

AWARD NUMBER: W81XWH-19-1-0778

TITLE: Early Intervention Stem Cell-Based Therapy (EISCBT) for Corneal Burns and Trauma

PRINCIPAL INVESTIGATOR: YIQIN DU

CONTRACTING ORGANIZATION:

University of Pittsburgh
Department of Ophthalmology
203 Lothrop St
Pittsburgh, PA 15213

REPORT DATE: October 2020

TYPE OF REPORT: Annual Report

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT:

Approved for Public Release; Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE Oct 2020		2. REPORT TYPE Annual		3. DATES COVERED 9/15/2019-09/14/2020	
4. TITLE AND SUBTITLE Early Intervention Stem Cell-Based Therapy (EISCBT) for Corneal Burns and Trauma				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-19-1-0778	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Yiqin Du E-Mail: yiqindu@pitt.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Pittsburgh Office of Research 123 University Place, B21 Pittsburgh, PA 15213				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT The goal of this project is to validate new stem cell markers which can be used to assess the corneal stromal stem cell (CSSC) regenerative potential and develop a regenerative corneal bandage (ReCoBand) with CSSC embedded in or with a regenerative extracellular matrix that can be applied in the battlefield to prevent permanent scarring of the cornea and treat the corneal scarring after trauma, blast, or burn wounds to preserve vision. In the first year of this project we have: (1) validated stem cell markers with which CSSC possess the regenerative potential, (2) characterized a transgenic mouse model which the fibrotic cells turning to green when the cornea is scarred, (3) confirmed that CSSC can prevent corneal scarring on this transgenic Col3a1-EGFP mouse model, (4) discovered one of the mechanisms that CSSC prevent corneal scar formation, (5) proved that ReCoBand with verified CSSC can prevent corneal scar formation in an alkali burn rabbit model. These accomplishments meet the proposed milestones and predict success in meeting the overall goals of the project by the end of the 2 year time-course.					
15. SUBJECT TERMS cornea, scarring, stem cells, regeneration, plastic-compressed collagen, anti-inflammatory, RNAseq, gene expression					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT Unclassified	18. NUMBER OF PAGES 68	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unclassified	c. THIS PAGE Unclassified			19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	5
2. Keywords	5
3. Accomplishments	5-8
4. Impact	8-9
5. Changes/Problems	10-11
6. Products	11-13
7. Participants & Other Collaborating Organizations	13-15
8. Special Reporting Requirements	15
9. Appendices	
Appendix 1. Figures	16-23
Appendix 2. Quad Chart	24
Appendix 3. Exp Eye Res paper published recently	25-34
Appendix 4. Eye and Vision paper accepted	35-69

1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Corneal trauma and chemical burns lead to corneal scarring, producing a long-term reduction in vision, frequently blindness. Corneal scarring and decompensation are the second-most common causes of poor vision among ocular injuries in combat, commonly caused by explosions with fragmentary munitions and by chemical and thermal exposure. Although corneal transplantation is effective at restoring vision, it is not an ideal solution for the war fighter with long-term medication and possible rejection. The **subject** of this project is to discover a portable and effective treatment potential which can be simply used in the clinic of a battle field to promote corneal regeneration and save soldiers' vision and preserve their normal ability in their routine life and as a soldier. The **purpose** is to validate new marker genes which can be used to assess the regenerative potential of corneal stromal stem cell (CSSC) strains and validate the effectiveness of a regenerative corneal bandage (ReCoBand) containing either CSSC or regenerative ECM in a large animal model. The scope of the research includes three Aims: (1) To validate new marker genes to assess the regenerative potential of CSSC by in vitro qPCR assessment and in vivo in our Col3-EGFP mouse model; (2) To evaluate the regenerative potential of CSSC in the ReCoBand with expressing of those identified new marker genes in an alkali burn rabbit model as immediate acute treatment and delayed treatment (1 week after alkali burn); (3) To evaluate the regenerative potential of a regenerative ECM in the ReCoBand in the same alkali burn rabbit model as acute and delayed treatment. The results will demonstrate how to select the correct CSSC with regenerative potential and demonstrate the regenerative potential of CSSC and ECM in the ReCoBand to treatment corneal blindness which can be used clinically, especially in a combat field to save soldiers' vision. If success, it will dramatically reduce the need for corneal transplantation and significantly save vision in a timely manner.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Cornea, Scarring, Blindness, Regeneration, Stem cells, Stem cell-based therapy, ReCoBand, ECM, Vision

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major goals of the project:

1. Validate new marker genes as a means of assessing the regenerative potential of CSSC
2. Proof of Concept and safety studies in a large animal model
3. Effectiveness of ECM in remediating corneal scars.

Milestones:

1. Identification of new stem cell markers to identify regenerative potential. (12m) **Achieved**
2. Proof of Concept for effectiveness of ReCoBand in a large animal model.(12m) **In progress (50%) (because of COVID-19 with campus shutting down, it's a little delayed)**
3. Determination of effectiveness of delayed application of ReCoBand-SC therapy. (14m) **Will start in a month after completion of #2**
4. Demonstrate Effectiveness of ECM in ReCoBand format (ECM-ReCoBand).(20m) **Will finish it on time.**
5. Demonstration of Proof of Concept of effectiveness of ECM in corneal regeneration in a ReCoBand device. (24m) **Will finish it on time.**

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

During the 1st year of the project, we have made progress toward the goals.

Specific Aim 1: Validate new marker genes as a means of assessing the regenerative potential of CSSC.

Task 1: Correlate new marker gene expression of CSSC cell lines with regenerative potential.

Subtask 1: DHRPO approval to use cell lines derived from deidentified cadaveric human tissues. (To be obtained before starting work on project) (0-1m).

Subtask 2: ACURO approval for mouse protocols. (0-6m)

Subtask 3: Screen of CSSC lines from 12 donors to assess correlation of regenerative potential with marker gene expression. (6-10m)

Milestone: Identification of new stem cell markers to identify regenerative potential. (12m)

Accomplishment: We have received all the approvals of the protocols. We have screened the CSSC lines from different donors. The new stem cell markers Alix1, MMP16, FGD5, TBX18, and LONRF3 can be used to identify good CSSC lines with regenerative potential. The CSSC lines HC436, HC461, HC439, HC466L, HC438, HC470L, HC454, HC462, HC463 are identified good cell lines. (**Appendix 1, Figure 1.**) We have characterized a novel transgenic mouse model, Tg9Col3a1-EGFP/DJ124Gsat, in which enhanced green fluorescence protein (EGFP) reporter expression is driven by the promoter of collagen 3a1 (*COL3a1*), a stromal fibrosis gene. Similar to wildtype, Col3a1-EGFP transgenic mouse corneas develop opacities after wounding by alkali burn and mechanical ablation, as examined under stereomicroscopy and Spectral Domain optical coherent tomography (SD-OCT). The time course induction of EGFP is aligned with Col3a1 upregulation and matched with the elevated expression of other fibrosis genes (α -smooth muscle actin, fibronectin and tenascin C). Increased number of EGFP+ cells and fluorescent intensities were correlated to corneal thickening and scar volume as evaluated by flow cytometry and ELISA. After treatment with CSSC or CSSC exosomes, EGFP expression is 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 and perfect tool for studying corneal fibrosis and scarring in vivo, and will be useful in monitoring the changes of corneal fibrosis over time. It also confirms the regenerative potential of the identified CSSC by those stem cell markers. (**Appendix 3**, published paper in Exp Eye Res, 2020, 200:108270. PMID: 32979396). Further, one of the mechanisms of CSSC regenerative effect on anti-corneal scarring was investigated and unveiled. Mouse macrophage RAW264.7 cells were induced to M1 proinflammatory phenotype by IL- γ and LPS; or induced to M2 anti-inflammatory phenotype by IL-4. After CSSC were co-cultured with M1 macrophages, CSSC had increased expression of TGF β 3 with increased regenerative potential. When CSSC were treated with TGF β 3 siRNA to knockdown the TGF β 3 expression, the CSSC lost their anti-corneal scarring ability in mice in vivo, while untreated CSSC showed dramatic reduction of scar formation in mice. It illustrates that identified CSSC maintain their anti-scarring effect to save the corneal transparency and vision partially mediated by TGF β 3. (**Appendix 4**, accepted manuscript together with the acceptance notification in Eye and Vision)

Specific Aim 2: Proof of Concept and safety studies in a large animal model

Task 2: Demonstrate ReCoBand effectiveness in Rabbit Corneal Burn Model

Subtask 1: ACURO approval for rabbit experiments (0-10m)

Subtask 2: Examine ability of ReCoBand containing CSSC (SCReCoBand) to regenerate rabbit cornea after alkali burns. (10-12m)

Milestone: Proof of Concept for effectiveness of ReCoBand in a large animal model. (12m)

