

# Bone marrow mesenchymal stem cells can differentiate and assume corneal keratocyte phenotype

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## Abstract

It remains elusive as to what bone marrow (BM) cell types infiltrate into injured and/or diseased tissues and subsequently differentiate to assume the phenotype of residential cells, for example, neurons, cardiac myocytes, keratocytes, *etc.*, to repair damaged tissue. Here, we examined the possibility of whether BM cell invasion *via* circulation into uninjured and injured corneas could assume a keratocyte phenotype, using chimeric mice generated by transplantation of enhanced green fluorescent protein (EGFP)<sup>+</sup> BM cells into keratocan null (*Kera*<sup>-/-</sup>) and lumican null (*Lum*<sup>-/-</sup>) mice. EGFP<sup>+</sup> BM cells assumed dendritic cell morphology, but failed to synthesize corneal-specific keratan sulfate proteoglycans, that is KS-lumican and KS-keratocan. In contrast, some EGFP<sup>+</sup> BM cells introduced by intrastromal transplantation assumed keratocyte phenotypes. Furthermore, BM cells were isolated from *Kera-Cre/ZEG* mice, a double transgenic mouse line in which cells expressing keratocan become EGFP<sup>+</sup> due to the synthesis of Cre driven by keratocan promoter. Three days after corneal and conjunctival transplantations of such BM cells into *Kera*<sup>-/-</sup> mice, green keratocan positive cells were found in the cornea, but not in conjunctiva. It is worthy to note that transplanted BM cells were rejected in 4 weeks. MSC isolated from BM were used to examine if BM mesenchymal stem cells (BM-MSC) could assume keratocyte phenotype. When BM-MSC were intrastromal-transplanted into *Kera*<sup>-/-</sup> mice, they survived in the cornea without any immune and inflammatory responses and expressed keratocan in *Kera*<sup>-/-</sup> mice. These observations suggest that corneal intrastromal transplantation of BM-MSC may be an effective treatment regimen for corneal diseases involving dysfunction of keratocytes.

**Keywords:** bone marrow • mesenchymal stem cells • cell therapy • cornea • keratocytes

## Introduction

In the 1970s, circumstantial evidence implicated that BM cells might egress from BM and migrate into injured/diseased tissues; thereafter, further studies suggested that BM cells might contribute to tissue repair [1–3]. These studies imply that injury to a tissue or organ might induce the egression and homing of BM stem cells that are capable of differentiation to assume the phenotype of residential cells and repair damaged tissue [4–12]. Subsequently, numerous studies have suggested that BM-derived stem cells can give rise to various cell phenotypes and transplantation with BM stem cells may be a promising strategy for tissue repair and regeneration. For example, introduction of BM-derived

endothelial and haematopoietic progenitors restores tissue vascularization after ischaemic events in retina, myocardium and adult mouse BM cells participate in the reconstruction of skin [13–17]. However, the hypothesis that BM stem cells can transdifferentiate to other cell types has been questioned. For example, genetically marked BM cells transplanted into the areas of induced muscle degeneration do not undergo myogenic differentiation rather they assume a cardiomyocyte phenotype and function *via* cell fusion, or they actually differentiate to haematopoietic cell lineages instead of cardiomyocytes [18,19]. The variegation may be derived from the fact that there are two major stem cell types in BM, that is MSC and HSC (haematopoietic stem cells). It is very likely that these two stem cell populations may assume different fates when they are transplanted *in vivo*.

There has been a growing interest in the application of MSC transplantation in regenerative medicine to repair and restore the function of diseased and injured tissues [20–24]. One of the most interesting characteristics of MSC is their ability to home into sites of damage or inflammation, when they are transplanted *via*

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intravenous infusion in patients [25]. This extraordinary ability of MSC has been demonstrated in the bone fracture, cerebral ischaemia and the infarcted heart as well [7, 8, 18, 26–28].

Our previous study showed that intrastromal xenograft transplantation of human umbilical MSC cured cloudy and thin corneas of *Lum*<sup>-/-</sup> mice; while transplanted human HSC failed to do so, instead they triggered severe inflammatory and immune responses [29]. This observation prompts us to investigate whether corneal keratocyte dysfunction can be restored by BM cells that egress from BM and home into the diseased cornea. To examine this hypothesis, chimera mice are prepared by transplantation of BM cells isolated from EGFP mice into *Kera*<sup>-/-</sup> or *Lum*<sup>-/-</sup> mice. Our results show that EGFP<sup>+</sup> BM cells appear in naive and/or injured corneas of chimera mice, but do not assume a keratocyte phenotype, whereas a direct transplantation of MSC into diseased corneas is necessary for assuming keratocyte phenotype by transplanted MSC.

## Materials and methods

### Animal welfare

All animal protocols conformed to the Statement of Association for Research in Vision and Ophthalmology for the Use of Animals in Ophthalmic and Vision Research and were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

### Isolation of BM cells

Experimental mice were killed by CO<sub>2</sub> asphyxiation. The femurs of 6- to 8-week-old male EGFP mice expressing EGFP by all cell types were excised, and all connective tissues attached to the bones were removed under a stereomicroscope. Following removal of both ends, a 21-gauge needle fitted to a 5 ml syringe was inserted into marrow cavity and marrow was flushed out with 5 ml of DMEM. Bone marrow cells were collected by centrifugation at 250 × *g* for 5 min. and resuspended in 500 μl of TAC buffer (170 mM Tris base and 160 mM ammonium chloride) for 3 min. at room temperature to lyse erythroid cells. After adding DMEM, BM cells were collected by centrifuging at 250 × *g* for 5 min. and the supernatant was discarded. Finally, the cells were re-suspended with 1 ml PBS. Cell density was determined by hemacytometer, and 10 μl of cell suspension was mixed with an equal volume of 0.4% trypan blue. In another series of experiments, BM cells were isolated from *Kera-Cre/ZEG* mice in which cells expressing keratocan were EGFP<sup>+</sup> due to the synthesis of Cre recombinase driven by the keratocan promoter [30, 31].

### Isolation of BM-MS

Briefly, according to the method of isolation of BM described above, BM cells from 8- to 12-weeks-old wild-type mice were cultured in alpha-MEM supplemented with 10% foetal bovine serum (Invitrogen Corporation, Carlsbad, CA, USA) in an incubator at 37°C and 5% CO<sub>2</sub>. Next day, non-

adherent cells were removed. The medium was changed every 3–4 days. The plastic adherent cells near confluence were harvested by Trypsin/EDTA, subcultured, and used for transplantation after six passages.

