneal injury on the homeostasis of endogenous MSCs, and the homing and engraftment potential of systemically administered MSCs to the cornea are still not well understood, however. In this study, we demonstrate that corneal injury leads to increased frequencies of circulating endogenous MSCs and elevated levels of the putative stem cell–specific chemoattractants in the peripheral blood. Furthermore, intravenously administered MSCs were observed to home specifically to the injured cornea and promote epithelial regeneration.

## Methods

### Animals

Six- to 8-week-old male C57BL/6 wild-type mice (Charles River Laboratories, Wilmington, MA) and green fluorescent protein (GFP) transgenic mice (Jackson Laboratory, Bar Harbor, ME) were used in these experiments. The protocol was approved by the Schepens Eye Research Institute Animal Care and Use Committee, and all animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Isolation, Expansion, and Characterization of MSCs

Bone marrow was harvested from euthanized B6 wild-type and GFP.B6 mice. Using the plastic adherence method of MSC cultivation, bone

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marrow cells were cultured in murine MSC-specific MesenCult basal media and supplement (Stem Cell Technologies, Vancouver, BC, Canada) according to manufacturer's instructions. Cells were passaged at every third day, and after 8 to 10 passages, MSCs were characterized phenotypically for the expression of MSC markers by flow cytometry as described later, and functionally by their in vitro differentiation into adipocytes using MesenCult adipogenic stimulatory supplements (Stem Cell Technologies). Oil-red-O (Sigma-Aldrich, St. Louis, MO) staining was used to confirm the differentiation of MSC into the adipocytes.

## Model of Corneal Injury and Treatment

Mice were subjected to corneal injury in the right eye under general anesthesia. Using a thermal cautery device (Aaron Medical Industries Inc., St. Petersburg, FL), epithelial cautery was performed on half of the cornea and limbus to induce epithelial injury. Dead corneal and limbal epithelia were mechanically scraped using a surgical blade followed by rinsing with 0.9% NaCl and application of topical antibiotic ointment. To study the kinetics of endogenous MSCs and chemoattractants, mice were killed at 24, 48, and 72 hours post injury to collect blood for flow cytometry and ELISA analyses as described later. To study the homing and therapeutic potential of MSCs, mice were randomly divided into cautery-alone or MSC-recipient groups, with $n = 6$ in each group. In vitro expanded and characterized MSCs, labeled with Alexa 647 Qtracker dots (Invitrogen, Grand Island, NY) or MSCs derived from GFP-B6 mice, were injected into the tail veins of mice 1 hour post injury. Mice were killed at days 3, 21, and 50, and corneas were harvested to examine the presence of MSCs.

## Evaluation of Corneal Epithelial Regeneration

Corneal epithelial regeneration following cautery was determined by fluorescein staining of the corneal surface to evaluate the area of epithelial defect, and by molecular analysis using real-time PCR as described later. Corneal fluorescein staining was performed using slit lamp biomicroscopy at baseline before the application of cautery, and then subsequently on the same mice at 24, 48, and 96 hours. Using a micropipette, 1 μL of 2.5% fluorescein (Sigma-Aldrich) was applied to the corneal surface. After 3 minutes, epithelial staining was scored in a masked fashion using slit lamp biomicroscopy under cobalt blue light and photographic images taken. The area of the epithelial defect (green color) was calculated using National Institutes of Health Image J (version 1.34s) software.

## Flow Cytometry

Flow cytometry was performed to characterize the phenotype of in vitro expanded MSCs and for the purpose of quantifying the frequencies of circulating MSCs in the peripheral blood following corneal injury. Cultured MSCs in single-cell suspension were stained with conjugated monoclonal antibodies to CD45, CD34, SCA-1, and CD29 or CD105. All the antibodies with their matched isotype controls were purchased from Biolegend (San Diego, CA). Stained cells were analyzed on an EPICS XL flow cytometer (Beckman Coulter, Brea, CA). For analyzing the frequencies of circulating endogenous MSCs, peripheral blood was collected at 24, 48, and 72 hours following injury. Red cells were lysed by the addition of red cell lysing buffer (Sigma-Aldrich) to blood samples, and the final washed single-cell suspension was used for antibody staining.