Accomplishment: We have received the ACURO approval for rabbit experiments. We used the validated CSSC HC462 on our initial rabbit experiment and will do extra CSSC lines to further confirm the results. We seeded CSSC into compressed collagen as ReCoBand at different concentrations (we used 900 and 1800 cells in the 6mm ReCoBand). The rabbits were divided into 5 groups: (1) Naïve (the untreated left eyes as naïve control), (2) wound only (alkali burn on the right eye: soaked ϕ 5mm filter paper in 1N NaOH and placed onto the central cornea for 30 seconds. Immediately washed the cornea with 20ml PBS to completely remove NaOH), (3) wound + compress collagen (immediately after wound and wash, put the ϕ 6mm compress collagen onto the wounded cornea with fibrinogen and thrombin to form fibrin gel to seal the collagen onto the cornea. Do tarsorrhaphy for a week to prevent the collagen scratched off by rabbits), (4) Wound + CSSC-900 cells in the compress collagen, (5) Wound + CSSC-1800 cells in the compress collagen. At one week, we used slit lamp to observe the corneas and could see the difference between the groups (**Appendix 1, Figure 2**). At two weeks, we also did slit lamp pictures to see the difference. At week 3, the animals were observed by slit lamp and OCT and then sacrificed and the corneas were detected by qPCR, WB, and hematoxylin and eosin (H-E) staining on cryosections. Both the slit lamp pictures (**Appendix 1, Figure 2**) and OCT show that alkali burn caused corneal scarring and thickening in wound only and wound+collagen groups, while ReCoBand treatment with both CSSC 900 and 1800 reduced the corneal scarring and corneal thickness at week 3 (**Appendix 1, Figure 3**). H-E staining shows the corneal thickness changes with some inflammatory cells remained in the corneas treated with wound and wound+collagen (**Appendix 1, Figure 4**). qPCR on the rabbit corneas shows the corneas with wound and wound+collagen had statistically significant increased expression of Col3a1, while wound+CSSC treated corneas had reduced Col3a1 expression, similar to that of naïve corneas (**Appendix 1, Figure 5**). Western blotting on the rabbit corneas of wound and wound+collagen had increased expression of fibrotic markers Col3a1, fibronectin (FN), and reduced expression of corneal stromal marker vimentin (**Appendix 1, Figure 6**). The results have proved the concept that ReCoBand with CSSC embedded in is effective in preventing of scar formation in a rabbit model with alkali burn, which is a severe condition and seen in a combat field and in routine life. Some of the changes are not statistically significant, such as the gene expression of decorin, keratocan, and SPARC. Because of limited sample size, we couldn't do statistical analysis on the Western blotting results. We have ordered more rabbits and we will do more experiments with different CSSC cell lines to confirm the results.

What opportunities for training and professional development has the project provided?

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. "Training" activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. "Professional development" activities result in increased knowledge or skill in one's area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

Dr. Irona Khandaker was a postdoctoral fellow on this project. She is the first author of the published paper in Exp Eye Res (Appendix 3). Now she moved to another position as a postdoc to further broaden her background aiming to become a research faculty in the field of vision regeneration.

Dr. Lin Weng was a research follow on this project. He is the first author of the accepted paper in Eye and Vision (Appendix 4). He went back to Shanghai and works in the vision regeneration field as a faculty in Shanghai Lanhe Optometry and Ophthalmology Clinic.

Dr. Gary Yam was recruited as a research associate professor for this project. He has been trained to take responsibilities and he has submitted his own DoD grant and he will become an independent researcher in the corneal regeneration field.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

The published paper and accepted paper cited the support of this grant which allows people know the scientific work, technologies, and the grant support. The animal model will be shared if other researchers are interested and ask for it. Due to COVID-19, there were no scientific conferences recently but we will submit abstracts to meeting when available.

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We have order new rabbits and we will use different CSSC cell lines to confirm the results we have achieved in the acute alkali burn model to confirm the CSSC regenerative potential to preserve vision. We will also use the verified CSSC lines to work on the delayed treatment (to put the ReCoBand with CSSC onto rabbits one week after alkali burn to allow the scar formed). We will also do the experiments on the same rabbit models using the ReCoBand with the regenerative ECM on it to fulfill the proposed Specific Aim 3.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

We identified the specific stem cell genes with which the corneal stromal stem cells (CSSC) have strong regenerative potential. This illustrates an important fact that stem cells vary markedly in their potential according to their source and that defining criteria for assessing that potency will be essential for translating their use to the clinic.

We characterized the Col3a-EGFP mice which shows green cornea which the cornea has scar. This can be used to track the scar changes with treatment in vivo by directly looking at the green or not green corneas.

The ReCoBand we discovered, which contains compress collagen and verified CSSC, has been confirmed to have the regenerative potential, even in a relatively severe alkali burn rabbit model. This exciting discovering illustrates a hope that a ReCoBand can be stored and used in a clinic at a combat field to save soldiers' vision right on the site.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

With the COVID-19, the project was delayed but we will work hard to catch up for the time line. For Western blotting, we have used several other antibodies but didn't get results. Those antibodies did not work on rabbits so we will order other antibodies which work on rabbits so we can verify the results with more appreciate antibodies. For qPCR, we also tested other fibrotic and keratocyte primers which did not get any amplifications. We will design more primers which can detect rabbit genes.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Nothing to Report

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

- **Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

One manuscript citing DoD support was published in Exp Eye Res as Appendix 2.
One manuscript citing DoD support was accepted in Eye and Vision as Appendix 3 with the acceptance letter.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Nothing to Report

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*

- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

Name: Mary Smith
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.

Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)

Name:	Yiqin Du
Project Role:	PI
Univ. Pitt. ID	2.73
Nearest person month worked:	YD designed the project and is directing it; she is in charge of animal protocols and regulations. She analyzes the results.
Contribution to Project:	James Funderburgh Co-investigator 37069
Name:	
Project Role:	
Univ. Pitt. ID	
Nearest person month worked:	0.46
Contribution to Project:	Dr. Funderburgh designed the project and helped to analyze the NanoString data
Name:	Gary Yam
Project Role:	Co-investigator
Univ. Pitt. ID	
Nearest person month worked:	1.70
Contribution to Project:	Dr. Yam does cell culture and animal work. He also helps to analyze data and write manuscripts.
Name:	Martha Funderburgh
Project Role:	Research Specialist
Univ. Pitt. ID	
Nearest person month worked:	5.73
Contribution to Project:	Ms. Funderburgh is in charge of record-keeping for human cells, helps regulatory approval of human and animal studies. She helps in passaging and cryopreserving cells. She does qPCR work.
Name:	Moir Geary
Project Role:	Animal Technician
Univ. Pitt. ID	
Nearest person month worked:	8.50
Contribution to Project:	Ms. Geary maintains the mouse breeding colony and genotyping. She helps for animal procedures, OCT and ELISA.
Name:	Irona Khandaker
Project Role:	Postdoctoral Researcher
Univ. Pitt. ID	
Nearest person month worked:	1.92
Contribution to Project:	Dr. Khandaker did cell culture work and wrote the manuscript.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Nothing to Report

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

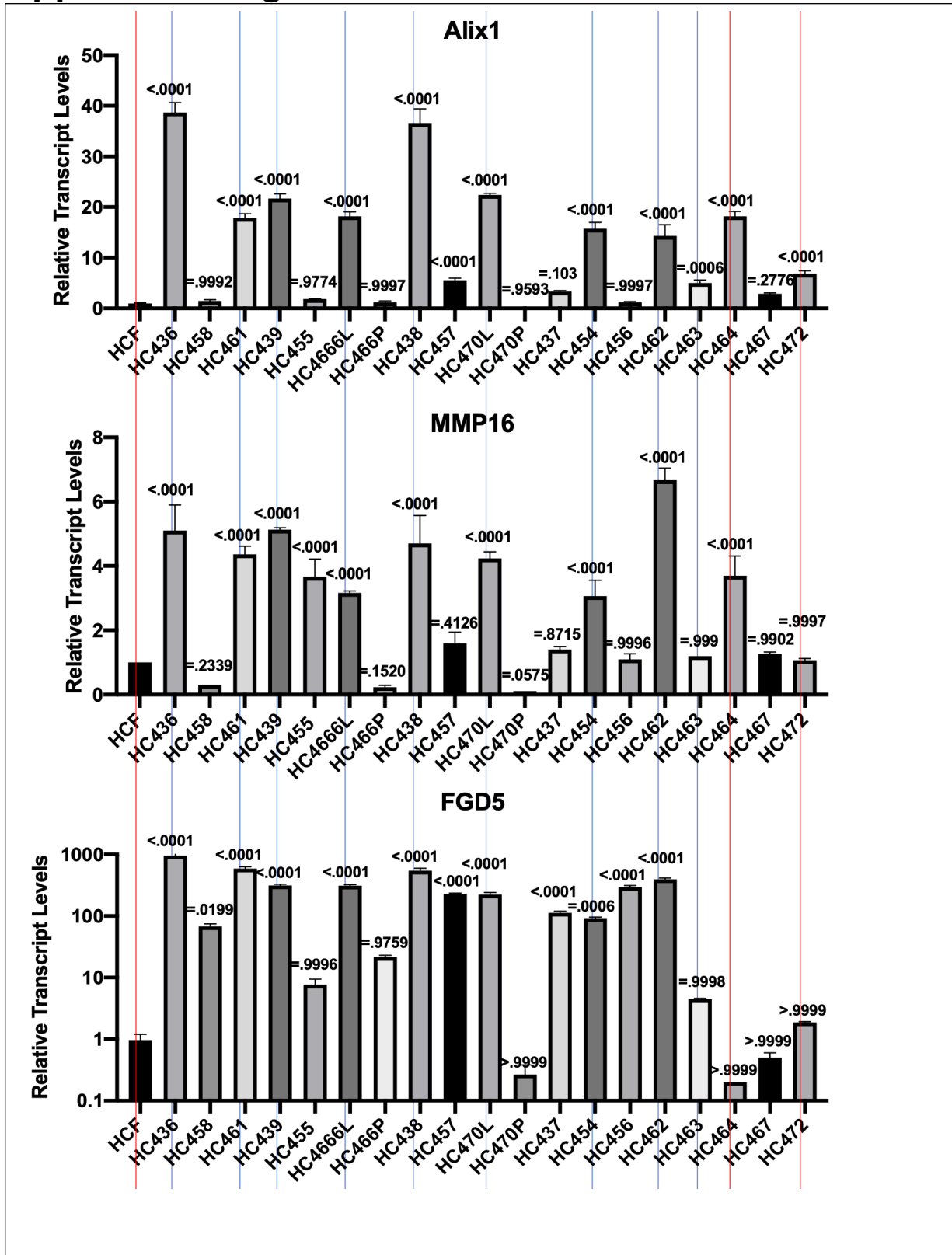
COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

Nothing to Report

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*

Appendix 1. Figures.