### Preparation of chimera mice

EGFP-BM cells ( $1 \times 10^6$  cells) were infused *via* tail vein injection into *Kera*<sup>-/-</sup> and *Lum*<sup>-/-</sup> recipient mice that were  $\gamma$ -irradiated with a total of 1200 rads (12 greys) delivered in two doses of 600 rads (6 greys) 3 hrs apart as previously described [32]. The experimental mice were allowed to recover for at least 2 weeks and then subjected to corneal epithelium debridement.

### Intrastromal transplantation of BM cells and BM-MS

Mice were anaesthetized by intraperitoneal injection of ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg). The eye was rinsed with PBS and topically anaesthetized with a drop of proparacaine. A small corneal intrastromal tunnel was created with a 32-gauge needle.  $2 \times 10^4$  BM cells or  $1 \times 10^4$  MSC in 2 μl of PBS were injected into each cornea with a 33-gauge needle (Hamilton Company, Reno, NV, USA). Afterwards, ophthalmic antibiotic ointment was applied to the eye and recipient animals were examined weekly by fluorescence stereomicroscopy [29].

### Immunofluorescence staining of cryosections and whole mount immunostaining

For conventional immunostaining, cryosections (10 μm thick) were used as described previously [29]. For whole mount immunostaining, corneas were incubated with primary antibody in 0.3% Triton X-100 in PBS overnight, and then washed with 0.02% Triton X-100 in PBS at room temperature and followed by incubation with fluorescence-conjugated secondary antibody in 0.02% Triton X-100 in PBS for 4–6 hrs. Corneas were subsequently washed with 0.02% Triton X-100 in PBS overnight. Finally, the cornea was mounted on a slide with Mowiol medium (Calbiochem, La Jolla, CA, USA) for confocal laser scanning microscopy or fluorescent microscopy.

### Western blot

Individual corneas were cut into small pieces under a stereomicroscope and suspended in 100 μl alkali extraction buffer containing 250 μ/ml Benzonase nuclease (EMD Chemicals, Gibbstown, NJ, USA) and  $1 \times$  protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) in 50 mM Tris (NaOH) pH 12 at 4°C overnight. After clarification by centrifugation, the supernatant was neutralized with 10 mM Tris/HCl (pH 6.0) to pH 7–8 and digested in 0.5 μ/ml Keratanase (SEIKAGAKU CORP., Tokyo, Japan) at 37°C overnight. The protein was separated on a 4–20% SDS-PAGE mini gel and then transferred to an immobilon transfer membrane (Millipore Corp., Bedford, MA, USA). The membrane was subjected to Western blot analysis as described previously [29]. Immune reactivity was visualized with fluorescence imaging (secondary fluorescence conjugates) using Odyssey Infrared Imaging System (Li-COR Biosciences, Lincoln, NE, USA).

## RT-PCR

Briefly, total RNA was isolated from BM cells and cornea using a reagent (Trizol Reagent, Invitrogen Life Technologies, Carlsbad, CA). cDNA was synthesized (AMV Reverse Transcriptase; Promega, Madison WI, USA) and subjected to RT-PCR for detection of keratocan and lumican mRNA with the following primer pairs: mKeraRT-F, 5'-CTAACCTGCAGCACCTTCACCTTGAT, and mKeraRT-R, 5'-GTTCTGCACGCAGTGTGGTGGGAC, for keratocan mRNA; primer mLumRT-F, 5'-ATAGTGGGGTACCTGGAAACTCGT, and mLumRT-R, 5'-CCAGGATCTTACAGAAGCTCTTCA, for lumican mRNA; primer mGAPDH-F, 5'-AAGGTGGTGAAGCAGGCATCTGAG, and mGAPDH-R, 5'-TCTTACTCCTGGAGGCCATGTAG, for GAPDH mRNA as control. PCR was performed as follows: 94°C for 5 min., followed by 34 cycles of 94°C for 30 sec., 65°C for 30 sec. and 72°C for 30 sec., and 72°C for 5 min. PCR products were analysed by electrophoresis in 3% agarose gels.

## Statistical analysis

Data were presented as mean  $\pm$  S.D. Two-side paired or non-paired Student's *t*-test was used to compare the mean values of two sets of data. ANOVA tests were used to analyse the mean values of multiple group data. Differences were considered significant at  $P < 0.05$ .

## Results

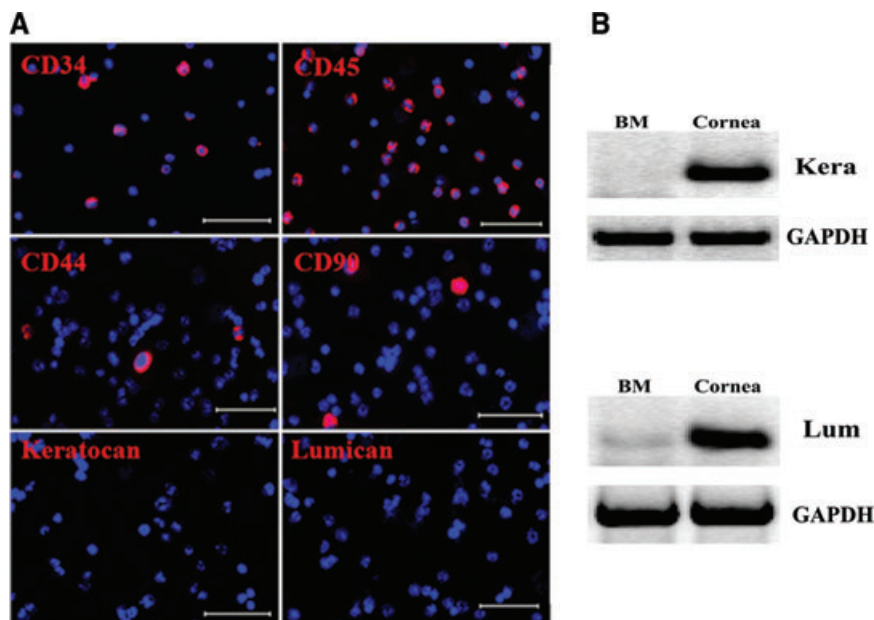
### The characteristics of BM cells

To characterize mouse BM cells, the expression pattern of several HSC and MSC markers was performed by fluorescent immunos-

taining analysis on smear slide before BM cell transplantation. Figure 1 showed representative images of fluorescence staining for CD34, CD45, CD90 and CD44. Table 1 demonstrated that more than 55% of BM cells were CD34<sup>+</sup>, a marker of HSC, and 65% CD45<sup>+</sup>, a marker of leucocytes; meanwhile, only a small number of cells expressed CD44 (2.4%) and CD90 (Thy1, a thymocyte marker) (3.3%), also markers of mesenchymal cell lineage (Fig. 1A). These indicate that cells of haematopoietic lineage are the major cell population used for BM transplantation. To verify the expression of keratocan and lumican by BM cells, immunostaining and RT-PCR were also performed before transplantation. Immunostaining showed a devoid of keratocan expression in BM cells; however, there was an average of three lumican-positive cells in six visual fields. RT-PCR revealed that BM cells lacked keratocan mRNA expression, but displayed a weak lumican band (Fig. 1B) consistent with the observation in which a very small number of lumican positive cells were found by immunofluorescence staining (Fig. 1A and Table 1).