## Real-Time PCR

Corneas were harvested at 96 hours after cautery from each group, and RNA isolated with RNeasy Micro Kit (Qiagen, Valencia, CA) and reverse transcribed using Superscript III Kit (Invitrogen). Real-time PCR was performed using Taqman Universal PCR Mastermix and preformulated primers for murine glyceraldehype-3-phosphate dehydrogenase (GAPDH), ABCG2, P63, Hes1, CEBPσ, K12, K19, TGF-β, and IL-1Ra (Applied Biosystems, Foster City, CA). The results were analyzed by the comparative threshold cycle method, using GAPDH as an internal control and normalized to expression levels in noncauterized cornea tissue.

## Fluorescence Microscopy

Freshly excised corneas were washed in PBS and fixed with 4% paraformaldehyde for 15 minutes. Whole corneas were then mounted onto slides with DAPI containing mounting medium (Vectashield; Vector Laboratories, Burlingame, CA), and visualized under epifluorescence microscope (Nikon, Melville, NY) at ×40 magnification to detect red Q-dot–labeled MSCs. In case of detection of GFP+MSC, paraformaldehyde-fixed corneas were permeabilized with 0.5% TritonX-100 for 10 minutes, and then immunostained with Alexa Fluor 488 conjugated anti-GFP (Invitrogen).

## ELISA

Levels of SDF-1 and substance P in peripheral blood were analyzed using commercially available murine SDF-1α ELISA kit (R&D Systems,

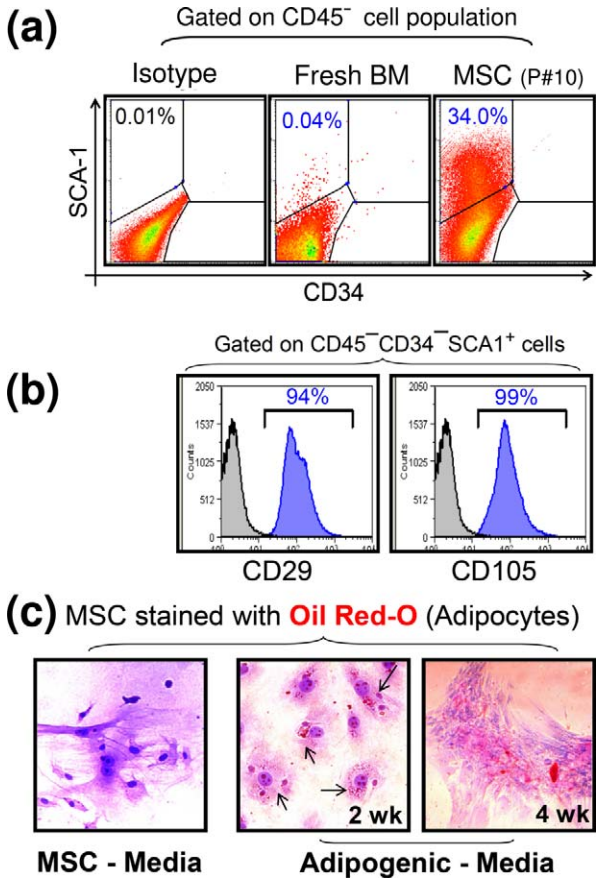


**Figure 1.** Expansion and characterization of MSCs. (**a**) Representative flow cytometric dot plots showing frequencies of $CD45^-CD34^-SCA1^+$ MSCs in fresh bone marrow (BM) and after 10 culture passages of BM in murine-specific MSC culture media. (**b**) Representative flow cytometric histograms confirming expression of CD29 (94%) and CD105 (99%) on $CD45^-CD34^-SCA1^+$ MSCs. (**c**) Microscopic images of MSCs cultured in adipogenic media and stained with Oil-Red-O dye. At 2 weeks, small red color vacuoles (*arrows*) were observed within the cytoplasm. By 4 weeks, mature adipocytes were seen, evident by the coalesced cells and large red vacuoles.