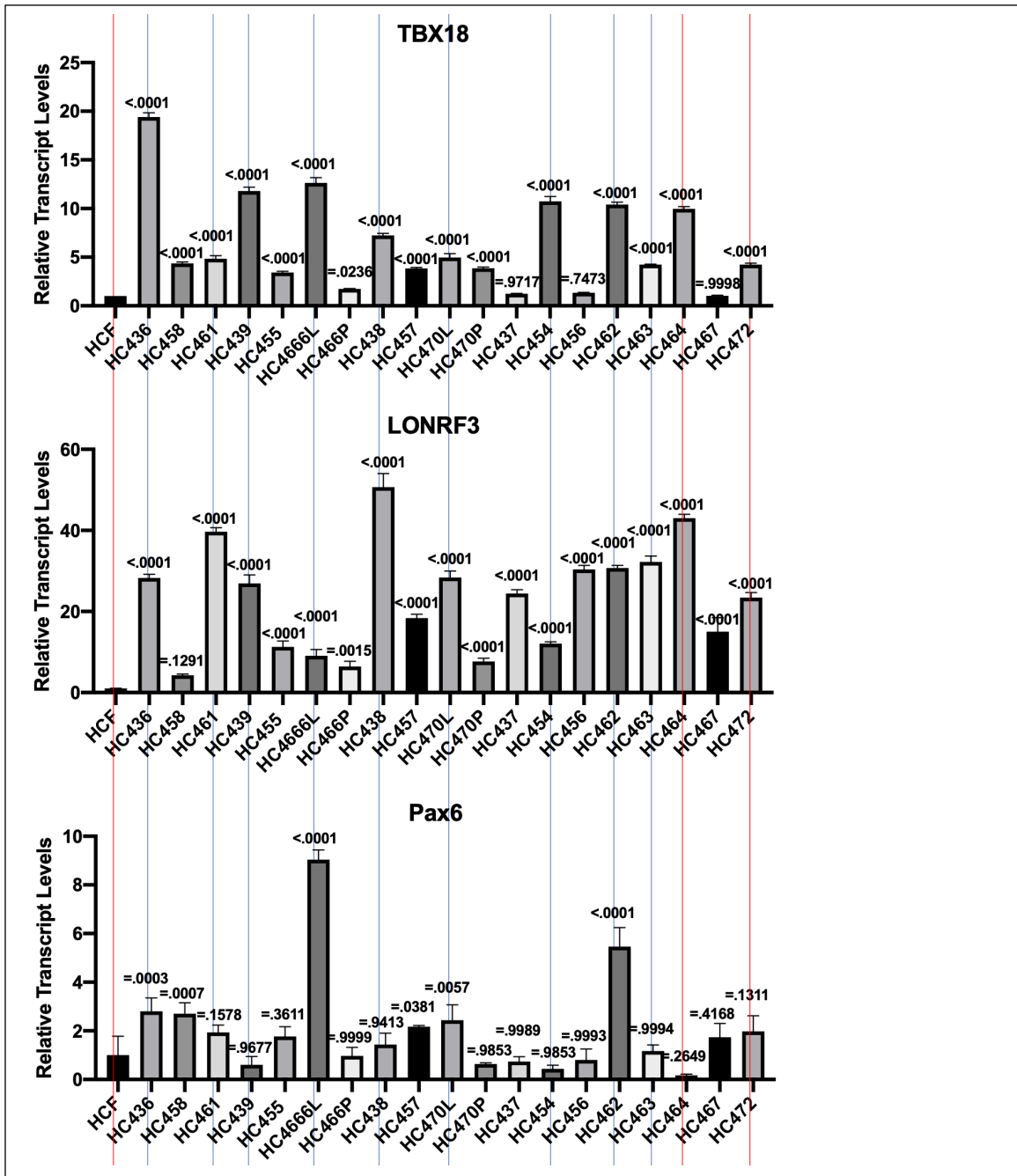


Figure 1. Differential gene expression of cultured CSSCs by qPCR. Human CSSCs and human corneal fibroblasts (HCF) as a negative control were cultured and lysed by RLT lysis buffer. RNA was extracted using RNeasy mini kit (Qiagen). cDNA was transcribed from the RNAs using cDNA MasterMix. qPCR was performed by direct dye binding using SYBR green (Applied Biosystems). Analysis was based on 18S and GAPDH as internal controls and all the gene expression was compared to HCF. One-way ANOVA followed by Dunnett's multiple comparisons test was used to detect the differences. $p < .05$ was considered as statistically significance. All the p values in comparison to HCF were labeled in the figures. Blue lines marked the cell strains had significant higher expression of genes Alix1, MMP16, FGD5, TBX18 and LONRF3. The Pax6 expression was not consistent with all the other genes. We will do mouse work to confirm the good cell stains. The red lines marked 2 CSSC strains with high expression of Alix1 but not all the genes. We will also test these two cell strains to see if Alix1 can stand alone as a gold standard for identifying good CSSCs. HCF will be used as negative control for in vivo test.

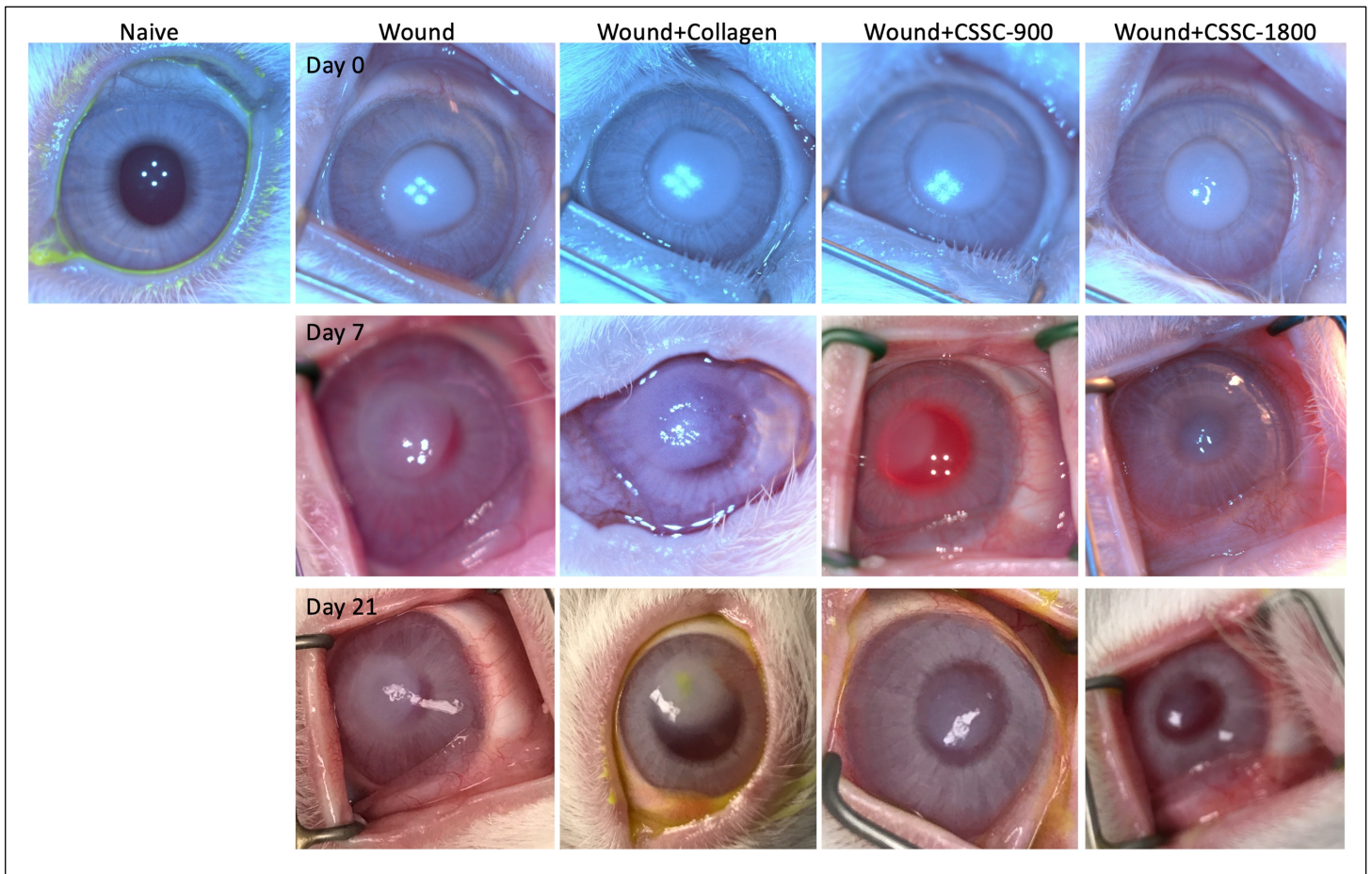


Figure 2. Slit lamp pictures of rabbit corneas with different treatments. Rabbit corneas were wounded by putting $\phi 5\text{mm}$ paper soaked with 1N NaOH on the center of the cornea for 30 seconds and completely washed with 20 ml PBS. Then the wounded corneas were no treatment (Wound), treated with $\phi 6\text{mm}$ compress collagen (Wound+Collagen), treated with $\phi 6\text{mm}$ compress collagen seeded containing 900 human CSSC cells (Wound+CSSC-900), or treated with $\phi 6\text{mm}$ compress collagen seeded containing 1800 human CSSC cells (Wound+CSSC-1800). Untreated corneas are as naïve control. The top line shows the eyes immediately after alkali burn with the central corneal swollen; the middle line shows the eyes at day 7 with different treatment indicating both 900 and 1800 CSSC treatment dramatically reduced the corneal swollen and scarring while the corneas with wound only or treated with blank compress collagen did not heal at day 7 with scar formation. The bottom line show the corneas at day 21 indicating the corneas with CSSC treatment had very thin haze while the wound only and wound with collagen had corneal scar.