### EGFP<sup>+</sup> BM cells in the cornea of chimeric mice did not assume keratocyte phenotype for the expression of keratocan and lumican

Following BM cell transplantation into sub-lethally irradiated *Kera*<sup>-/-</sup> and *Lum*<sup>-/-</sup> mice through tail vein infusion, a small number of EGFP<sup>+</sup> cells were present in the corneal limbal area within 1 week (Fig. S1A). EGFP<sup>+</sup> cells migrated into the corneal periphery and assumed a dendritic morphology in 2 weeks (Fig. S1B). After 4 weeks, green cells were homogenously distributed in both the periphery and the centre of the cornea and displayed large and dendritic cell morphology (Fig. S1C and D).



**Fig. 1** The characteristics of BM cells. (A) Immunostaining indicated that most BM cells injected expressed CD34 and CD45 (HSC markers), and a few cells were positive to CD90 and CD44; these cells were devoid of the expression of keratocan and lumican. (B) RT-PCR revealed that BM cells lacked keratocan mRNA expression, but displayed a very weak lumican band before transplantation. Scale bar: 50  $\mu$ m; Blue: DAPI.

**Table 1** The distribution of cell types isolated from mouse bone marrow

Marker	% of total nucleated cells (mean ± S.D.)
CD34	55.3 ± 1.0
CD45	63.5 ± 1.7
CD44	2.4 ± 0.2
CD90	3.3 ± 0.3
Lumican	+
Keratocan	-

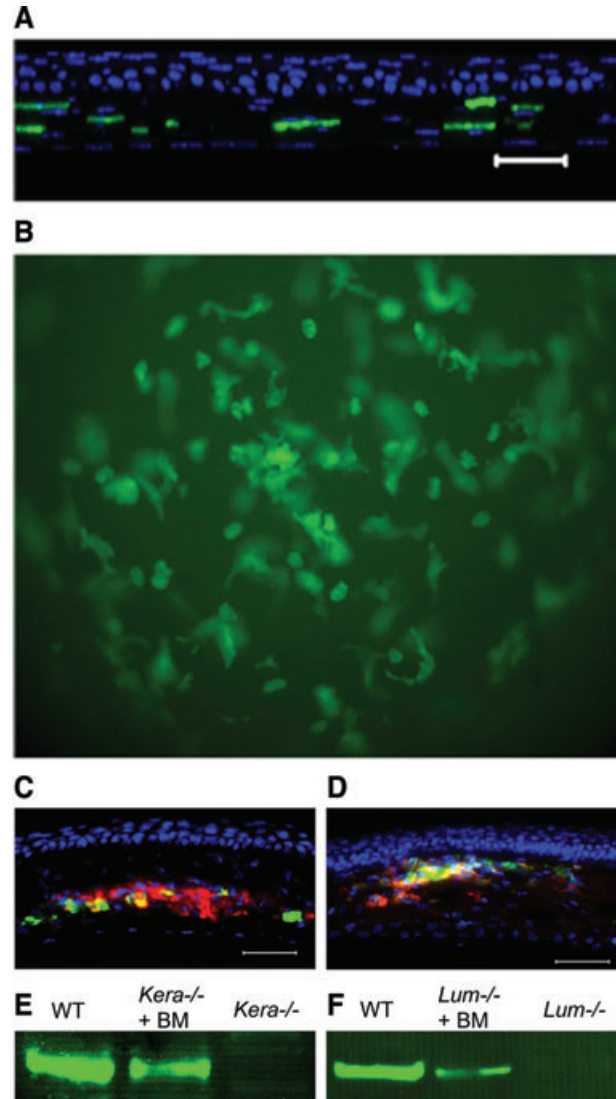
*Note:* More than 55% of BM cells injected expressed haematopoietic stem cell markers, CD34 and CD45 (red), and only few BM cells expressed mesenchymal stem cell markers, CD44 and CD90 (red), before the transplantation.

To determine the expression of keratocan and lumican by BM-derived cells in chimeric *Kera*<sup>-/-</sup> and *Lum*<sup>-/-</sup> mice, immunostaining with antibodies directed against keratocan and lumican was performed on tissue isolated from mice 6 and 12 weeks after BM transplantation. The results indicate that no keratocan or lumican is detected in cornea of chimeric *Kera*<sup>-/-</sup> and *Lum*<sup>-/-</sup> mice, respectively (Fig. S1E and G).

To examine whether transdifferentiation of BM cells may occur in injured cornea of chimeras, a 2 mm central corneal epithelial debridement wound was created using an ALGERBRUSH-II in chimeric *Kera*<sup>-/-</sup> and *Lum*<sup>-/-</sup> mice 6 weeks after BM cell transplantation and allowed to heal for 6 weeks, a time point the transplanted BM would have stably re-established immune system of the experimental animals that had received sub-lethal irradiation. Immunostaining failed to detect the presence of keratocan or lumican in healed corneas (Fig. S1F and H), suggesting that cells that egressed from BM in response to injury did not transdifferentiate and assume a keratocyte phenotype. Although our data revealed that a large number of BM-derived cells appeared in the mouse corneal stroma, we never found any CD90/Thy-1 (also expressed by MSC) positive BM-derived cells in the cornea before or after corneal epithelial debridement (data not shown); in contrast, these green cells expressed CD45 (a pan-leucocyte marker) (data not shown). Thus, we conclude that BM-MSC rarely egress from BM and home into the injured and un-injured cornea. We therefore examined if a direct introduction of BM cells into the corneal stroma is essential to allow for differentiation into a keratocyte phenotype to heal injured/diseased corneas similar to what we have shown with umbilical MSC [29].