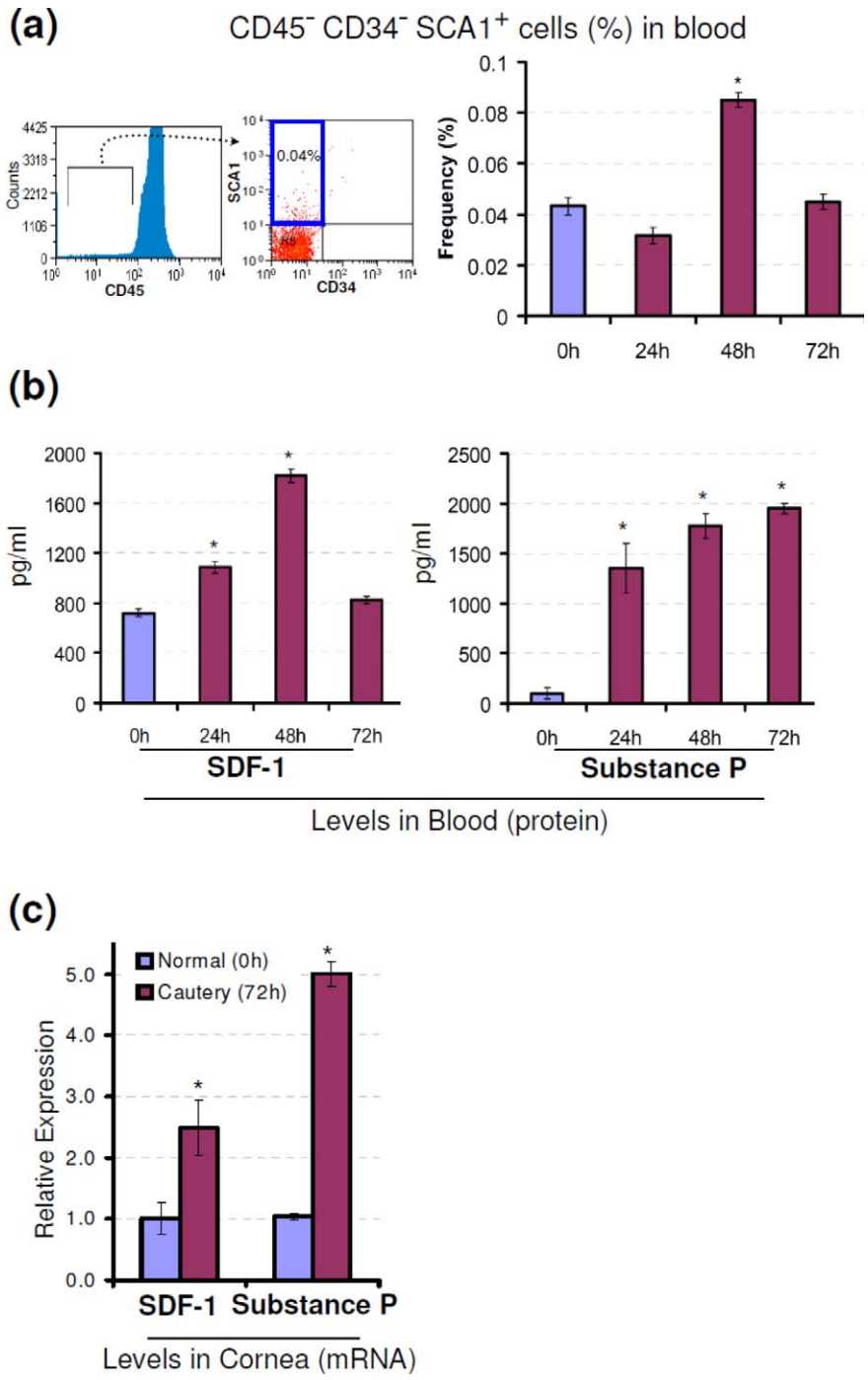


**Figure 2.** Corneal injury leads to increased frequencies of circulating MSCs in peripheral blood with an associated increase in the levels of potential stem cell-specific chemoattractants. (**a**) A significant 2-fold increase in the frequencies of $CD45^{-}CD34^{-}SCA1^{+}$ MSCs was observed in peripheral blood at 48 hours after injury. (**b**) ELISA quantification of chemoattractants in peripheral blood. A progressive and significant increase in the protein levels of SDF-1 was observed, which peaked at 48 hours, and returned to baseline by 72 hours. In contrast, a rapid rise in substance P levels was demonstrated by 24 hours, with levels continuing to increase at all time points evaluated ($n = 3$ mice/time point, $^{*}P < 0.05$). (**c**) Relative mRNA expression of chemoattractants in normal and cauterized cornea at 72 hours following the induction of injury. A significant increase in the expression levels of both SDF-1 and substance P was observed in the injured cornea.

Minneapolis, MN) and substance P assay kit (R&D Systems) as per the manufacturer's instructions.

### Statistical Analysis

A two-tailed Student's *t*-test was performed and *P* values less than 0.05 were regarded as statistically significant. Results are presented as the mean ± SEM of at least three experiments.

## Results

### Characterization and Expansion of MSCs

To study MSCs in corneal injuries, we first standardized the methodology for in vitro culture and characterization of MSCs according to the criteria outlined in the Mesenchymal and Tissue Stem Cell Committee of the International Society for

Cellular Therapy statement.[13] MSCs were cultured and expanded using the plastic adherence method, and after 10 subculture passages, most of cells were spindle shaped and had a fibroblast-like morphology. Cell surface markers were assessed using flow cytometry to phenotypically characterize in vitro expanded MSCs. All the cells were negative for the expression of hematopoietic markers CD45 and CD34, and were positive for the expression of MSC-associated markers, including SCA1, CD29, and CD105 (Figs. 1a, 1b), consistent with previous reports.