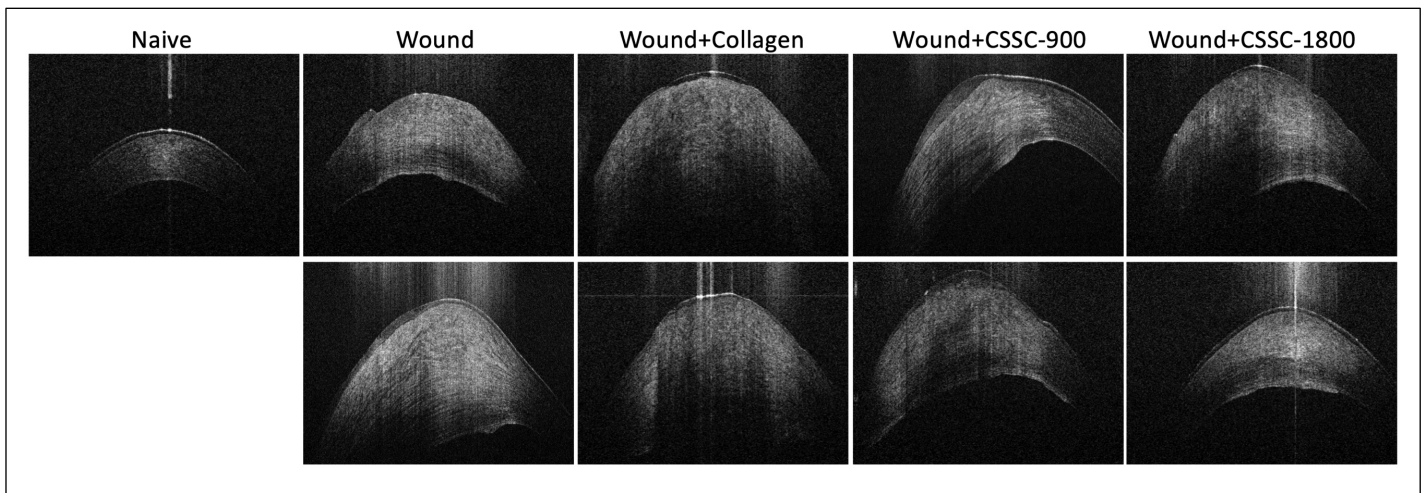


Figure 3. Optical coherence tomography (OCT) scanning of rabbit corneas with different treatments. Rabbit corneas were scanned with OCT at day 21. The figure shows two different corneas in each group except the naïve group with only one cornea. It shows the corneas treated with CSSC had reduced corneal thickness as compared to the wound and wound+collagen groups. One cornea with 1800 CSSC treatment still had swollen cornea. More experiments with different CSSC cell lines are planned to confirm the results.

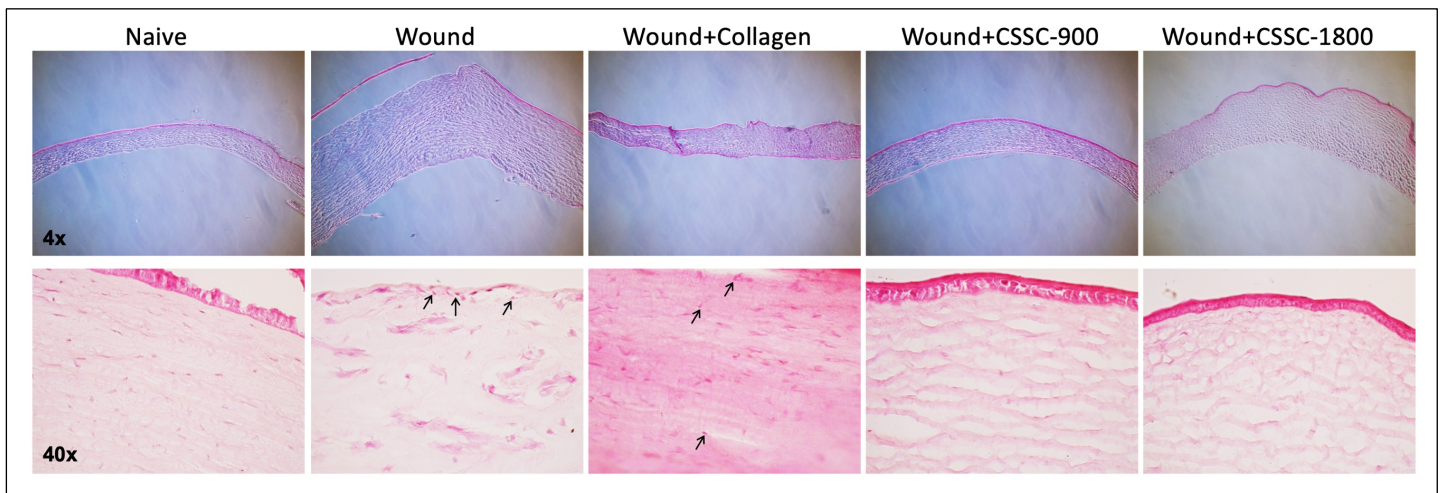


Figure 4. Hematoxylin and Eosin staining on cryosectioned rabbit corneas with different treatments. Rabbit corneas were fixed and cryosectioned at day 21. The H-E staining figures show the corneas at 4x (top) and 40x oil (bottom). The 4x figures show the corneal thickness with very swollen cornea in the wound group while the corneas of wound with CSSC treatment had reduced swollen. The cornea of the Wound+collagen was in normal thickness because the central cornea was damaged during sectioning and more corneas are to be sectioned and stained. 40x oil figures show the wound and wound+collagen corneas had inflammatory-like cells with small and round nuclei (arrows) while naïve and CSSC treated corneas had almost no inflammatory-like cells.

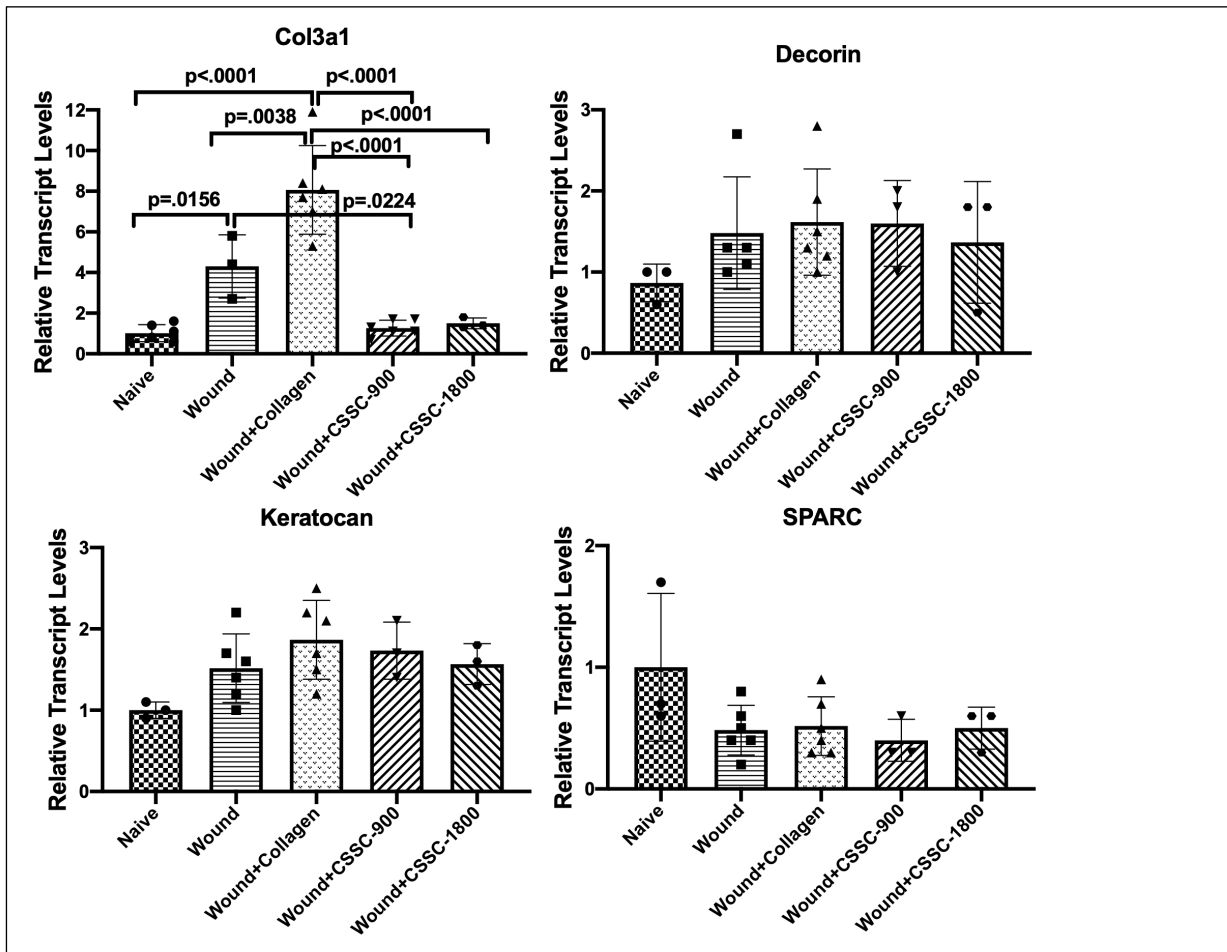


Figure 5. qPCR of the rabbit corneas with different treatments. Rabbit corneas were lysed and extracted for RNAs and then reverse transcription to cDNAs. qPCR results show the fibrotic marker Col3a1 (the same as our Col3a1-EGFP mice, Col3a1 indicating fibrosis and scar formation) has significantly increased expression in the corneas of wound and wound+collagen, while Col3a1 expression in the corneas treated with both 900 and 1800 corneas dramatically reduced as compared to that of wound and wound+collagen, and similar to that of naïve corneas. The other fibrotic markers Decorin and SPARC and the corneal keratocyte marker keratocan did not show significant difference. More experiments are needed and new markers specific for rabbits are needed. One-way ANOVA followed by Tukey posttest using PRISM. $P < .05$ was considered to be statistically significant.