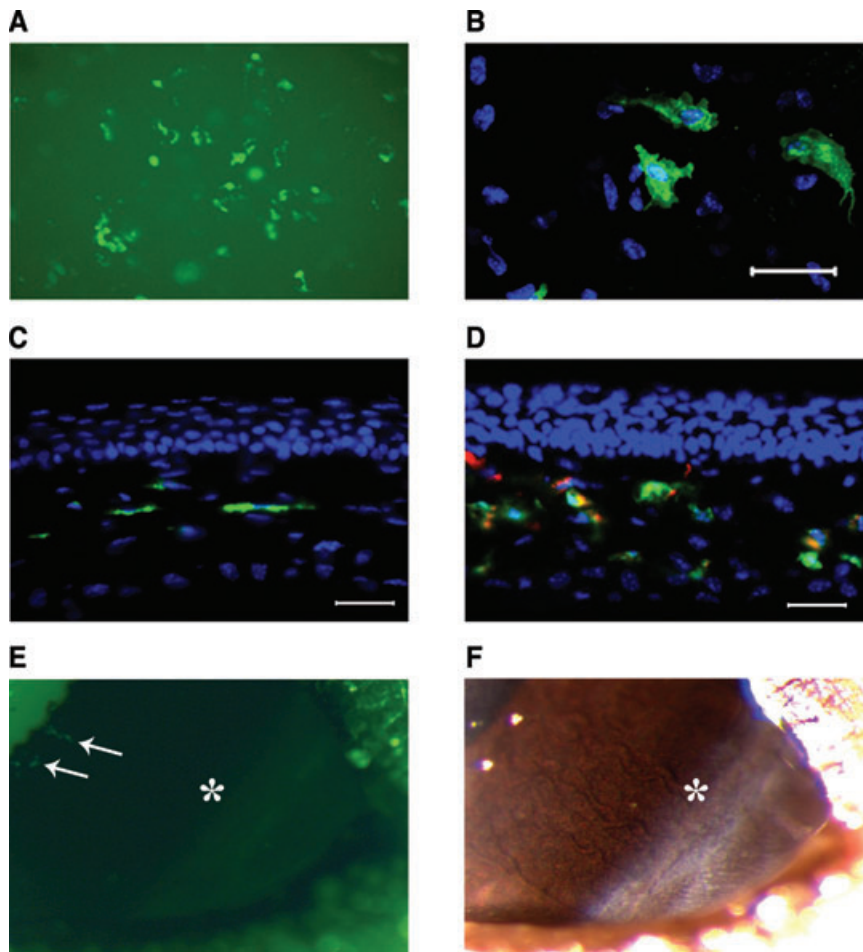
### The environment of corneal stroma induced the synthesis of extracellular matrix by intrastromally transplanted BM cells

Our previous studies have demonstrated that umbilical MSC can differentiate and assume a keratocyte phenotype when they are



**Fig. 2** Green BM-derived cells synthesized keratocan and lumican in the corneal stroma after intrastromal transplantation. (A) Cryosection prepared from eyes after 1 week of corneal intrastromal transplantation with BM cells, green BM cells uniformly distributed in corneal stroma and never migrated into corneal epithelium and anterior chamber. (B) One week after the transplantation, most of BM-derived cells changed their round cell shape to be dendritic under a fluorescent stereomicroscopy (20×). (C, D) Immunostaining on cryosections revealed that corneal stroma of *Kera*<sup>-/-</sup> and *Lum*<sup>-/-</sup> mice respectively, expressed keratocan and lumican (red) surrounding green BM-derived cells. (E, F) Following the treatment with keratanase and/or endo-β-galactosidase, a single 45 kD protein was respectively detected in *Kera*<sup>-/-</sup> or *Lum*<sup>-/-</sup> mouse cornea 1 week after corneal intrastromal transplantation of BM by Western blot with anti-keratocan and lumican antibodies. Scale bar: 50 μm; Blue: DAPI.

transplanted into the corneal stroma [29]. We thereby directly injected BM cells into the cornea of experimental mice to examine if the stromal tissue environment might provide a niche essential

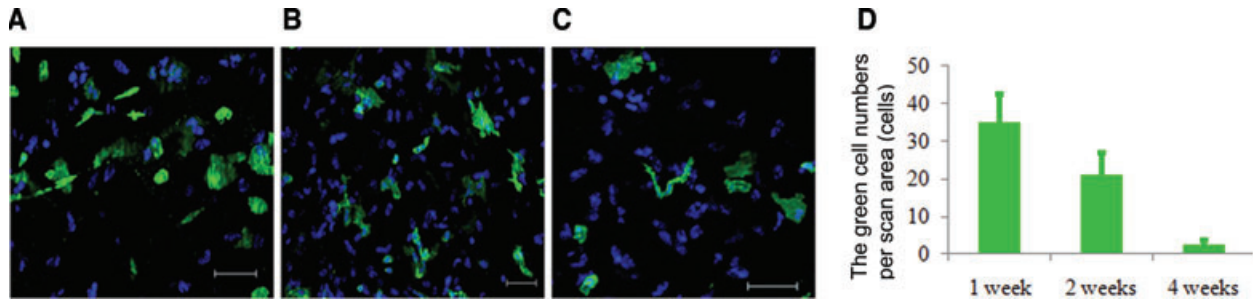


**Fig. 3** *Kera-Cre* transgene of BM-derived cells was turned-on in the stroma of *Kera*<sup>-/-</sup> mouse after intrastromal transplantation. (A) Green cells presented in the cornea of *Kera*<sup>-/-</sup> mouse 1 week after corneal intrastromal transplantation of BM cells isolated from *Kera-Cre/ZEG* mouse (20×, fluorescent stereomicroscopy image). (B) Confocal microscopy displayed that green cells display flat and dendritic cell morphology. (C, D) Fluorescent microscopy images of cryosection revealed that green cells of (A) and (B) were located in corneal stroma (C: control) and green cells expressed keratocan (red). (E) Following transplantation of BM cells from *Kera-Cre/ZEG* mice into the corneal stroma and sub-conjunctiva of *Kera*<sup>-/-</sup> mouse at same time, EGFP<sup>+</sup> cells (white arrows) presented in the cornea other than conjunctiva 1 week after transplantation. (\*: limbal region; 1.6×, fluorescent stereomicroscope image). (F) The same region with image (E) was taken by a stereomicroscope at same time (\*: limbal region, 1.6×). Scale bar: 50 μm; Green: EGFP; Red: Keratocan; Blue: DAPI.