[13,14] Cultured MSCs were further assessed for their functionality by determining their ability to differentiate into adipocytes. Following 2 weeks of culturing MSCs in adipogenic media, small red vacuoles were detected using Oil-red-O staining. By week 4, adipocytes were coalesced together and large-sized lipid vesicles were evident, indicating differentiation into mature adipocytes (Fig. 1c).

## Corneal Injury Alters Homeostasis of Circulating Endogenous MSCs

It has been established that increased frequencies of MSCs are found in the peripheral blood after the stimulus of an injury.[15,16] We herein aimed to determine if corneal injury could have an impact on bone marrow homeostasis and alter the frequencies of circulating endogenous MSCs. Flow cytometric studies performed on peripheral blood of mice with corneal injury showed significantly increased (2-fold) frequencies of $CD45^{-}CD34^{-}SCA1^{+}$ cells at 48 hours post injury compared with naïve mice (Fig. 2a). Furthermore, substance P and stromal cell–derived factor-1 (SDF-1; also known as chemokine C-X-C motif ligand 12 [CXCL12]) have been reported to possess stem cell chemoattractant properties, which can facilitate mobilization of endogenous MSCs from bone marrow into peripheral blood.[7,17] We therefore subsequently quantified the protein levels of substance P and SDF-1 in the peripheral blood following corneal injury (Fig. 2b). ELISA analyses revealed a gradual increase in SDF-1 levels, which peaked at 48 hours post injury (2.5-fold). In contrast, a rapid increase (15-fold) was observed in substance P levels by 24 hours, with levels continuing to rise until 72 hours. To confirm the injured corneal tissue as the potential source of these MSC chemoattractants, we characterized mRNA expression of substance P and SDF-1 in corneal tissue at 72 hours post cautery (Fig. 2c). Significantly increased expression levels were evident at this time point (~2.5- and ~5-fold increase in SDF-1 and substance P respectively). Taken together, these data suggest that corneal injury could lead to an increased expression of stem cell chemoattractants that mobilize endogenous MSCs into peripheral blood.

