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A novel transgenic mouse model for corneal scar visualization

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Abstract

Corneal opacities affect vision for millions of individuals worldwide. Fibrotic scar tissues accumulate in reaction to inflammatory responses and remain permanently in corneal stroma, and conventionally correctable only by donor corneal transplantation. Numerous studies have explored innovative approaches to reverse corneal scarring through non-surgical means; however, existing mouse models limit these studies, due to the lack of visibility of scar tissue in mouse corneas with steep curvature. Here, we reported that corneal scarring was modelled using a transgenic mouse line, Tg(Col3a1-EGFP)DJ124Gsat, in which enhanced green fluorescence protein (EGFP) reporter expression was driven by the promoter of collagen 3a1 (*COL3A1*), a stromal fibrosis gene. Similar to wildtype, Col3a1-EGFP transgenic corneas developed opacities after wounding by alkali burn and mechanical ablation, respectively, as examined under stereomicroscopy and Spectral Domain optical coherent tomography. The time course induction of EGFP was aligned with *Col3a1* upregulation and matched with the elevated expression of other fibrosis genes (α -smooth muscle actin, fibronectin and tenascin C). Measured by flow cytometry and enzyme-linked immunosorbent assay, increased number of EGFP expressing cells and fluorescent intensities were correlated to corneal thickening and scar volume. After treatment with human corneal stromal stem cells or their exosomes, EGFP expression was downregulated together with the reduction of scar volume and fibrosis gene expression. These results have demonstrated that the transgenic mouse line, Tg(Col3a1-EGFP)DJ124Gsat, can be a valuable tool for the detection of corneal fibrosis and scarring *in vivo*, and will be useful in monitoring the changes of corneal fibrosis over time.

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Author contributions

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Keywords

Cornea scarring; fibrosis; collagen 3a1; transgenic; enhanced green fluorescence protein; stromal cell therapy

1. Introduction

Loss of vision affects over 2.2 billion people worldwide and over 1 billion cases are preventable or treatable (World Report on Vision, World Health Organization, 2019). Corneal opacification due to the accumulation of fibrotic scar tissue as a result of infection or injury is a leading cause of corneal blindness (Fuest et al. 2016; Stern et al. 2018). The standard treatment for persistent scar tissues is full- or partial-thickness corneal transplantation of donor tissues. Despite remarkable advances in surgical techniques over the past decade, there are limitations hindering its long-term success, including the global restriction of transplantable donor materials, risks of allogenic graft rejection, limited graft survival, and the side-effects of long-term use of immunosuppressants. Hence, there is a compelling need to develop new strategies to address these problems. Cell-based regenerative therapy, using corneal stromal stem cells (CSSC) and stromal keratocytes, has shown success at clearing the scarred stromal tissue and restoring the corneal clarity, in animal models (Du et al. 2009; Basu et al. 2014; Shojaati et al. 2018; Yam et al. 2018; Ghoubay et al. 2020). In an ongoing clinical trial using donor-derived CSSC to treat existing corneal scars from acute burns, non-healing ulcer, and chronic post-keratitis scars, the clinical outcome at 2-year post-treatment showed improved best-corrected visual acuity and clear corneas, irrespective to the original indication (Basu et al. 2019). More recently, CSSC-derived extracellular vesicles (EVs), or exosomes, when applied to a mouse model of anterior stromal wound, reduced corneal inflammation and blocked the scar formation, in a manner similar to that of intact CSSC (Shojaati et al. 2019). The effect of such treatment could be related to specific microRNAs transported inside the vesicles, and the mechanism is currently investigated. In addition, combining CSSC with bioengineered products offers potential for tissue and organ repair. When CSSC were grown on substrates with aligned microgrooves, they formed a scaffold-free corneal stroma-like tissue and became transparent on murine corneal stroma (Syed-Picard et al. 2018). CSSC on polyglycolic acid fibrous scaffold or on silk substrates produced well-organized multilamellar stromal-like tissues, and could be developed for corneal stromal repair (Wu et al. 2012; Wu et al. 2013; Ghezzi et al. 2017). Similarly, human stromal keratocytes bio-printed in collagen-based bioinks as a 3D biomimetic construct showed the mechanical strength and transparency similar to native stroma and the laden cells maintained keratocyte phenotypes with collagen deposition (Duarte Campos et al. 2019). Hence, more innovative approaches are looming and we need appropriate *in vivo* corneal wound models in order to validate their treatment efficacy of single or even combined therapies, before designing their clinical applications.

Attempts to understand the pathogenesis of corneal scarring and other fibrotic corneal disorders have relied on animal models (Trinkaus-Randall et al. 1991; Cowell et al. 1999; Chan and Werb 2015). One unifying factor of many studies on stromal fibrosis is the role played by type III collagen (Col3), a major fibrillar collagen consisting of three identical α

chains. This molecule is found to be widely associated with tissue defects, ranging from its reduction in dermal and vascular fragility (D'Hondt et al. 2018) to an excessive production in scar deposits and fibrotic tissues. When compared to other collagen types in cornea, Col3 is found in both neonate corneas and scar tissue from adult corneas (Ahmadi and Jakobiec 2002). In wounded corneas, *COL3A1* mRNA expression is elevated in both stromal fibroblasts and myofibroblasts, and Col3 protein is significantly deposited in the healing stromal matrix and scar tissues, such as in keratoconus and host-graft junction (Newsome et al. 1981; Karamichos et al. 2010). Numerous studies have been carried out to elucidate the role of this important molecule in corneal fibrosis, but all are hindered by the difficulties in visualizing the fibrotic tissue accumulation and scar development *in vivo*, particularly in mouse corneas with steep curvature.