and sufficient for the differentiation of BM cells into keratocyte phenotypes. BM cells isolated from EGFP mice were directly transplanted into the corneal stroma of *Kera*<sup>-/-</sup> or *Lum*<sup>-/-</sup> mice. After intrastromal transplantation, green BM-derived cells uniformly distributed in the corneal stroma and never migrated into the corneal epithelium and anterior chamber (Fig. 2A). One week after transplantation, most of BM-derived cells assumed dendritic cell morphology (Fig. 2B). To determine whether BM cells can differentiate to assume a keratocyte phenotype, keratocan and lumican expression was examined by immunostaining and Western blot analysis in the tissue from *Kera*<sup>-/-</sup> and *Lum*<sup>-/-</sup> mice. Immunostaining results showed that keratocan and lumican were detected around green cells in the corneal stroma of *Kera*<sup>-/-</sup> and *Lum*<sup>-/-</sup> mice, respectively (Fig. 2C and D). Western blot with anti-keratocan and lumican antibodies identified a single 45 kD core protein presented after the treatment with keratanase and/or endo-β-galactosidase in *Kera*<sup>-/-</sup> and *Lum*<sup>-/-</sup> corneas 1 week after transplantation, respectively (Fig. 2E and F); without enzyme treatment, Western blots showed smear immune reactivity (data not shown). These results indicate that some BM cells transplanted

can differentiate into keratocytes and synthesize corneal stroma-unique KS-keratocan and KS-lumican.

To further elucidate that BM can give rise to cells with a keratocyte phenotype, BM cells were isolated from a double transgenic mouse line, *Kera-Cre/ZEG* mice in which cells expressing keratocan become EGFP<sup>+</sup> due to the excision of *LacZ* gene from the dual reporter *ZEG* gene by Cre-recombinase driven by the keratocan promoter [30, 31], and intrastromally transplanted into *Kera*<sup>-/-</sup> mice. Before transplantation, a smear of BM cells from a *Kera-Cre/ZEG* mouse was examined and were negative for EGFP expression. BM cells from *Kera-Cre/ZEG* mice were intrastromally transplanted into *Kera*<sup>-/-</sup> mouse. Interestingly, EGFP<sup>+</sup> cells appeared in the corneal stroma 72 hrs after transplantation (Fig. 3A and B), before assuming a dendritic morphology. The observation suggests that expression of keratocyte unique gene, keratocan, precede morphological change that might require the coordinated expression of many more genes. Under confocal microscopy, the EGFP<sup>+</sup> cells displayed a flat and dendritic cell morphology (Fig. 3B); immunostaining further demonstrated that these green cells expressed keratocan (Fig. 3D). These results suggest that the



**Fig. 4** The numbers of BM-derived cells decreased with time extension after the transplantation. **(A)** One week after the injection, a large number of BM-derived cells were distributed in corneal stroma and displayed a variety of cell morphology. **(B)** Two weeks after the transplantation, green cells displayed dendritic cell shape in corneal stroma; however, the numbers of BM-derived cells obviously decreased. **(C)** Four weeks after intrastromal injection, only several green cells continuously stayed in the cornea. **(D)** The number of BM-derived cells was determined by counting green cells in serial Z-stack images and the results revealed that the number of transplanted green cells rapidly decreased and almost all green cells were exhausted 4 weeks after the transplantation.  $P < 0.001$ ; scale bar: 50  $\mu\text{m}$ ; Green: EGFP; Blue: DAPI.

Cre gene was turned on in BM-cells derived from *Kera-Cre/ZEG* mouse in the corneal stroma; implying certain BM cell types are capable of assuming keratocyte phenotype. In contrast, when BM cells from *Kera-Cre/ZEG* mice were transplanted into sub-conjunctival stroma of *Kera<sup>-/-</sup>* mouse concurrent with corneal intrastromal transplantation, EGFP<sup>+</sup> cells were found in cornea but not in conjunctiva from 72 hrs to 2 weeks after transplantation (Fig. 3E and F). This indicates that corneal stromal environment provides the niche for differentiation of BM cells into the keratocyte phenotype.

### Transplanted BM cells were rejected by recipient

To elucidate whether intrastromal transplantation of allogeneic BM cells can be used to heal corneal diseases, a crucial requirement is that BM cells can survive and function in an allogeneic cornea. To address this question, excised *Kera<sup>-/-</sup>* mouse cornea that had been treated with EGFP<sup>+</sup> BM cell transplantation were stained with DAPI and scanned by a ZEISS Observer.Z1 fluorescent microscope with ApoTome Mode and the number of transplanted cells was determined by counting green cells in serial Z-stack images. The results revealed that the number of transplanted green cells rapidly decreased and almost all green cells vanished within 4 weeks after transplantation (Fig. 4).

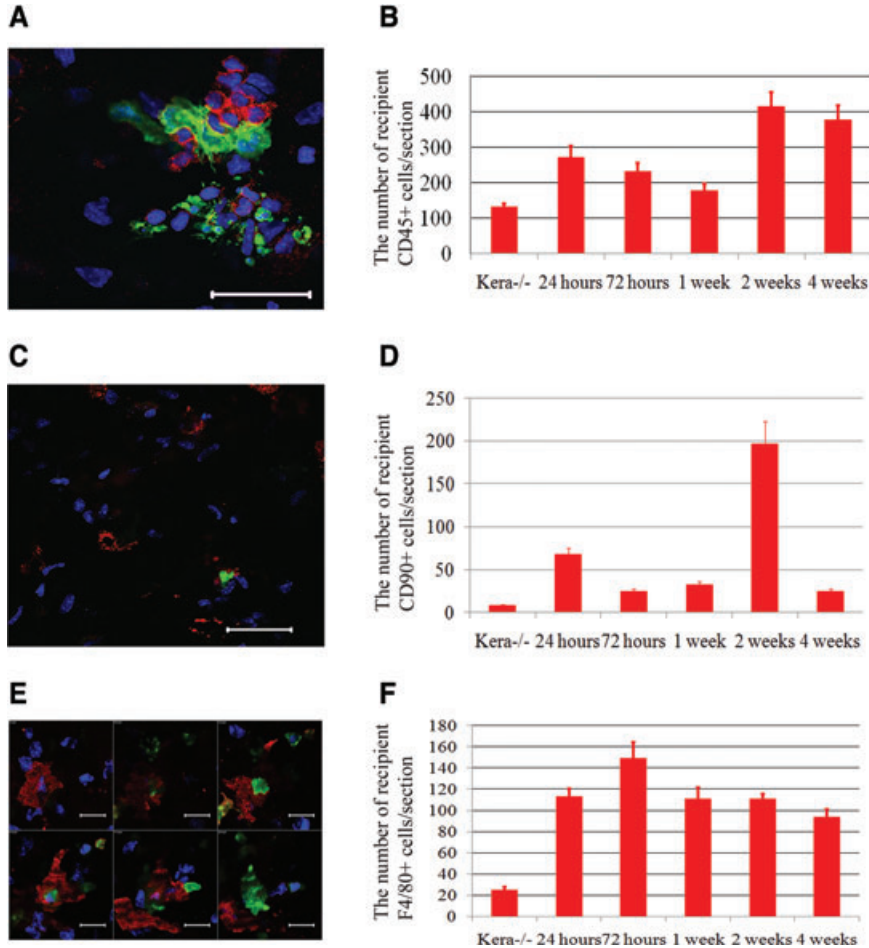
The decrease of transplanted cells might be due to host immune rejection, because the recipient mice were not lethally irradiated prior to intrastromal transplantation and host immune system remained intact. To further elucidate that the disappearance of transplanted BM cells were caused by host inflammation and immune rejection response, immunostaining was performed at different time points after transplantation of BM cells into *Kera<sup>-/-</sup>* mouse cornea. Results of immunostaining with anti-CD45 antibody, a pan-leucocyte marker, showed that a large number of recipient CD45<sup>+</sup> leucocytes invaded the corneal stroma, which surrounded and engulfed green cells 1 week after the trans-