## MSCs Home Specifically to the Injured Cornea

We sought to ascertain whether MSCs have the potential to home to corneal tissue by tracking the presence of intravenously injected MSCs in both injured and contralateral healthy corneas. MSCs homing to cornea following injury was investigated by injecting red Q-dot–labeled or GFP+ bone marrow–derived MSCs (GFP+MSCs). MSCs were injected 1 hour post injury, and epifluorescence microscopy on corneal wholemounts was used to detect the labeled cells (Fig. 3a). At 72 hours after injury, substantial numbers of Q-dot–labeled cells were visible specifically in the injured corneas, but not in the contralateral cornea (Fig. 3b). Next, to determine if MSCs remained detectable in the injured cornea at a later time point, we injected GFP+MSCs and evaluated the presence of these cells up to day 50. GFP+MSCs were similarly observed in the injured cornea at this time point, but not on the contralateral side (Fig. 3b), suggesting that MSCs not only home to the cornea in response to an injury, but also indicating that these cells persist long term at the site of injury.

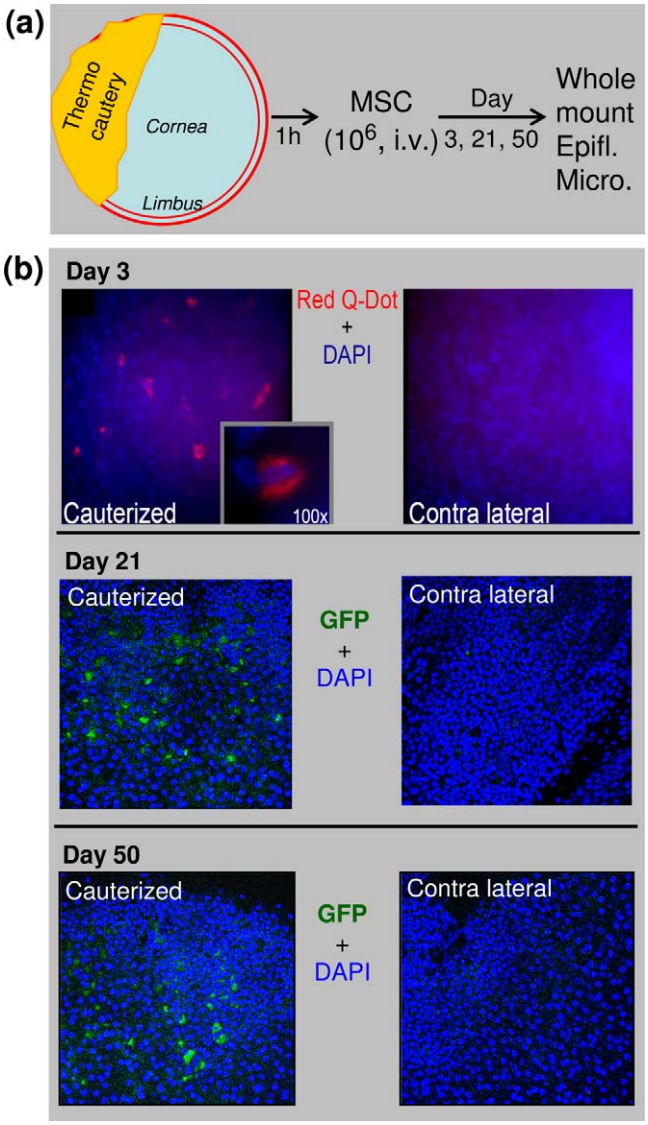


**Figure 3.** MSCs home and persist in the cornea following injury. (**a**) Schematic diagram of the experimental design to study MSCs homing to the injured cornea. Thermocautery was applied to half of the cornea and limbus to induce epithelial injury. After 1 hour of corneal injury, in vitro expanded and characterized MSCs were injected intravenously to one group of mice ($n = 6$), and then at days 3, 21, and 50, corneas were harvested to examine the presence of MSCs. (**b**) Epifluorescence micrographs (×40) of corneal wholemounts showing presence of red quantum-dot–labeled MSCs at day 3 in the epithelium of the cauterized cornea but not in the contralateral cornea. Similarly at days 21 and 50, GFP+ MSCs were observed in the cauterized cornea, but remain absent from the contralateral side.

## MSCs Promote Corneal Epithelial Regeneration following Injury

We finally studied whether homing and engraftment of MSCs to injured cornea can promote epithelial regeneration. To determine the extent of epithelial regeneration following corneal injury in MSC-injected or control mice, we used corneal fluorescein staining to determine the extent of the epithelial defect. A smaller epithelial defect, suggestive of more rapid epithelial regeneration, was apparent by 96 hours in the