Herein, we report an existing Swiss Webster mouse line, Tg(Col3a1-EGFP)DJ124Gsat, with a genotype containing multiple copies of a modified BAC (bacterial artificial chromosome) in which enhanced green fluorescent protein (EGFP) reporter expression is driven by *COL3A1* promoter, i.e. inserted between the promoter and the first exon of *COL3A1* gene. The heterozygous mice express EGFP-tagged Col3a1, enabling *in vivo* real-time visualization of *COL3A1* expression along with corneal fibrosis and scarring after injuries. Also, following the application of CSSC and exosomes on corneal wounds, we were able to detect the downregulated expression of *COL3A1* and other fibrosis genes, together with a resolution of visible scars and EGFP fluorescence. Our results have demonstrated that Col3a1-EGFP transgenic mice can be a valuable tool for studying the pathophysiology and development of corneal fibrosis and scarring, as well as for the evaluation of potential treatments.

2. Materials and Methods

2.1. Transgenic mice

This study was carried out in strict accordance with the guidelines for Care and Use of Laboratory Animals of NIH and The Association for Research in Vision and Ophthalmology Statement for Use of Animals in Ophthalmic and Vision Research. The protocol was approved by the Institutional Animal Care and Use Committee of University of Pittsburgh (Protocol 18022511). The transgenic mouse strain, Tg(Col3a1-EGFP)DJ124Gsat, RRID:MMRRC_010616-UCD, was obtained from the Mutant Mouse Resource and Research Center (MMRRC), a NCRR-NIH funded strain repository, and was donated to the MMRRC by the NINDS-funded GENSAT BAC transgenic project (<https://www.mmrrc.org/about/generalInfoBkgnd.php>). Mice recovered from a cryo-archive had health surveillance performed on recipient females. The genotype was modified to contain multiple copies of a modified BAC (clone RP23-324D9) in which EGFP reporter gene was inserted immediately upstream of the coding sequence of *COL3A1* (Fig. 1A). The construct was injected into pronuclei of FVB/N fertilized oocytes. Hemizygous progeny were mated to Swiss Webster mice for each generation thereafter. Mice were housed in an AALAC-approved ABSL2 facility with a 12-h light/12-h dark cycle, and provided an unrestricted standard diet. All procedures were ensured to minimize pain and suffering of animal subjects. Both genders with correct genotype were used in experiments.

2.2. Genotyping

At postnatal day 14–17, ear punch biopsy was obtained for the extraction of genomic DNA using REDEExtract-N-Amp™ Tissue PCR kit (Sigma-Aldrich, St Louis, MO, US). PCR was performed using the primer pair to specifically amplify Col3a1-EGFP transgene (sense: 5'-ctg agc tgc ttc ttc ctc tct cc-3' and antisense: 5'-tag cgg ctg aag cac tgc a-3'), under the condition of 94°C for 30 sec, 60°C for 30 sec, and 72°C for 1 min (35 cycles) with a final extension at 72°C for 10 min. After resolving in Criterion TBE polyacrylamide gel electrophoresis (BioRad, Hercules, CA, US), the PCR product ~300 bp indicated the correct Col3a1-EGFP genotype (Fig. 1B).

2.3. Corneal wound models

Mice at 7 to 8 weeks of age were anesthetized with intraperitoneal injection of ketamine (50 mg/kg) and xylazine (5 mg/kg). The eyes received topical proparacaine hydrochloride (0.5%, Alcaine®, Alcon, Fort Worth, TX, US) for local analgesia. After saline rinses, the eyes were subjected to corneal injury.

2.3.1. Anterior stromal ablation (Supplementary Fig. 1A): Removal of central corneal epithelium (2 mm diameter) was done by high speed rotation of AlgerBrush II (Accutome Inc, Malvern, PA, US) and completed by scraping using a surgical blade #15. The basement membrane and anterior stromal layers were then removed by a second application of AlgerBrush (Boote et al. 2012; Hertsberg et al. 2017). After an extensive rinse with normal saline, the ablated stromal surface was briefly dried with sterile cotton spears before topical proparacaine for analgesia.

2.3.2. Alkali burn (Supplementary Fig. 1B): Filter paper (Whatman no. 3) as a 2 mm diameter of circle was soaked in sterile sodium hydroxide (NaOH, 1 N; Sigma-Aldrich), drip-dried and placed on the mouse central cornea for 30 sec. After extensive rinses with normal saline (20 ml volume), briefly dried, the injured cornea received topical proparacaine for analgesia.

Power analysis from our previous study showed that at least 6 eyes were required for statistical significance in visible scar assay (Basu et al. 2014; Hertsberg et al. 2017).