plantation (Fig. 5A). It appeared that differentiation of BM cells to assuming a keratocyte phenotype might occur concurrently with the invasion of inflammatory cells. However, the reason for such coincidence remains unknown. Afterwards, many transplanted BM cells were phagocytosed by host CD45<sup>+</sup> cells; 4 weeks after the transplantation, numerous fragments of green cell debris remained in the corneal stroma. Figure 5B summarizes the leucocyte density at different time points; the results indicate that there are more CD45<sup>+</sup> cells at every time point after transplantation compared to uninjured *Kera<sup>-/-</sup>* mouse corneas.

T cells and macrophages are known to play pivotal roles in graft rejection. To detect the presence of T cells and macrophages, anti-CD90 (Thy-1) and anti-F4/80 antibodies were used to detect the presence of pan-T cells and macrophages after transplantation. The results revealed that the number of pan-T cells and macrophages were significantly increased at different time points after transplantation (Fig. 5C–F). This is concurrent with the time period of graft rejection.

### MSC isolated from BM assume keratocyte phenotypes and suppress host immune and inflammatory responses

We previously demonstrated that the xenograft transplantation of human umbilical HSC into mouse corneas triggered immense immune rejection response. In contrast, transplantation of umbilical MSC survived and healed cloudy and thin corneas of *Lum<sup>-/-</sup>* mice [29]. Thus, the synthesis of KS-keratocan and KS-lumican following direct BM cell transplantation is likely derived from cells of mesenchymal stem cell lineage rather than haematopoietic stem cell lineage. We therefore hypothesize that transplantation of BM-MSK may be used as treatment regimens for congenital corneal disease caused by genetic mutation. To examine the hypothesis, MSC were isolated from BM of wild type mice as described in Materials and methods. These plastic adherent cells



**Fig. 5** BM cell transplantation induced the infiltration of immune and inflammatory cells into corneal stroma. **(A)** Confocal image revealed that CD45<sup>+</sup> leucocytes (red) invaded the corneal stroma of *Kera*<sup>-/-</sup> mouse to surround and engulf green cells in one week after the transplantation. **(B)** A large number of recipient leucocytes (CD45<sup>+</sup>) continuously invaded corneal stroma up to 4 weeks of transplantation. **(C and D)** The numbers of T cells (red CD90/Thy-1<sup>+</sup> shown in C) significantly increased and peaked at 2 weeks after the transplantation and then decreased to a lower level in 4 weeks of transplantation. **(E)** A serial confocal images verified that green cells were phagocytosed by recipient macrophages (red) in the corneal stroma of *Kera*<sup>-/-</sup> mouse. **(F)** There was always a large number of recipient macrophage infiltrations in the corneal stroma after the transplantation in comparison to normal cornea. *P* < 0.001; scale bar: 20 μm; Green: EGFP; Red: F4/80; Blue: DAPI.

were CD90<sup>+</sup>, CD44<sup>+</sup>, CD34<sup>-</sup>, CD45<sup>-</sup> and keratocan negative prior to transplantation (Fig. 6A).

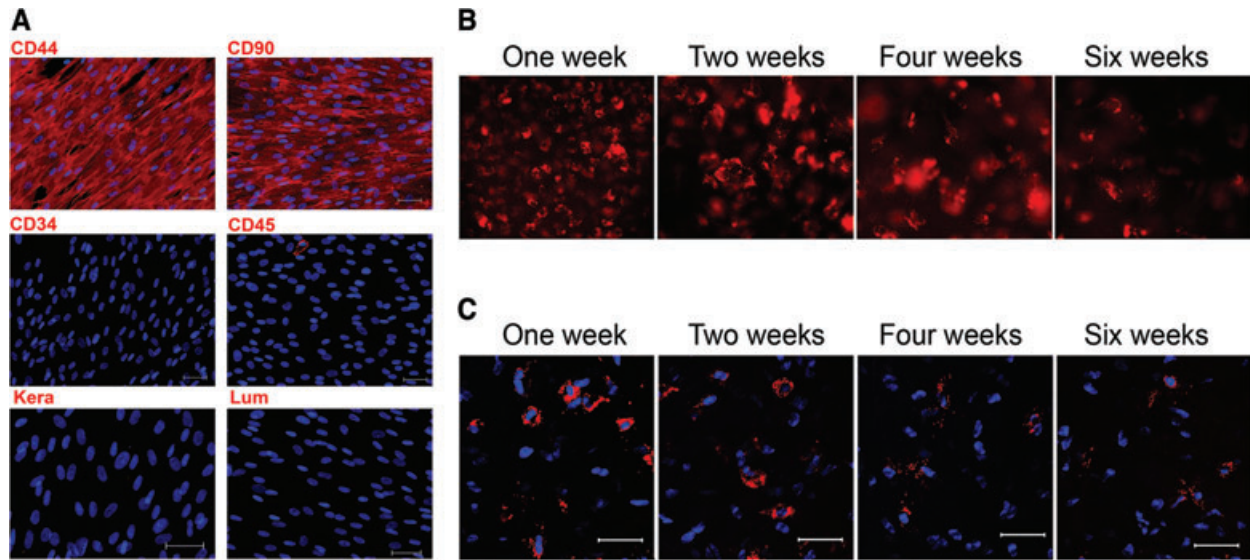
For tracing the morphological changes of BM-derived MSC in the corneal stroma, MSC were *ex vivo* labelled with Dil (red fluorescence) prior to transplantation and examined by fluorescent stereomicroscopy. Following the transplantation of Dil-labelled MSC, most of MSC initially stayed near the site of injection and then gradually migrated to the corneal periphery. Morphologically, MSC began to display dendritic morphology within 1–2 weeks after transplantation. Four and six weeks later, transplanted MSC were homogeneously distributed in the entire cornea as revealed by fluorescent stereomicroscopy (Fig. 6B). The morphological change was better illustrated by confocal microscopy with the whole mount *Kera*<sup>-/-</sup> mouse cornea (Fig. 6C) and also the intensity of dye decreased, suggesting that BM-MSCs might undergo cell division after transplantation, same as what had been shown in the transplantation of umbilical MSC into the mouse cornea [29].