MSC-injected mice compared with the cautery-only group (Fig. 4a). To further characterize the impact of MSCs on epithelial regeneration at the molecular level, mRNA expression of both stem cell and differentiated corneal epithelial cell markers were quantified in both groups of cauterized corneas. Corneas from mice receiving MSCs had substantially increased (~2-fold) mRNA expression of ABCG2, P63, Hes1, and CEBPσ stem cell markers, and K12 and K19 epithelial cell markers compared with control cautery-only mice that had not received MSCs (Fig. 4b). MSCs are known to possess potent anti-inflammatory properties.[6] To ascertain whether MSC administration is associated with an increase in anti-inflammatory factors in the injured cornea, we characterized mRNA expression of TGF-β and IL-1Ra in the two groups of cauterized corneas. Increased expression levels of both TGF-β and IL-1Ra were evident in corneas from MSC recipient mice compared with control mice.

## Discussion

We show herein that corneal injury leads to the mobilization of endogenous MSCs and elevation of stem cell chemotactic factors in the peripheral blood, and that systemically administered MSCs home specifically to the injury site and promote corneal epithelial regeneration.

Our characterization of increased frequencies of MSCs in the peripheral blood following corneal injury is the first report showing the impact of corneal injury on endogenous $CD34^{-}CD45^{-}Sca\text{-}1^{+}$ MSC homeostasis and is consistent with previous published findings of injury to other tissues (non-ocular) stimulating enhanced mobilization of MSCs.[15,16] The frequencies of circulating MSCs peaked at 48 hours after corneal injury, suggesting that this insult provides a transient stimulus for MSC release. Furthermore, the release of potential stem cell–specific chemoattractants from injured tissues have been implicated in inducing the mobilization of endogenous