2.4. Human corneal stromal stem cell (hCSC) culture and application

The research followed the tenets of the Declaration of Helsinki and was approved by the University of Pittsburgh Institutional Review Board and Committee for Oversight of Research and Clinical Training Involving Decedents (CORID protocol #161). Human corneal rims from de-identified donors younger than 70 years old with consent for research use, were obtained from The Center for Organ Recovery and Education (Pittsburgh, PA, US). Tissues were preserved in Optisol GS (Bausch&Lomb Inc., Rochester, NY) and used within 9 days post-enucleation. After the removal of corneal epithelium and endothelium by gentle scraping and rinses, the anterior limbal stroma (1–2 mm wide, 0.5 mm deep) was isolated and cut into small pieces for digestion with collagenase A (0.5 mg/ml, Sigma-Aldrich) for 16 hours at 37°C (Basu et al. 2014). The dissociated cell suspension was passed through a cell strainer (70 µm pore size, Corning, NY, US), and single cells were cultured

with stem cell growth medium (JM-H) (Du et al. 2005) with 2% pooled human serum (Innovative Res, Novi, MI, US) on culture surface coated with FNC (AthenaES, Baltimore, MD). Cells in holoclones were expanded to passage 3 for experiments. The cells expressed markers specific for mesenchymal stem cells (MSC), including CD73, 90 and 105, as demonstrated by flow cytometry (Kumar et al. 2018; Shojaati et al. 2019). To prepare CSSC for application on mouse corneas after wounding, the cells were suspended at a concentration of 10^6 cells/ml, and mixed 1:1 (vol/vol) with ice-cold human fibrinogen (70 µg/ml, Sigma-Aldrich). The cell suspension (1 µl) and thrombin (0.5 µl, 100 U/ml, Sigma-Aldrich) were added to the wound site. After gelation, a second drop of fibrinogen and thrombin was applied to cover the first gel. The eyes received topical gentamicin (0.3%, Genoptic®, USP, Rockville, MD, US).

2.5. Harvest and purification of CSSC-derived extracellular vesicles (EVs) and treatment

Human CSSC at passage 3 were cultured for EV isolation. At confluence, cells were rinsed and replenished with culture medium pre-cleared of particulate materials by centrifugation at 100,000 g for 18 hours. After 3 days, the conditioned media were collected, filtered through 0.22 µm pores, concentrated with Amicon Ultra 100k cutoff ultrafilter at 4,000 g. The EV fraction was precipitated using Total Exosome Purification kit (Thermo Fisher Sci, Waltham, MA US) and the reconstituted suspension was serially centrifuged, first at 10,000 g for 1h, then the supernatant spun at 100,000 g for 3 hours (Shojaati et al. 2019). The pellet was resuspended in PBS and protein concentration was measured by UV absorbance at 260 and 280 nm. Particle size and concentration were determined by Tunable Resistive Pulse Sensing using a qNano Gold instrument (Izon Sci, Medford, MA, US). The EV expression of calnexin and CD63 was checked by western blotting (Shojaati et al. 2019). To treat the injured mouse corneas, 10^{10} EVs per ml (0.5 mg/ml protein) were resuspended in human fibrinogen (70 µg/ml). The EV/fibrinogen mixture (1 µl) and thrombin (0.5 µl, 100 U/ml) were applied to the wound surface. A second drop of fibrinogen and thrombin was applied after gelation to cover the first layer. The eyes received topical gentamicin (0.3%).

2.6. Bone marrow-mesenchymal stem cell (BMSC) culture and intrastromal injection to mouse corneas

Col3a1-EGFP mice aged 6 months were anesthetized with intraperitoneal ketamine and xylazine, and terminated by cervical dislocation. After the whole body soaked in 70% (vol/vol) ethanol for 2 minutes, the claws were dissected at the ankle and carpal joints, and incisions were made at the shoulder and hip joints. The whole skin, muscles, ligaments, tendons and all soft tissues were carefully removed from humeri and femurs. The bones were transferred to sterile PBS supplemented with antibiotics and antimycotics (200 U/ml penicillin, 200 µg/ml streptomycin sulfate and 50 µg/ml Amphoptericin B, Invitrogen) for 10 minutes before being blotted dry with sterile gauze. The bone ends were cut and the bone cavity was inserted with a 23-gauge (G) needle attached to a 5 ml syringe with culture medium, α -Minimal Essential Medium (α MEM, Thermo Fisher Sci) supplemented with 15% foetal bovine serum (FBS, Gibco) (Huang et al. 2015). The marrow was slowly flushed out and the marrow mass was gently dissociated by repeated pipetting to obtain single cell suspension for culture. The culture was washed daily to remove blood cells and

macrophages. Cells at passage 3 were characterized for marker expression of MSC (CD73, CD90, CD105), macrophages (CD11b) and hematopoietic stem cells (CD34).

BMSCs were suspended at 10^4 cells/ μ l PBS for cell injection to corneal stroma. Wildtype mice (n=8) were anesthetized by intraperitoneal ketamine and xylazine and the right eyes were rinsed with normal saline, followed by topical proparacaine hydrochloride for local analgesia. A stromal tunnel was created with a 31-G needle at the anterior corneal region, and BMSC suspension (1 μ l) was injected through a 33-G blunt needle attached to a Hamilton syringe (Hamilton Co., Reno, NV, US) (Yam et al. 2018). Blebs were formed to indicate successful injection and the depth was visualized using a Spectral domain optical coherence tomography (SDOCT, Bioptigen, Durham, CA, US). Four mouse corneas were injected with BMSC and the remaining 4 corneas with PBS injection served as controls. At 5 days post-injection, the mouse corneas were harvested for fluorescence stereomicroscopy (SZX16, Olympus) and histochemistry.