To verify that BM-MSCs could indeed assume a keratocyte phenotype, the synthesis of KS-keratocan was determined by

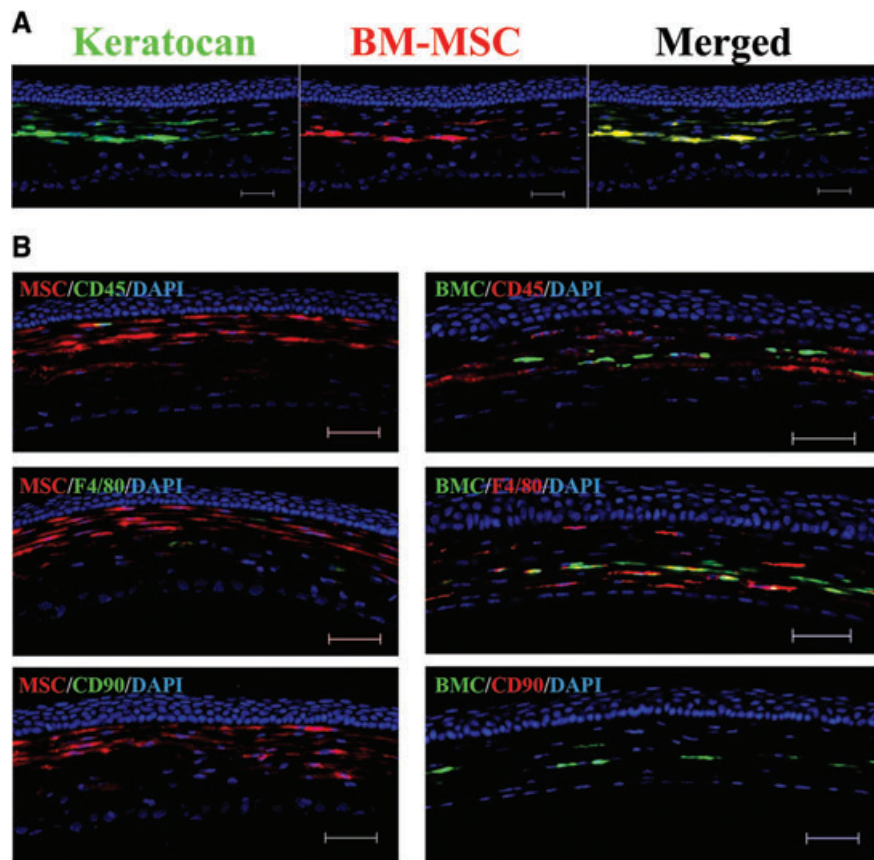
immunostaining with anti-human keratocan antibody in *Kera*<sup>-/-</sup> mouse cornea. Results displayed that BM-MSCs expressed keratocan in 3 days after transplantation as shown in Figure 7A. Whether transplanted cells can be used for cell therapy, a critical criterion is that the cells escape from the host immune rejection. To address this point, we did immunostaining on cryosections at different time-points following BM-MSCs transplantation. Compared to BM-transplanted *Kera*<sup>-/-</sup> mouse cornea, there is very few inflammatory cell infiltration 1 week after BM-MSCs transplantation (Fig. 7B).

## Discussion

In this study, we have demonstrated that EGFP<sup>+</sup> BM-derived cells of chimeric mice generated by BM transplantation *via* tail vein infusion can egress and migrate into injured and un-injured cornea, where they display dendritic cell morphology but failed to synthesize corneal-specific keratan sulfate proteoglycans, that is



**Fig. 6** BM-MSC assumed the phenotypes of keratocytes after corneal intrastromal transplantation. **(A)** The plastic adherent cells isolated from bone marrow were positive to CD44 and CD90/Thy-1, and negative to CD34 and CD45, and also did not express keratocan and lumican before the transplantation. **(B)** The images of *in vivo* fluorescent stereomicroscopy showed that, DiI-labelled BM-MSC (red) displayed a round-like cell shape at the first week after the transplantation; later, the cells extended cell body to be dendritic cells (magnification, 20 $\times$ ). **(C)** Confocal microscopy revealed that BM-MSC-derived cells displayed dendritic cell characters and the density of dye labelling became lower with the time points. Scale bar: 50  $\mu$ m; Red: antibody; Blue: DAPI.



**Fig. 7** Transplanted BM-MSC synthesized keratocan in *Kera*<sup>-/-</sup> mouse cornea without an obvious infiltration of immune and inflammatory cells. **(A)** Immunostaining with anti-keratocan antibody displayed that keratocan (green) distributed surrounding transplanted BM-MSC (red) in the corneal stroma of *Kera*<sup>-/-</sup> mouse. **(B)** Compared to BM-transplanted *Kera*<sup>-/-</sup> mouse cornea, there was an obvious increase in inflammatory cell infiltration after BM cell transplantation, but very few inflammatory cell infiltration after BM-MSC transplantation at same stage. Scale bar: 50  $\mu$ m; Green: Keratocan; Red: BM-MSC; Blue: DAPI.