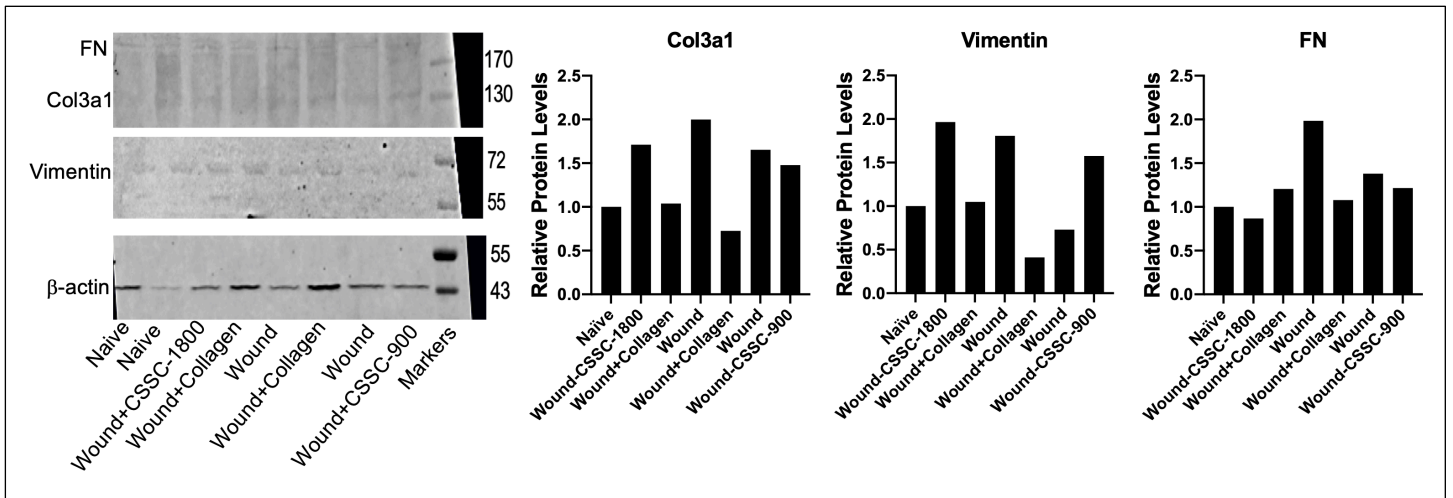


Figure 6. Western blotting results detecting protein levels in the rabbit corneas with different treatments. Rabbit corneas were lysed in RIPA and underwent Western blotting to detect the fibrotic and corneal stromal protein expression. The gel bands were quantified using ImageJ and normalized to β -actin as internal control. The graphs show relative protein levels after quantification. Because of the limited sample size, no statistical analysis was made and more experiments will be done to further confirm the conclusion.

Appendix 2 Quad Chart

Early Intervention Stem Cell-Based Therapy (EISCBT) for Corneal Burns and Trauma
Log# VR180189, W81XWH1910778



PI: Yiqin Du

Org: University of Pittsburgh

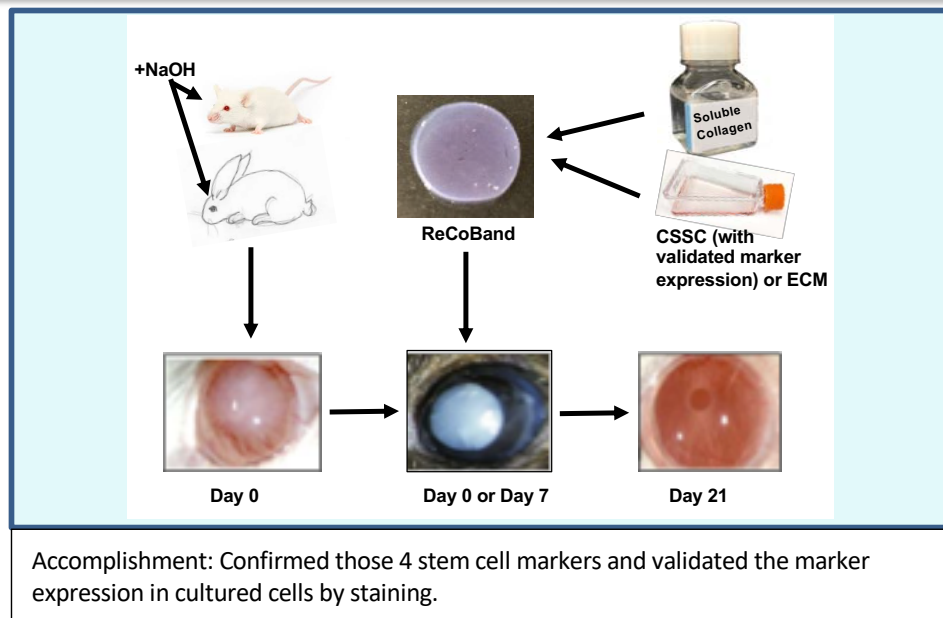
Award Amount:

Study/Product Aim(s)

- **Aim 1.** Validate new marker genes as a means of assessing the regenerative potential of CSSC.
- Aim 2.** Provide proof of Concept and safety studies in a large animal model (rabbit alkali burn wounds)
- Aim 3.** Compare the regenerative efficacy of ECM in a ReCoBand device with that of CSSC

Approach

These experiments will test scar suppression/tissue regeneration of two promising therapeutic agents (CSSCs in ReCoBand and ECM ReCoBand) in a rabbit chemical burn model to provide Proof of Concept for their efficacy.



Timeline and Cost

Activities	CY	19	20	21	22
Correlate marker gene expression with CSSC regenerative potential in vivo.					
Demonstrate ReCoBand effectiveness in Rabbit Corneal Burn Model					
Delayed application of ReCoBand					
Effectiveness of ECM in the ReCoBand format					
Estimated Budget (\$K)		\$42	\$434	\$284	\$000

Updated: (October 14, 2020)

Goals/Milestones

CY19 Goal – Submission protocols for ACURO approval; Identification of new stem cell markers

- ☒ Both mouse and rabbit protocols submitted and rabbit ACURO approved; NanoString assay and staining to test the markers.

CY20 Goals – Validation new markers correlated to CSSC regenerative potential; Proof of concept and safety studies in a rabbit model.

- ☐ Investigate the specific marker expression CSSCs have strong scar suppressing effect in a mouse model.
- ☐ Use the confirmed CSSC lines to test the concept in a rabbit alkali burn modelmodel

CY21 Goal – Proof of Concept in a rabbit model with delay application and validation of ECM effect

- ☐ Validate CSSC ReCoBand with delayed application after wound
- ☐ Compare regenerative effect of ECM and CSSC

Comments/Challenges/Issues/Concerns

Budget Expenditure to Date

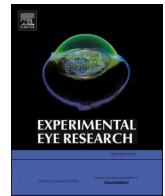
Projected Expenditure: \$366k

Actual Expenditure: \$260k



Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexerA novel transgenic mouse model for corneal scar visualization[☆]

Irona Khandaker, James L. Funderburgh, Moira L. Geary, Martha L. Funderburgh, Vishal Jhanji, Yiqin Du^{**}, Gary Hin-Fai Yam^{*}

Department of Ophthalmology, University of Pittsburgh School of Medicine, Pittsburgh, PA, 15213, United States

ARTICLE INFO

Keywords:

Cornea scarring
Fibrosis
Collagen 3a1
Transgenic
Enhanced green fluorescence protein
Stromal cell therapy

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 coherence 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.

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 allogeneic 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; Shojati 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

Abbreviations: 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.

[☆] This paper is dedicated to the memory of Professor James L Funderburgh.

^{*} Corresponding author. Department of Ophthalmology, University of Pittsburgh, 203 Lothrop Street, Pittsburgh, PA, 15213, USA.

^{**} Corresponding author. Department of Ophthalmology, University of Pittsburgh, 203 Lothrop Street, Pittsburgh, PA, 15213, USA.

E-mail addresses: duy@upmc.edu (Y. Du), yamg@pitt.edu (G. Hin-Fai Yam).

<https://doi.org/10.1016/j.exer.2020.108270>

Received 18 June 2020; Received in revised form 10 September 2020; Accepted 20 September 2020

Available online 23 September 2020

0014-4835/© 2020 Elsevier Ltd. All rights reserved.