2.7. *In vivo* corneal scanning with fluorescence stereomicroscopy and optical coherence tomography and measurements

At regular time intervals, mice were anesthetized by intraperitoneal ketamine and xylazine injection. Whole cornea was scanned using stereomicroscopy (SZX16) with real-time fluorescence detection using factory-engineered fluorescence filters (excitation at 426–466 nm and emission at 460–500 nm). The cross-sectional corneal structure was examined with SDOCT with a pachymetry scan of central 4 mm diameter cornea. Scanning data were analysed in a masked fashion. Images were processed with NIS Elements software (Nikon, Melville, NY, US).

Central corneal thickness (CCT) was measured as the mean of three measurements taken at the center (0 mm) and at 0.5 mm on either side (Yam et al. 2018). Scar volume analysis was conducted with ImageJ (NIH) and MetaMorph 7.7.3 (Molecular Devices Inc., San Jose, CA, US) (Basu et al. 2014). Threshold images were generated after digitally removing the corneal epithelium from the stroma. Control eyes were used to set the threshold for the calculation of the percentage of scar volume changes in injured corneas.

2.8. *Ex vivo* assessment of corneal EGFP expression

At time intervals post-wounding and treatment, mouse eyes were enucleated and corneal image was obtained using a dissecting microscope with indirect illumination. Fluorescence images were taken with a Nikon Eclipse TE2000-E inverted DIC fluorescence microscope. Images were analyzed for EGFP expressing cells using Adobe Photoshop CC (Adobe, San Jose, CA, US) and ImageJ (NIH) using the automatic cell counting tool after background subtraction and binary image conversion, following the software's instruction. The threshold particle size was set at 3 μ m and circularity at 0.3 (Basu et al. 2014).

2.9. Enzyme-Linked Immunosorbent Assay (ELISA)

Central corneas (n=6 per group) were carefully dissected to isolate from sclera, trimmed into pieces and placed in Tissue Extraction Buffer, supplied from a GFP *in vitro* CatchPoint SimpleStep ELISA kit (Abcam), and disrupted by sonication. The homogenate was

centrifuged and aliquots of clear supernatant were bound by antibody cocktail. After rinses, signals were developed with CatchPoint HRP Development solution and intensities were measured with a multiplate reader set at 530/570/590 nm Excitation/Cut-off/Emission wavelengths. Each sample was analysed in triplicate and EGFP intensity was determined from a standard curve.

2.10. Quantitative polymerase chain reaction (qPCR)

Transgenic mouse corneas with and without injuries (n=6 per group) were collected at time intervals, placed in RLT buffer (Qiagen) and disrupted in MagNA Lyser Green Beads kit (Roche, Indianapolis, IN, US) using a MagNA Lyser Instrument (Roche). The lysate was processed with Qiasredder and RNeasy Miniprep (Qiagen, Germantown, MD, US) for total RNA extraction. RNA was precipitated by ethanol and quantified using NanoDrop One (Thermo Fisher). After reverse transcription using SuperScript III RT-PCR kit (Thermo Fisher) and random hexanucleotide primers, cDNA was assayed for target gene expression with specific target primers (Supplementary Table 1) using SYBR Green Real-Time Master Mix (Life Technologies, Carlsbad, CA, US) in a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, US). Experiments were run in triplicate. The relative RNA abundance was assayed by 2^{-CT} method after normalization with housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18S genes and fold changes were expressed as mean $\pm$ standard deviation (SD).

2.11. Western blotting

The central corneas were excised, transferred to tissue lysis buffer (100 mM Tris-HCl, 50 mM EDTA, 1% NP-40) with freshly added Complete™ protease inhibitor cocktail (Roche) and 1 mM phenylmethylsulfonylfluoride (PMSF, Sigma-Aldrich), and disrupted using MagNA Lyser Green Beads kit (Roche). Soluble proteins were denatured in sodium dodecylsulfate (SDS, 2%, Sigma-Aldrich) and β -mercaptoethanol (1%, Sigma-Aldrich), resolved by 4 to 20% SDS-polyacrylamide gel electrophoresis (SDS-PAGE, BioRad, Hercules, CA), together with Precision Plus Protein Standards (BioRad, #161-0375). After transfer to PVDF-FL membrane (BioRad), the samples were blocked with 5% bovine serum albumin (BSA) in PBS, and incubated with rabbit polyclonal antibody against mouse Col3a1 (Novus Biol., Centennial, CO, US), followed by goat anti-rabbit IRDye-labeled IgG (LI-COR Biosciences, Lincoln, NE, US). Staining signals were detected and digitized using an Odyssey scanner (LI-COR). Experiments were conducted with protein samples from individual corneas.

2.12. Flow cytometry

Transgenic mouse corneas (n=3 per group) were collected at 3 days post-injury and cut into pieces for collagenase A digestion. After passage through a 70 μ m cell strainer and a 40 μ m cell strainer sequentially, single cell suspension was blocked in Fc-Block (BioLegend), then incubated with primary antibodies (PerCP-conjugated CD45, PE-conjugated Ly-6G/Ly6C and BV421-conjugated CD86, Supplementary Table 2) for 30 min on ice in dark. After washes, the cells were fixed with 1% paraformaldehyde (Sigma-Aldrich) before analysis using a FACS Aria III Flow Cytometer (BD Biosciences). Each corneal sample was spiked with 20,000 fluorescent counting beads (CountBright absolute counting beads, Thermo

Fisher Sci) and the absolute number of positively labeled cells was calculated following the manufacturer's instruction.