KS-lumican and KS-keratocan. In contrast, some BM cells that are directly transplanted into the corneal stroma assume a keratocyte phenotype, as evidenced by dendritic cell morphology, synthesis of corneal stromal-specific proteins, and expression of *Kera-Cre* transgene following BM cell transplantation. Although these results demonstrate a potential for cell therapy with direct transplantation of BM cells, subsequent inflammatory and immune response eventually leading to the elimination of implanted cells preclude the possibility of a direct allogeneic BM cell transplantation as a treatment regimen for corneal disease. It should be noted, however, that autologous transplantation of BM cells would ameliorate corneal diseases because this type of autograft would likely not trigger immune rejection.

There is evidence showing that BM cells can migrate into tissues under normal conditions, such in skin, brain, liver and cornea [17, 33–36]. These circulating cells can home into damaged tissues and are involved in tissue defence and inflammatory response [17, 37, 38]. In addition, BM-derived cells not only contribute to inflammatory response, but also give rise to tissue-specific cell phenotypes and participate in tissue regeneration and restoration of function. For example, Eglitis and Mezey show that BM-derived cells express the astroglial marker, glial fibrillary acidic protein in brain, and Theise *et al.* find that BM cells could transdifferentiate and replenish a large number of hepatic parenchymal cells in humans [33, 34]. Although cornea is devoid of blood vessels, studies have demonstrated the presence of lymphoid and myeloid cell types, for example, circulating BM-derived CD45<sup>+</sup> leucocytes belonging to haematopoietic cell lineage in corneal stroma besides keratocytes. The observation shows that these cells display a dendritic morphology and about half of them co-expressed F4/80 (a macrophages marker) [35, 36]. However, the normal cornea does not contain T cells, as evidenced by the absence of CD90<sup>+</sup> cells in the stroma.

BM contains two major stem cell populations, that is HSC and MSC. The latter is multi-potent and capable of differentiating into various progenitor cells of connective tissues and these fibroblast-like cells are defined by multiple markers (CD90<sup>+</sup>, CD44<sup>+</sup>, CD73<sup>+</sup>, CD105<sup>+</sup>, CD45<sup>-</sup>, CD34<sup>-</sup>, CD14<sup>-</sup>) and only represent a minor cell population in BM, representing about 0.001–0.01% of the nucleated cells [4, 39–47]. Our previous study demonstrates that umbilical MSC transplanted into the corneal stroma of *Kera*<sup>-/-</sup> and *Lum*<sup>-/-</sup> mice can assume a keratocyte phenotype [29]. In this study, although BM cells show dendritic cell morphology after transplantation *via* tail vein infusion, they never express keratocan and lumican in both un-injured and injured corneas of chimeric mice. In contrast, after direct transplantation of BM cells into the corneal stroma, some BM-derived cells display the characteristics of keratocytes, namely dendritic cell morphology and the synthesis of KS-keratocan and KS-lumican. Taken together, our results suggest that BM-derived MSC do not normally egress and home into the corneal stroma under normal circumstances or upon injury. As mentioned earlier, there is circumstantial evidence that BM-derived cells may repair the injured tissue; however, our observation implies that the capacity of

spontaneous repair *via* egression of BM-MSc may be minimal in respect to the injured cornea. Pluripotency of MSC has attracted great interest for stem cell therapy. However, whether transplanted cells can be used for cell therapy, a critical issue is that the cells must escape from the host immune rejection and survive in the tissue. Our previous study demonstrates that human umbilical MSC transplanted into the corneal stroma of *Kera*<sup>-/-</sup> and *Lum*<sup>-/-</sup> mice can survive for more than 3 months with little or no noticeable graft rejection; whereas transplanted HSC trigger severe host inflammatory and immune rejection responses [29]. When un-fractionated BM cells are transplanted, those cells that differentiate and assume a keratocyte phenotype are consumed due to host immune rejection triggered by the presence of haematopoietic cells present in the allogeneic transplantation. In contrast, transplanted allogeneic MSC survive in the cornea up to 6 weeks without triggering noticeable inflammation and host immune rejection response. Coincidentally, our recent data from another experiment transplanting human umbilical MSC into an alkali-burned mouse cornea show that MSC transplantation can significantly suppress the infiltration of inflammatory cells (our unpublished observation). Although several *in vitro* studies have demonstrated that cellular and molecular mechanisms by which MSC suppress the host immune and inflammatory response benefited from the expression of TNF- $\alpha$  stimulated genes (TSG6 and TSG14) [48, 49], iNOS (inducible nitric oxide synthetase) [50], IDO (Indoleamine-pyrrole 2,3-dioxygenase) [51] by transplanted MSC. Our *in vivo* study has showed that MSC expressed TSG14 (PTX3) in alkali-burned mouse cornea 72 hrs after the transplantation. Therefore, this may explain why very few inflammatory cells are presented in the corneas of *Kera*<sup>-/-</sup> mice after MSC transplantation as seen in this study (Fig. 7). Based on our finding, these long-term survived BM-MSc might continuously secrete keratocan to meet the requirement of corneal stroma; thus, transplantation of BM-MSc could be an effective treatment regimens for corneal diseases. Moreover, it should also be noted that autologous BM cell transplantation may be a potential alternative treatment regimen for severely traumatized corneas, for example chemical burns, which frequently lead to limbal stem cell deficiency because of persistent inflammation.

In conclusion, we demonstrate that BM-derived haematopoietic cells can home into naive un-injured and injured corneas *via* circulation, but these cells cannot give rise to keratocytes using chimeric mice *via* tail vein transplantation of EGFP<sup>+</sup> bone marrow cells. However, direct-introduction of BM cells into the corneal stroma is rejected soon after transplantation albeit some BM-derived cells assume a keratocyte phenotype, for example synthesis KS-keratocan and KS-lumican. In comparison, BM-derived MSC can survive in the corneal stroma up to 6 weeks and assume a keratocyte phenotype after intrastromal transplantation. This study suggests that allogeneic corneal intrastromal transplantation of BM-MSc may be an effective treatment regimen for the treatment of corneal diseases involving dysfunction of keratocytes, which is similar to that of umbilical mesenchymal stem cell transplantation into lumican null mice [29]. It should be noted that

autograft of BM cells may also be feasible to treat traumatized corneas, for example chemical burns.

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## Conflict of interest

The authors indicate no potential conflicts of interest.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1** Green BM-derived cells displayed dendritic cells morphology in corneas of chimeras after tail vein transplantation of EGFP<sup>+</sup> BM cells, but failed to synthesize corneal unique keratan sulfate proteoglycans, that is KS-lumican and KS-keratocan.

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