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 micro-RNAs 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, 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 (Trinka-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 REDExtract-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 tct tct cc-3' and antisense: 5'-tag cgg ctg aag cac tgc a-3'), under the condition of 94 °C for 30 s, 60 °C for 30 s, 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 7–8 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 s. 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 (hCSCC) 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

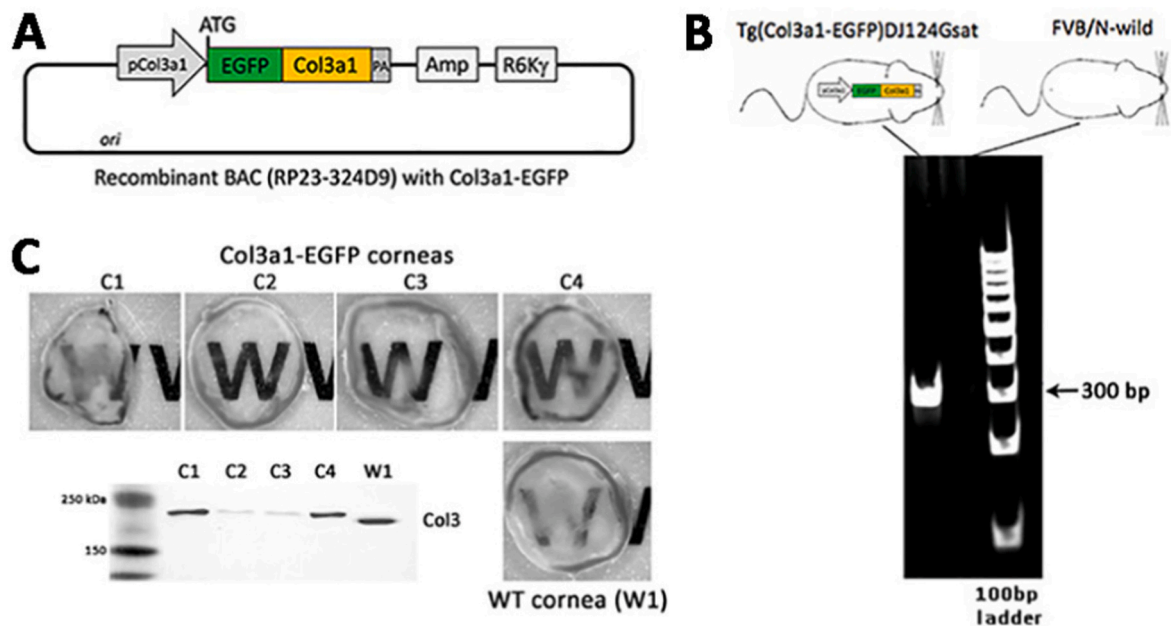


Fig. 1. (A) Transgene construct. The coding sequence of EGFP, followed by a polyadenylation signal, was inserted into the mouse genomic bacterial artificial chromosome (BAC) RP23-324D9 at the ATG initiation codon of Col3a1 gene, resulting in the expression of EGFP reporter driven by Col3a1 promoter. (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.

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 h 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 h. After 3 days, the conditioned media were collected, filtered through 0.22 µm pores, concentrated with Amicon Ultra 100 k cutoff ultrafilter at 4000 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 1 h, then the supernatant spun at 100,000 g for 3 h (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 min, 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 Amphopterin B, Invitrogen) for 10 min 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/µl 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 analysed 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 Qias shredder 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 $2^{-\Delta\Delta CT}$ method after normalization with housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18 S 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 4–20% 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'-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.

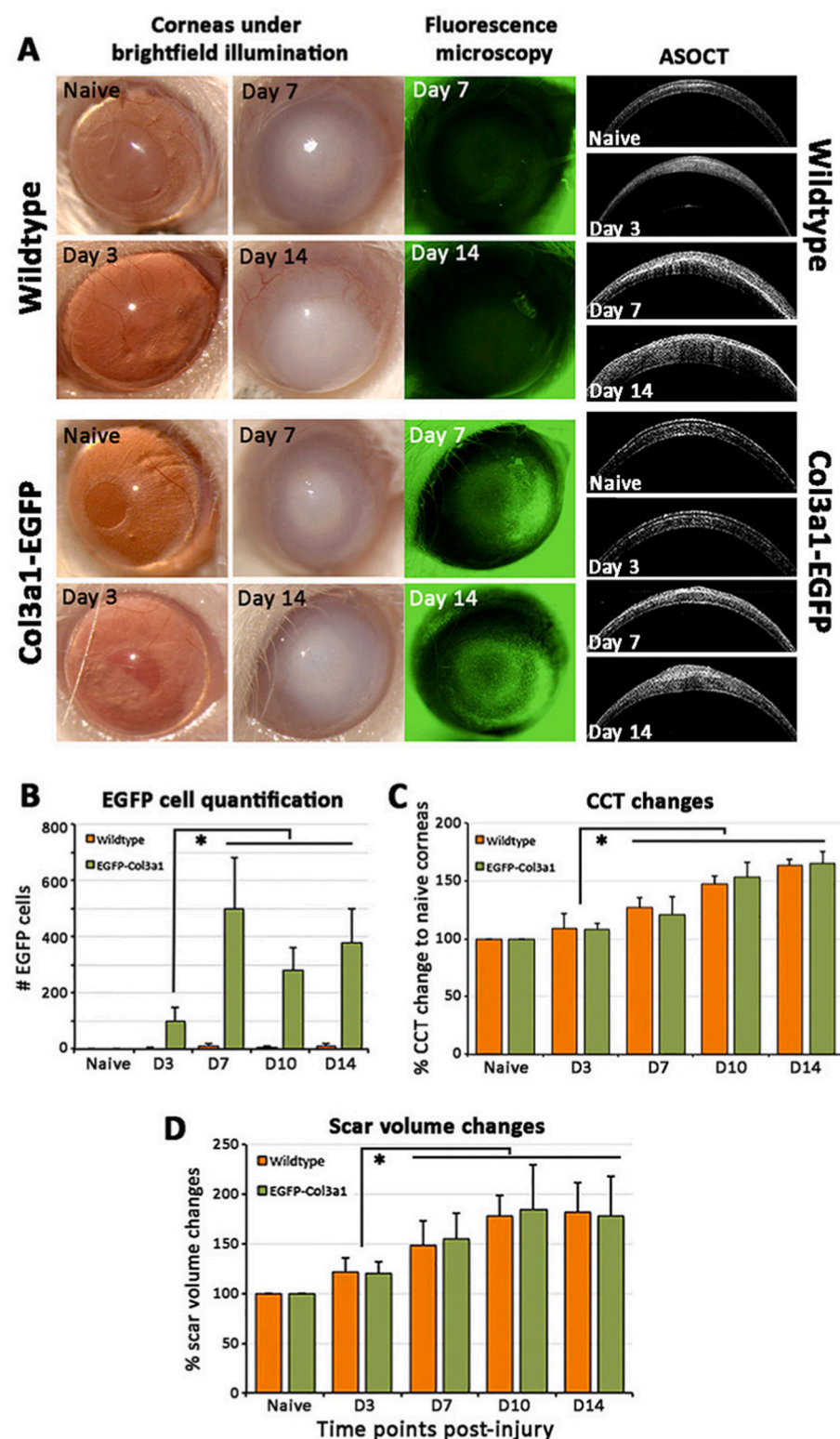


Fig. 2. Mouse corneal changes after alkali-burn injury. (A) Ocular examination using brightfield and fluorescence stereomicroscopy, anterior segment optical coherence tomography (ASOCT) showed that both wildtype and Col3a1-EGFP transgenic mouse corneas developed corneal haze and scarring, corneal thickening 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 10–14 post-injury, compared to naive 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. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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 (Fig. 2C and D). The mean percentage changes of scar volume at day 7–14 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

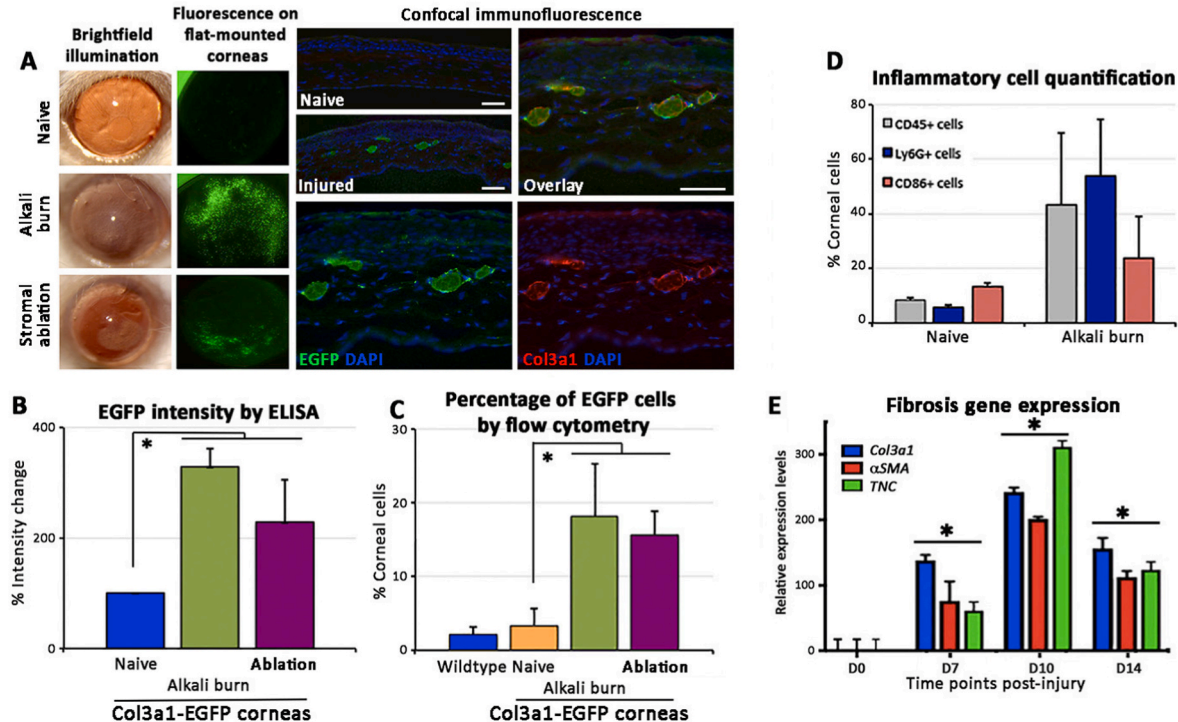


Fig. 3. Col3a1-EGFP transgenic mouse corneal changes after alkali-burn or stromal debridement injury. (A) Ocular examination using brightfield stereomicroscopy showed corneal haze and scarring after injuries for 14 days. Flat-mount corneal samples displayed fluorescence dots in central corneas. Cryosections 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.