2.13. Immunohistochemistry

Mouse corneas were fixed in 2% neutral-buffered paraformaldehyde and embedded for cryosectioning at 8 μ m thick. Sections were blocked and permeabilized by 0.5% Triton X-100 (Sigma-Aldrich), 1% BSA and 2% normal goat serum (NGS, Thermo Fisher), followed by incubation with primary antibodies (Supplementary Table 2). The signal was visualized by incubation with AlexaFluor-592-conjugated IgG secondary antibody (Jackson ImmunoRes Lab, West Grove, PA, US). Samples were washed, mounted with Fluormount-G containing 4',6-diamidino-2-phenylindole (DAPI) (Thermo Fisher) and viewed under confocal microscopy (FluoView 1000, Olympus, Center Valley, PA, US), equipped with CellSens Dimension 2.1 imaging software v.2.1 (Olympus).

2.14. Statistical analysis

All experiments were done in triplicate and the animal number was 6 or more in each group. Data were presented as mean $\pm$ SD. Mean value was compared using unpaired two-tailed Student's t-test or ANOVA with a post hoc Bonferroni test using GraphPad Prism 7. Non-parametric comparison was done by Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Col3a1-EGFP fusion protein expression in transgenic mouse corneas after wounding

Transgenic and wildtype mouse corneas were subjected to anterior stromal ablation using Algerbrush. At day 7 post-injury, individual corneas showing different scarring levels were harvested to obtain soluble protein lysates for Col3a1 detection by western blotting. Corneas with intense scarring displayed stronger Col3a1 band at >200 kDa region, when compared to corneas with mild opacities (Fig. 1C). Also, the Col3a1-EGFP fusion protein expressed in the transgenic corneas displayed with a greater molecular mass than Col3a1 protein from wildtype corneas.

3.2. Scarring resolution of wildtype and transgenic corneal stroma after injury

At regular intervals after stromal injury by alkali burn ($n=6$ per time point), wildtype mouse corneas were examined under stereomicroscopy with brightfield illumination (Fig. 2A). Both central and peripheral corneal regions developed opacification. Before injury, the corneas were completely transparent and the underlying iris structures were fully visible. At day 3 post-wounding, minor haze developed and the fine iris details were mildly obscured. By day 7, the corneas became opaque and appeared whitish in color with the visibility of iris and lens being obstructed. At day 14, the opacification was more intense, and corneal neovascularization with blood vessels ingrown from the peri-corneal plexus was observed. All wildtype corneas did not show any fluorescence.

Similar alkali-burn injury was performed on transgenic Col3a1-EGFP mice. This model offers the advantage of visualizing the fibrosis-related *COL3A1* induction through EGFP

expression under *in vivo* fluorescence microscopy. To confirm that the genetic alterations in Col3a1-EGFP mice did not affect the corneal opacification in response to injury, we compared the opacity development with that of the wildtype mice. Under stereomicroscopy with brightfield illumination, the corneas injured by alkali burn in the central 2 mm (diameter) area exhibited visible haze progression at day 7 and 14 and loss of visibility of the underlying structures (iris and lens), similar to that in the wildtype mice (Fig. 2A). At these time points, fluorescence patches derived from the transgene emerged in the central corneal region (Fig. 2A). Such fluorescent dots and clones were not observed in any wildtype corneas. Automatic cell counting using ImageJ showed increased numbers of green cells at various time points post-injury (Fig. 2B). The cell numbers at day 7, 10 and 14 were significantly greater than that of the early injury time point (day 3) and in naïve corneas ($P<0.05$, Mann-Whitney U test).

Serial cross-sectional scanning of mouse corneas was performed using ASOCT (Fig. 2A). Compared to non-injured (naïve) group, alkali-burnt transgenic corneas at day 3 showed greater stromal reflectivity (Fig. 2A). At day 7 and 14, the corneas were thicker with stronger stromal reflection. Similar changes were noted in wildtype corneas. The mean CCT gradually increased after injury and there was no significant difference between wildtype and Col3a1-EGFP mice at all time points (Fig. 2C). Thresholding and quantitative assessment of stromal hyper-reflective pixels in serial sections of central corneal region (determined by the absence of iris structure) allowed scar volume analysis. Increased mean CCT and stromal volume with light scattering were observed in both wildtype and transgenic corneas, obvious from day 7 after alkali burn (Figs. 2C and D). The mean percentage changes of scar volume at day 7 to 14 were significantly greater than that of day 3 and in naïve corneas ($P<0.05$, paired Student's t-test). Hence, Col3a1-EGFP transgenic mice had similar stromal response as wildtype mice after corneal injury.

3.3. Col3a1-EGFP mice displayed *in vivo* corneal fluorescence after injuries

To examine how the transgenic mice respond to different traumatic injuries on corneas, we performed stromal ablation and alkali burn, respectively. In a set of 6 transgenic mice, both types of injury caused visible corneal scarring under brightfield imaging (Fig. 3A), while the naïve corneas remained transparent. Confocal microscopy on flat-mounted samples provided a high-resolution perspective of green cell distribution in wounded corneas (Fig. 3A). Quantification of day-14 corneas showed 768 ± 484 green fluorescence dots after alkali burn and 430 ± 267 dots after ablation wounding. There was no statistically significant difference between injury types. On the other hand, these mice demonstrated no appreciable changes in body weight, mobility, abnormal bleeding during and after injury, light and pain sensitivity, nor in eating and daily behaviors.