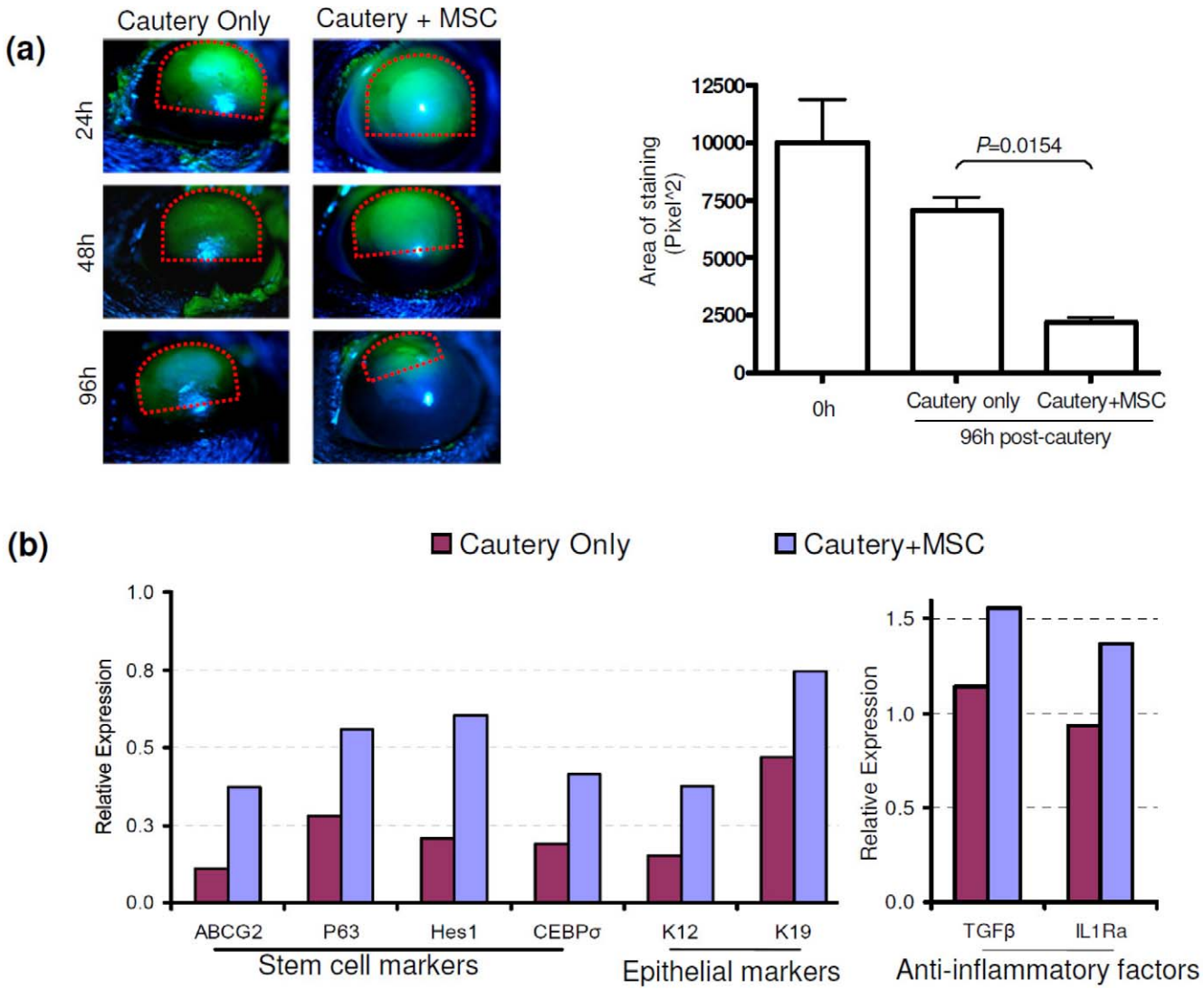


**Figure 4.** MSCs promote corneal epithelial regeneration. (**a**) *Left panel*: Representative slit lamp biomicroscopy images of fluorescein-stained corneas at 24, 48, and 96 hours post injury. A smaller area of fluorescein staining, suggestive of a faster rate of regeneration, was seen at 48 and 96 hours post injury in MSC-injected mice compared with the cautery-only group ($n = 6$ mice). *Right panel*: The mean area of fluorescein staining was significantly less in the MSC recipient mice compared with the cautery-only mice at 96 hours ($P < 0.02$). (**b**) Corneas isolated from MSC-injected mice at 96 hours had a higher mRNA expression of markers of stemness and epithelial cells compared with cautery-only mice. Additionally, higher mRNA expression levels of the anti-inflammatory factors TGF-β and IL-1Ra were observed in corneal tissue derived from MSC-recipient mice. The results were normalized to mRNA expression levels in the cornea of normal mice (defined as 1 on the y-axis).