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 ($329 \pm 34\%$) compared to that after ablation ($229 \pm 77\%$). 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 ($15.7 \pm 3.2\%$ for the ablated corneas and $18.2 \pm 7.1\%$ for alkali-burn corneas, versus $3.4 \pm 2.3\%$ 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) (Fig. 5A and 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; Hertsberg 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; Shojati 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; Hertsberg 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*

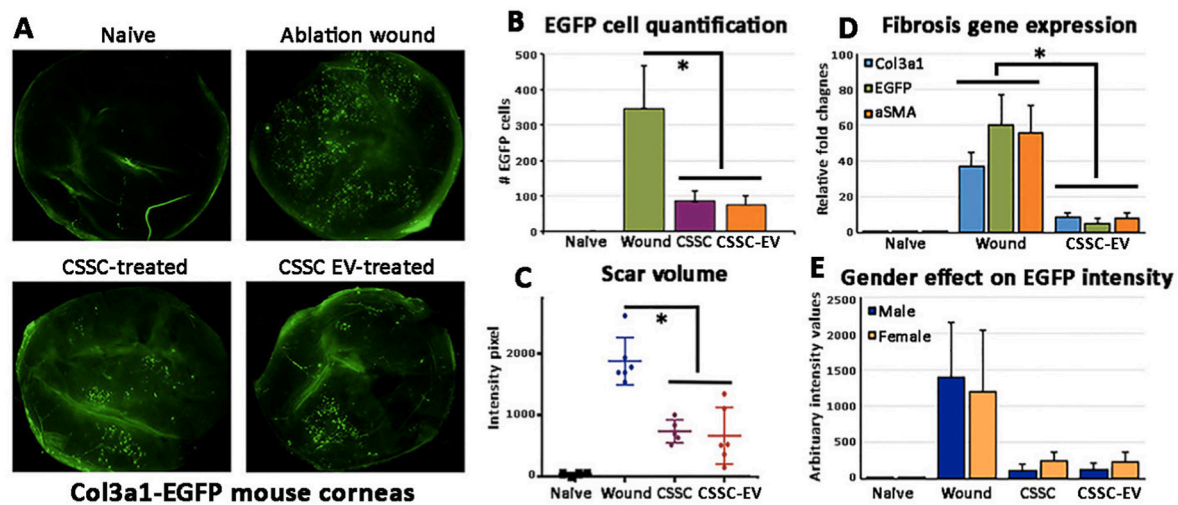


Fig. 4. Healing response of Col3a1-EGFP transgenic mouse corneas to stem cell treatment. (A) Fluorescence microscopy of flat-mounted corneas showed reduced fluorescent dots at day 7 after treatment with human corneal stromal stem cells (CSSCs) and the derived extracellular vesicles (EVs), respectively. (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, αSMA) 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.

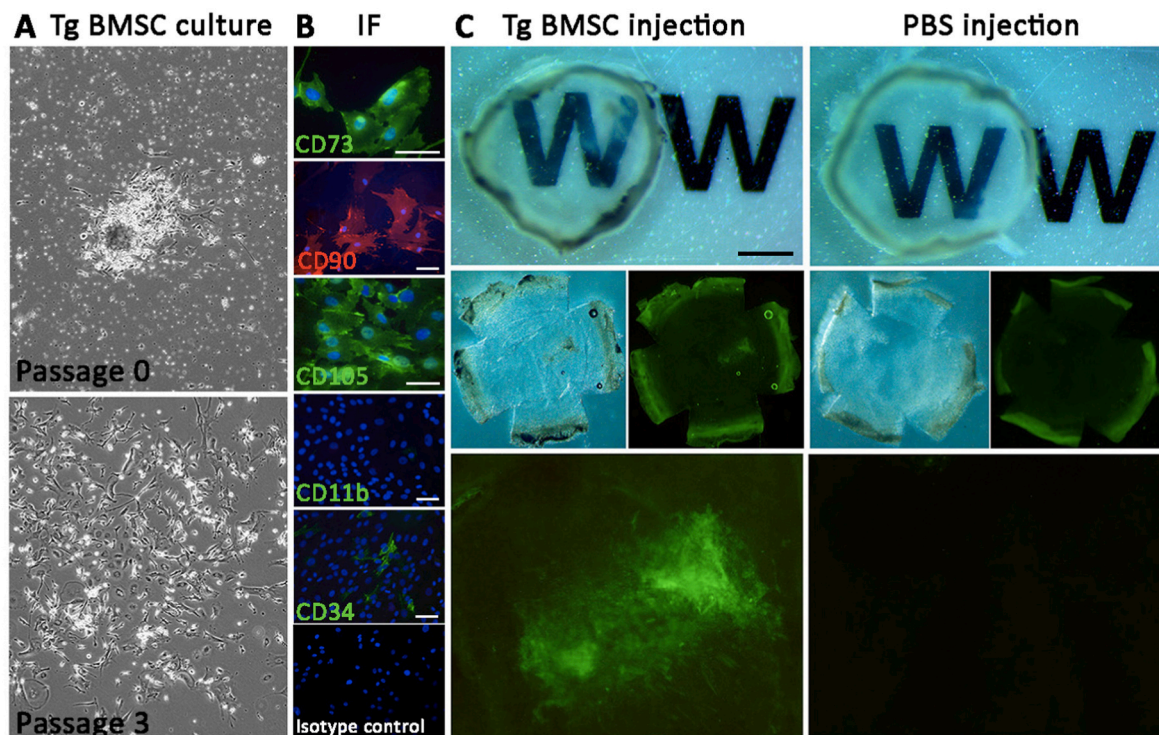


Fig. 5. (A–B) Bone marrow MSC from transgenic mice (Tg-BMSC) were cultured for pure MSC population with characterized expression of CD73, CD90 and CD105 (MSC markers), and negligible or mild expression of CD11b (macrophage marker) and CD34 (hematopoietic lineage marker) by immunofluorescence (IF). (C) 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). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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; Hertsberg 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 (Col3a1-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.

Funding agency statement

The funding agency had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Financial disclosure

All authors have no proprietary or commercial interests in any materials involved in this article.

Data availability statement

The data in this study are available from the corresponding author upon reasonable request.

Author contributions

Conception and design: JF, MF, YD. Collection, assembly and analysis of data: JF, IK, MG, MF, GY. Financial support: JF, YD. Manuscript writing and editing: GY, JF, MF, VJ, IK, YD. All authors approved the manuscript.

Acknowledgements

We thank K Davoli for histological processing of corneal samples and K Lathrop and A Kumar for guidance in microscopy. This work was supported by Department of Defense Grant W81XWH1910778 (JF, YD), NIH Grants RO1 EY016415 (JF, YD), P30 EY008098, Stein Innovator Award from Research to Prevent Blindness (JF), Eye and Ear Foundation of Pittsburgh, and Louis J Fox Center for Vision Restoration.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.exer.2020.108270>.

References

- Ahmadi, A.J., Jakobiec, F.A., 2002. Corneal wound healing: cytokines and extracellular matrix proteins. *Int. Ophthalmol. Clin.* 42 (3), 13–22.
- Basu, S., Damala, M., Tavakkoli, F., Mitragotri, N., Singh, V., 2019. Human limbus-derived mesenchymal/stromal stem cell therapy for superficial corneal pathologies: two-year outcomes. *Invest. Ophthalmol. Vis. Sci.* 60, 4146.
- Basu, S., Hertsberg, A.J., Funderburgh, M.L., Burrow, M.K., Mann, M.M., Du, Y., Lathrop, K.L., Syed-Picard, F.N., Adams, S.M., Birk, D.E., Funderburgh, J.L., 2014. Human limbal biopsy-derived stromal stem cells prevent corneal scarring. *Sci. Transl. Med.* 6 (266), 266ra172.
- Boote, C., Du, Y., Morgan, S., Harris, J., Kamma-Lorger, C.S., Hayes, S., Lathrop, K.L., Roh, D.S., Burrow, M.K., Hiller, J., Terrill, N.J., Funderburgh, J.L., Meek, K.M., 2012. Quantitative assessment of ultrastructure and light scatter in mouse corneal debridement wounds. *Invest. Ophthalmol. Vis. Sci.* 53 (6), 2786–2795.
- Chan, M.F., Werb, Z., 2015. Animal models of corneal injury. *Bio Protoc* 5 (13), e1516.
- Chandler, H.L., Tan, T., Yang, C., Gemensky-Metzler, A.J., Wehrman, R.F., Jiang, Q., Peterson, C.M., Geng, B., Zhou, X., Wang, Q., Kaili, D., Adesanya, T.M., Yi, F., Zhu, H., Ma, J., 2019. MG53 promotes corneal wound healing and mitigates fibrotic remodeling in rodents. *Commun Biol* 2, 71.
- Chen, W.S., Cao, Z., Leffler, H., Nilsson, U.J., Panjwani, N., 2017. Galectin-3 inhibition by a small-molecule inhibitor reduces both pathological corneal neovascularization and fibrosis. *Invest. Ophthalmol. Vis. Sci.* 58 (1), 9–20.
- Coulson-Thomas, V.J., Caterson, B., Kao, W.W., 2013. Transplantation of human umbilical mesenchymal stem cells cures the corneal defects of mucopolysaccharidosis VII mice. *Stem Cell.* 31 (10), 2116–2126.
- Cowell, B.A., Wu, C., Fleiszig, S.M., 1999. Use of an animal model in studies of bacterial corneal infection. *ILAR J.* 40 (2), 43–50.
- D'Hondt, S., Guillemin, B., Syx, D., Symoens, S., De Rycke, R., Vanhoutte, L., Toussaint, W., Lambrecht, B.N., De Paepe, A., Keene, D.R., Ishikawa, Y., Bachinger, H.P., Janssens, S., Bertrand, M.J., Malfait, F., 2018. Type III collagen affects dermal and vascular collagen fibrillogenesis and tissue integrity in a mutant Col3a1 transgenic mouse model. *Matrix Biol.* 70, 72–83.
- Dominici, M., Le Blanc, K., Mueller, L., Slaper-Cortenbach, L., Marini, F., Krause, D., et al., 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8, 315–317.
- Du, Y., Carlson, E.C., Funderburgh, M.L., Birk, D.E., Pearlman, E., Guo, N., Kao, W.W., Funderburgh, J.L., 2009. Stem cell therapy restores transparency to defective murine corneas. *Stem Cell.* 27 (7), 1635–1642.
- Du, Y., Funderburgh, M.L., Mann, M.M., SundarRaj, N., Funderburgh, J.L., 2005. Multipotent stem cells in human corneal stroma. *Stem Cell.* 23 (9), 1266–1275.
- Duarte Campos, D.F., Rohde, M., Ross, M., Anvari, P., Blaesser, A., Vogt, M., Yam, G.H., Mehta, J.S., Fischer, H., Walter, P., Fuest, M., 2019. Corneal bioprinting utilizing collagen-based bioinks and primary human keratocytes. *J. Biomed. Mater. Res.* 107 (9), 1945–1953.
- Fuest, M., Yam, G.H., Peh, G.S., Mehta, J.S., 2016. Advances in corneal cell therapy. *Regen. Med.* 11 (6), 601–615.
- Ghezzi, C.E., Marelli, B., Omenetto, F.G., Funderburgh, J.L., Kaplan, D.L., 2017. 3D Functional corneal stromal tissue equivalent based on corneal stromal stem cells and multi-layered silk film architecture. *PloS One* 12 (1), e0169504.
- Ghoubar, D., Borderie, M., Grieve, K., Martos, R., Bocheux, R., Nguyen, T.M., Callard, P., Chedotal, A., Borderie, V.M., 2020. Corneal stromal stem cells restore transparency after N2 injury in mice. *Stem Cells Transl Med* (in press).
- Hertsberg, A.J., Shojaati, G., Funderburgh, M.L., Mann, M.M., Du, Y., Funderburgh, J.L., 2017. Corneal stromal stem cells reduce corneal scarring by mediating neutrophil infiltration after wounding. *PloS One* 12 (3), e0171712.
- Huang, S., Xu, L., Sun, Y., Wang, K., Li, G., 2015. An improved protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow. *J Orthop Translat* 3 (1), 26–33.
- Kao, W.W., Kao, C.W., Kaufman, A.H., Kombrinck, K.W., Converse, R.L., Good, W.V., Bugge, T.H., Degen, J.L., 1998. Healing of corneal epithelial defects in plasminogen- and fibrinogen-deficient mice. *Invest. Ophthalmol. Vis. Sci.* 39 (3), 502–508.
- Kao, W.W., Liu, C.Y., 2002. Roles of lumican and keratocan on corneal transparency. *Glycoconj. J.* 19 (4–5), 275–285.
- Karamichos, D., Guo, X.Q., Hutcheon, A.E., Zieske, J.D., 2010. Human corneal fibrosis: an *in vitro* model. *Invest. Ophthalmol. Vis. Sci.* 51 (3), 1382–1388.