3.4. Corneal scarring in Col3a1-EGFP mice

On cryosections, EGFP-expressing cells were detected in the transgenic mouse corneas after stromal ablation or alkali injuries at day 14, but not in naïve corneas (Fig. 3A). Punctate EGFP signals were distributed in the central stroma. Confocal immunofluorescence showed that Col3a1 signals were predominantly colocalized with EGFP. Quantitative EGFP ELISA on corneal lysates showed that both injury types induced significant increase in EGFP

intensities over the non-injured corneas ($P<0.05$) (Fig. 3B). Between them, corneas with alkali burn showed greater but insignificant induction of EGFP intensities ($329\pm34\%$) compared to that after ablation ($229\pm77\%$). Flow cytometry assay of single corneal cell suspensions also demonstrated greater numbers of EGFP expressing cells after both injuries, when compared to non-injured corneas ($P<0.05$) (Fig. 3C). Overall, the wounding significantly induced green fluorescent cells ($15.7\pm3.2\%$ for the ablated corneas and $18.2\pm7.1\%$ for alkali-burn corneas, versus $3.4\pm2.3\%$ for naïve corneas) ($P<0.05$). In corneas collected at day 3 post-alkali burn, flow cytometry detected a significant proportion of cells expressing Ly6G, CD45 and CD86, indicative of neutrophils and monocytes (Fig. 3D). This represented an increase of Ly6G+ cells by 9.8-fold, CD45+ cells by 5.3-fold and CD86+ cells by 1.8-fold in alkali-burnt corneas. The expression of fibrosis-related genes (Col3a1, α SMA and tenascin C, TNC) was also upregulated from day 7 through 14, when experiment was ended (Fig. 3E). By immunofluorescence, the expression of fibronectin, TNC, and α SMA, was detected in the wound region (Supplementary Fig. 2). The staining signals were closely associated with the EGFP-expressing cells. On the other hand, keratocan was expressed in the stromal region adjacent to the wound site, hence not in close association with EGFP-expressing cells.

In order to clarify that Col3a1 expression was not limited to corneal cells of transgenic mouse model, we isolated bone marrow MSC (BMSC), enriched in cell culture and injected into wildtype corneas to examine any changes of Col3a1 expression. After 3 passages to expand and enrich MSC, the cells were characterized for the positive expression of CD73, CD90 and CD105, while CD34 (hematopoietic lineage marker) and CD11b (macrophage marker) were mildly or negligibly expressed (following the minimal criteria of MSC identification by International Society of Cellular Therapy) (Dominici et al. 2006) (Figs. 5A–B). At day 5 after intrastromal injection of 10^4 transgenic BMSCs into wildtype mouse corneas, visible scars were observed and appeared with green fluorescence under fluorescence stereomicroscopy (Fig. 5C). Image of higher magnification showed the scar consisting of a disorganized mixture of cells and stromal matrix. In contrast, PBS-injected corneas had no scar formation.

3.5. Healing responses of Col3a1-EGFP transgenic mouse corneas to stem cell treatments

Using different wounding models on wildtype corneas, we previously showed that treatment with human CSSC and the derived EVs prevented corneal fibrosis and scarring and reduced corneal inflammation (Du et al. 2009; Basu et al. 2014; Hertsberg et al. 2017; Shojati et al. 2019). To determine whether Col3a1-EGFP mice would provide an *in vivo* indicator of regenerative treatment for corneal scarring, we applied human CSSC and EV treatments, respectively, to corneas after ablation wounding. The injured mouse corneas were randomly chosen for CSSC or CSSC-derived EV treatment. After 7 days, the non-treated injured corneas developed stromal scarring with increased EGFP expression, as shown by the dense appearance of green fluorescence dots, whereas naïve corneas had negligible fluorescence (Fig. 4A). Injured corneas treated with CSSCs or with EVs showed reduced fluorescence. The number of EGFP positive cells was significantly decreased in treated corneas, when compared to corneas with wounding only ($P<0.05$) (Fig. 4B). Similarly, the scar volumes as

measured with serial OCT images were also significantly reduced in treated corneas (Fig. 4C). The expression of fibrosis and scar markers, *Col3a1* and α SMA, as well as *EGFP* expression, was significantly reduced after EV treatments, as compared to wound controls (Fig. 4D). Lastly, we did not observe any gender effect on the treatment outcome. Corneas from both male and female transgenic mice exhibited similar healing response, as determined by the reduced EGFP intensity (Fig. 4E).

4. Discussion

In this study, we used an existing mouse line, Tg(Col3a1-EGFP)DJ124Gsat, carrying the Col3a1-EGFP transgene, to provide a real-time visualization of corneal fibrosis development and to confirm its use in our established corneal injury models. When the mouse corneas were wounded either by alkali burn or mechanical ablation, the temporal changes of EGFP expression were aligned with Col3a1 up-regulation, as well as matching with the induction of other fibrosis genes (FN, α SMA and TNC). Our quantitative analyses showed that the amount of EGFP-expressing cells and fluorescent intensities in wounded corneas increased in conjunction with corneal thickening and scar volume changes. When the wounded corneas were treated by either human CSSC or the derived EVs, the EGFP expression was reduced together with fibrosis genes and scar intensities. This transgenic mouse model thus provides a valuable tool for *in vivo* real-time visualization of corneal fibrosis and scarring.