MSCs into peripheral blood,[18] which is consistent with our results that indicate the coincidence of increased frequencies of circulating MSCs with elevated levels of SDF-1 and substance P after corneal injury. In a similar manner to leukocyte trafficking, the directional migration of MSCs to inflamed and injured tissues is influenced largely by the release of chemoattractants from these affected sites.[19] Our data of elevated levels of SDF-1 and substance P in the cauterized cornea is also suggestive of their role in the mobilization and recruitment of MSCs to the injured cornea. Raised levels of SDF-1 in the peripheral blood and injured tissues are seen in both human and animal models following injury and trauma, and have been implicated in the homing of MSCs to injured tissues, in addition to its function of facilitating MSC mobilization from the bone marrow.[20,21] Similarly, a recent study highlights an important function of neuropeptide substance P as a systemically acting injury-inducible messenger that mobilizes and recruits CD29+ stromal-like cells to the site of injury, resulting in accelerated wound healing.[7]

Using a combination of Q-dot–labeled and GFP+MSCs, we demonstrate MSCs homing to the injured cornea. Although relative specificity for the injured tissue was confirmed by the absence of MSC infiltration into the contralateral uninjured cornea, previous reports have suggested that an element of nonspecific MSC infiltration may occur, evident by small numbers of MSCs migrating into noninjured solid organs.[22,23] Our finding of MSCs homing to the site of injury and promoting repair of the injured cornea corroborates previous reports on MSC infiltration after systemic administration in solid organ injury, including in models of myocardial infarction, cerebral ischemia, pulmonary fibrosis, and nephropathy[8]; however, controversy still remains as to whether these MSCs are capable of persisting long term in the injured cornea.[24] Our observation of MSCs remaining detectable in the cornea up to 50 days post injury is the first report showing long-term survival of MSCs in corneal tissue. In contrast, however, a recent study on the systemic administration of human MSCs in a rat corneal injury model has suggested that the enhanced wound healing was the result of paracrine effects of MSCs from a distant site, rather than their engraftment into corneal tissues.[25] These differences may be in part explained by the usage of human MSCs in animal models, and the potential issues with integration and engraftment of cells across the xeno-species barrier.

It is well established that MSCs possess a series of anti-inflammatory and immunomodulatory properties.[6] Our findings of elevated expression of anti-inflammatory factors in injured corneas from mice receiving intravenously administered MSCs are consistent with these findings. It has been proposed that the suppression of inflammation during the acute phase of injury by the application of MSCs lessens the severity of loss of LSCs, and therefore improves epithelial regeneration. In support of MSCs promoting epithelial wound healing indirectly via suppressing inflammation is the demonstration of high quantities of the anti-inflammatory cytokines IL-10 and TGF-β found in the injured corneas after the application of MSCs to the ocular surface.[26] In addition to this, it has recently been suggested that the MSC-secreted TNF-α stimulated gene 6 (TSG-6) protein has an important role in directly mediating the anti-inflammatory effects of MSCs in corneal wound healing.[25,27] Finally, our observation of increased IL-1Ra expression in corneas from MSC recipient mice is in accord with a previous report demonstrating IL-1Ra expressing populations of murine MSC, and the role of MSC-derived IL-1Ra in promoting lung injury repair.[28] Given the absence of a comparable effect seen after treatment with immunosuppression,[11] however, it is unlikely that the immunomodulatory capabilities of MSCs can fully explain their impact on corneal epithelial regeneration.

In summary, the present study elucidates the dynamics of endogenous MSC trafficking and MSC-mediated regeneration in cornea. However, further investigation is required to delineate the mechanisms through which MSCs facilitate epithelial regeneration, particularly the relative contribution of paracrine effects versus direct differentiation of MSCs. The demonstration that MSCs home to the injured cornea, survive, and promote corneal epithelial regeneration may have therapeutic implications for corneal injuries affecting the LSC compartment, for which only limited treatment options are available.

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