An appropriate animal model with tracking ability offers the opportunity to study the real-time development of fibrosis as well as the interplay of inflammatory responses that drive the pathological fibrotic remodeling in cornea. More importantly, such a model can be useful to compare different injury models, identify dose-severity association, examine treatment effects, and detect putative treatment targets for the validation before human trials. Previous animal studies using environmental and genetic models have generated important insights into the pathophysiology of corneal injuries, inflammation, fibroproliferation and scarring. Utilizing mice with plasminogen knockout has demonstrated the role of plasmin in fibrinolysis during the healing process (Kao et al. 1998). Lumican knockout mice show cloudy corneas with disorganized collagenous matrix characterized by large fibril size and altered fibril spacing (Kao and Liu 2002). In addition, animal models are useful in the therapeutic investigations on corneal fibrosis and scarring. Different studies examined the treatments with recombinant proteins or inhibitors on chemically-injured mouse corneas (Chen et al. 2017; Hertsensberg et al. 2017; Chandler et al. 2019); and cell-based treatments using keratocytes in rat corneas with stromal haze induced by phototherapeutic keratectomy (Yam et al. 2018), application of CSSCs and their EVs, as well as umbilical mesenchymal stem cells to mouse stromal injury models by mechanical ablation, lumican knockout and mucopolysaccharidosis VII transgene, respectively (Du et al. 2009; Liu et al. 2010; Coulson-Thomas et al. 2013; Basu et al. 2014; Shojaati et al. 2018).

Here, the Col3a1-EGFP mouse model offers a real-time visualization of corneal fibrosis development, using our established corneal injury methods of alkali burn and anterior stromal ablation (Du et al. 2009; Boote et al. 2012; Basu et al. 2014; Hertsensberg et al. 2017). We validated that the transgenic mice respond to corneal injuries similar as wildtype. Both strains exhibited corneal haze, starting at day 3 and more intense scarring by day 7

until day 14 post-injury. They showed similar time-course in the loss of corneal clarity, as well as increase of corneal thickness and scar volume. This indicated that the genetic modification of Col3a1-EGFP did not affect the corneal wound healing response. Only the Col3a1-EGFP corneas displayed fluorescence under *in vivo* assessment, flat-mounted fluorescence microscopy and on cryosections. The quantification assays showed that the injured corneas had increased amounts of both EGFP cells and intensity, which were not detectable in naïve corneas. Additionally, the up-regulation of inflammatory cells and fibrosis genes were consistent with our previous findings (Du et al. 2009; Boote et al. 2012; Hertsenbergh et al. 2017; Shojaati et al. 2018). The Col3a1-EGFP induction was not restricted to the corneal cells. Intrastromal injection of BMSCs, obtained from humeri and femurs of transgenic mice, to wildtype corneas also induced green fluorescence as a wound response due to the mechanical creation of stromal tunnel. Hence this transgenic mouse model can be used to study tissue fibrosis and scarring in different organ systems after injury.

In this study, we provided direct and real-time evidence that stem cell-based treatment using human CSSCs or CSSC-derived EVs reduced corneal fibrosis and prevented scar formation. The substantial reduction of EGFP cells in treated corneas was likely attributed to a diminished Col3a1 expression. This was in accordance with the reduced scar volume and fibrosis marker expression. Notably, our results are comparable with the reported studies using wildtype mouse models in regards to stromal regeneration and restoration of corneal clarity (Du et al. 2009; Basu et al. 2014; Shojaati et al. 2019). There was no statistical difference in EGFP intensity changes between male and female mice after corneal injury and cell treatment. There have been some reports of gender differences in relation to corneal epithelial wound healing that could be hormonally regulated (Krishnan et al. 2012; Wang et al. 2012). *In vitro* study using rabbit corneal stromal fibroblasts treated with female sex hormones (17 β -estradiol and progesterone) also showed an inhibition of interleukin-1 β -induced collagen degradation (Zhou et al. 2011). However, these differences were not observed in our experimental paradigm as the cell-based treatment reduced Col3 expression in haze formation instead of resolving the existing collagen fibrils. Further work examining the treatments on established corneal haze/scarring using this transgenic mouse model will provide more understanding on this phenomenon.

A limitation of this study was that the expression of EGFP reporter did not reflect post-transcriptional events regulating the abundance of protein produced from the endogenous *Col3a1* gene. Rather, the EGFP expression reflects more closely the relative transcription levels of the inserted gene. Though GFP fluorescence intensity is directly proportional to its mRNA abundance in cells, the expression of EGFP reporter could in some cases lag behind the production of endogenous protein, or persist for a short period beyond the endogenous product when the gene is turned off (Soboleski et al. 2005). Also, the inserted gene could be subjected to gene-silencing effects in later offspring and this requires to refresh breeder or perform reversible complementation by inducible expression of transgene (Muller 1999). In addition, the resolution of *in vivo* corneal fluorescence signal, which was captured by a fluorescence stereomicroscope in this study, can be enhanced using Spectralis OCT with excitation under fluorescein mode and optimal intensity setting (Yam et al. 2018).

In this study, we visualized the deployment of Col3a1-EGFP expression in mouse cornea after injury. This transgenic line, Tg(Col3a-EGFP)DJ124Gsat, thus enables an *in vivo*, actual time assessment of corneal fibrosis/scarring in conjunction with other phenotypic features and fibrosis gene expression, providing a valuable tool to evaluate corneal scar treatment and beyond.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used:

BMSC	bone marrow mesenchymal stem cell
CCT	central corneal thickness
Col3a1	collagen 3a1
CSSC	corneal stromal stem cells
EGFP	enhanced green fluorescence protein
EV	extracellular vesicle
SDOCT	Spectral domain optical coherence tomography
TNC	tenascin C

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Highlights:

- Corneal scarring of Tg(Col3a1-EGFP)DJ124Gsat mice was similar to wildtype mice
- EGFP induction was aligned with *COL3A1* upregulation after corneal injuries
- EGFP-expressing cells and fluorescent intensity were correlated to corneal scarring
- Tg(COL3a1-EGFP)DJ124Gsat mice allow real-time assessment of corneal scarring

Figure 1.

(A) Transgene construct. The coding sequence of EGFP, followed by a polyadenylation signal, was inserted into the mouse genomic bacterial artificial chromosome (BAC) RP23-324D9 at the ATG initiation codon of Col3a1 gene, resulting in the expression of EGFP reporter driven by Col3a1 promoter. (B) Genotyping identify mice carrying transgene versus wildtype. The amplicon size of transgene is ~300 bp. Molecular size 100 bp ladder indicates the size distribution. (C) Western blotting of Col3a1 expression in corneal lysates from Col3a1-EGFP transgenic and wildtype corneas. Protein samples from individual corneas at day 5 post-injury by anterior stromal ablation showed Col3a1 expression levels in association to the scarring severity. The Col3a1-EGFP fusion protein band migrated slower than Col3a1, indicating a greater molecular mass due to EGFP fusion.

Figure 2. Mouse corneal changes after alkali-burn injury.

(A) Ocular examination using brightfield and fluorescence stereomicroscopy, anterior segment optical coherence tomography (ASOCT) showed that both wildtype and Col3a1-EGFP transgenic mouse corneas developed corneal haze and scarring, corneal thickening with increased stromal reflectivity up to day 14 post-injury. Transgenic corneas exhibited green fluorescent dots and clusters. (B) Cell quantification from fluorescence images illustrated an increase of EGFP-expressing cells in transgenic corneas after injury. (C) Mean central corneal thickness (CCT) measured from ASOCT images showed both wildtype and transgenic mice with corneal thickening, and CCT increased by 50% at day 10 to 14 post-injury, compared to naïve corneas. (D) Volume measurement of stromal light scattering from serial ASOCT images showed similar increase of scar volume in wildtype and transgenic mouse corneas. Data are presented as mean and SD. * $P < 0.05$, One-way ANOVA, Mann-Whitney U test.

Figure 3. Col3a1-EGFP transgenic mouse corneal changes after alkali-burn or stromal debridement injury.

(A) Ocular examination using brightfield stereomicroscopy showed corneal haze and scarring after injuries for 14 days. Flat-mount corneal samples displayed fluorescence dots in central corneas. Cryosections showed EGFP fluorescence inside the corneal stroma, in colocalization with the immunostaining signal of Col3a1. (B) ELISA showed significant increase of EGFP intensity after injuries. (C) Flow cytometry detected significant increases of EGFP-expressing cells after injuries. (D) Flow cytometry result showing an induction of inflammatory cells expressing CD45, CD86 and Ly6G at day 3 post-injury, compared to naïve. (E) RT-PCR analysis detected the upregulated expression of fibrosis genes (COL3a1, α SMA and tenascin C, TNC), starting from day 7 up to 14 after alkali-burn injury. Data are presented as mean and SD. * $P < 0.05$, One-way ANOVA, Mann-Whitney U test. Scale bars: 30 μ m.

Figure 4. Healing response of Col3a1-EGFP transgenic mouse corneas to stem cell treatment. (A) Fluorescence microscopy of flat-mounted corneas showed reduced fluorescent dots at day 7 after treatment with human corneal stromal stem cells (CSSCs) and the derived extracellular vesicles (EVs), respectively. (B) Cell quantification from fluorescence images showed both treatments significantly reduced the number of EPGP-expressing cells. (C) The scar volumes were also significantly reduced after treatments. (D) qPCR detected significant suppression of fibrosis genes (COL3a1, α SMA) and EGFP, after treatment with CSSC-derived EVs, compared to non-treated corneas. (E) There was no difference of EGFP intensity changes in wounded corneas with or without treatment between male and female transgenic mice. Data are presented as mean and SD. * $P < 0.05$, One-way ANOVA, Mann-Whitney U test.

Figure 5.

(A-B) Bone marrow MSC from transgenic mice (Tg-BMSC) were cultured for pure MSC population with characterized expression of CD73, CD90 and CD105 (MSC markers), and negligible or mild expression of CD11b (macrophage marker) and CD34 (hematopoietic lineage marker) by immunofluorescence (IF). (C) At 5 days after intrastromal injection of BMSC into wildtype corneas, visible scar on BMSC-injected corneas was detected. Green fluorescence patches were visualized due to the induced Col3a1 expression. Control corneas with PBS injection did not show any scarring. Scale bars: 20 μ m (B); 500 μ m (C).