- Krishnan, T., Prajna, N.V., Gronert, K., Oldenburg, C.E., Ray, K.J., Keenan, J.D., Lietman, T.M., Acharya, N.R., 2012. Gender differences in re-epithelialisation time in fungal corneal ulcers. *Br. J. Ophthalmol.* 96 (1), 137–138.
- Kumar, A., Xu, Y., Yang, E., Du, Y., 2018. Stemness and regenerative potential of corneal stromal stem cells and their secretome after long-term storage: implications for ocular regeneration. *Invest. Ophthalmol. Vis. Sci.* 59 (8), 3728–3738.
- Liu, H., Zhang, J., Liu, C.Y., Wang, L.J., Sieber, M., Chang, J., Jester, J.V., Kao, W.W., 2010. Cell therapy of congenital corneal diseases with umbilical mesenchymal stem cells: lumican null mice. *PloS One* 5 (5), e10707.
- Muller, U., 1999. Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. *Mech. Dev.* 82 (1–2), 3–21.
- Newsome, D.A., Foidart, J.M., Hassell, J.R., Krachmer, J.H., Rodrigues, M.M., Katz, S.I., 1981. Detection of specific collagen types in normal and keratoconus corneas. *Invest. Ophthalmol. Vis. Sci.* 20 (6), 738–750.
- Shojaati, G., Khandaker, I., Funderburgh, M.L., Mann, M.M., Basu, R., Stolz, D.B., Geary, M.L., Dos Santos, A., Deng, S.X., Funderburgh, J.L., 2019. Mesenchymal stem cells reduce corneal fibrosis and inflammation via extracellular vesicle-mediated delivery of miRNA. *Stem Cells Transl Med* 8 (11), 1192–1201.
- Shojaati, G., Khandaker, I., Sylakowski, K., Funderburgh, M.L., Du, Y., Funderburgh, J.L., 2018. Compressed collagen enhances stem cell therapy for corneal scarring. *Stem Cells Transl Med* 7 (6), 487–494.
- Soboleski, M.R., Oaks, J., Halford, W.P., 2005. Green fluorescent protein is a quantitative reporter of gene expression in individual eukaryotic cells. *Faseb. J.* 19 (3), 440–442.
- Stern, J.H., Tian, Y., Funderburgh, J., Pellegrini, G., Zhang, K., Goldberg, J.L., Ali, R.R., Young, M., Xie, Y., Temple, S., 2018. Regenerating eye tissues to preserve and restore vision. *Cell Stem Cell* 23 (3), 453.
- Syed-Picard, F.N., Du, Y., Hertsberg, A.J., Palchesko, R., Funderburgh, M.L., Feinberg, A.W., Funderburgh, J.L., 2018. Scaffold-free tissue engineering of functional corneal stromal tissue. *J. Tissue Eng Regen Med* 12 (1), 59–69.
- Trinkaus-Randall, V., Leibowitz, H.M., Ryan, W.J., Kupferman, A., 1991. Quantification of stromal destruction in the inflamed cornea. *Invest. Ophthalmol. Vis. Sci.* 32 (3), 603–609.
- Wang, S.B., Hu, K.M., Seamon, K.J., Mani, V., Chen, Y., Gronert, K., 2012. Estrogen negatively regulates epithelial wound healing and protective lipid mediator circuits in the cornea. *Faseb. J.* 26 (4), 1506–1516.
- Wu, J., Du, Y., Mann, M.M., Yang, E., Funderburgh, J.L., Wagner, W.R., 2013. Bioengineering organized, multilamellar human corneal stromal tissue by growth factor supplementation on highly aligned synthetic substrates. *Tissue Eng.* 19 (17–18), 2063–2075.
- Wu, J., Du, Y., Watkins, S.C., Funderburgh, J.L., Wagner, W.R., 2012. The engineering of organized human corneal tissue through the spatial guidance of corneal stromal stem cells. *Biomaterials* 33 (5), 1343–1352.
- Yam, G.H., Fuest, M., Yusoff, N.Z., Goh, T.W., Bandeira, F., Setiawan, M., Seah, X.Y., Lwin, N.C., Stanzel, T.P., Ong, H.S., Mehta, J.S., 2018. Safety and feasibility of intrastromal injection of cultivated human corneal stromal keratocytes as cell-based therapy for corneal opacities. *Invest. Ophthalmol. Vis. Sci.* 59 (8), 3340–3354.
- Zhou, H., Kimura, K., Orita, T., Nishida, T., Sonoda, K.H., 2011. Inhibition by female sex hormones of collagen degradation by corneal fibroblasts. *Mol. Vis.* 17, 3415–3422.

Appendix 4

Acceptance email

From:

Sent: Monday, October 12, 2020 9:13 PM

To:

Subject: Decision has been reached on your submission to Eye and Vision - EAVI-D-20-00301R3

EAVI-D-20-00301R3

The anti-scarring effect of corneal stromal stem cell therapy is mediated by transforming growth factor β 3

Lin Weng; James L Funderburgh; Irena Khandaker; Moira L Geary; Tianbing Yang; Rohan Basu;
Martha L Funderburgh; Yiqin Du; Gary Hin-Fai Yam, PhD
Eye and Vision

Dear Dr Yam,

We are pleased to inform you that your manuscript "The anti-scarring effect of corneal stromal stem cell therapy is mediated by transforming growth factor β 3" (EAVI-D-20-00301R3) has been accepted for publication in Eye and Vision.

Before publication, our production team will check the format of your manuscript to ensure that it conforms to the standards of the journal. They will be in touch shortly to request any necessary changes, or to confirm that none are needed.

We look forward to publishing your manuscript and we do hope you will consider Eye and Vision again in the future.

Best wishes,

The Eye and Vision Editorial Team

e-mail: editorial@eandv.org

Web: <https://nam12.safelinks.protection.outlook.com/?url=http%3A%2F%2Fwww.eandv.org%2F&data=02%7C01%7Cgary.yam%40pitt.edu%7Cfc536c58de434336791208d86f1d9c28%7C9ef9f489e0a04eeb87cc3a526112fd0d%7C1%7C0%7C637381520250532244&sdata=R%2FBg5nok03v9YJw%2BRNgIf3UrsrkNLPEWH5p9Wyz6lRo%3D&reserved=0>

Appendix 4

Accepted manuscript

The anti-scarring effect of corneal stromal stem cell therapy is mediated by transforming growth factor β 3

Lin Weng^{1,2}, James L Funderburgh^{1#}, Irena Khandaker¹, Moira L Geary¹, Tianbing Yang¹, Rohan Basu¹, Martha L Funderburgh¹, Yiqin Du¹, Gary Hin-Fai Yam^{1*}

¹Department of Ophthalmology, University of Pittsburgh School of medicine, Pittsburgh, PA 15213, United States

²Shanghai Lanhe Optometry and Ophthalmology Clinic, Shanghai 200032, P. R. China

[#]This paper is dedicated to the memory of Professor James L. Funderburgh

Short title: TGF β 3 in corneal stromal stem cells

Number of Tables: 1

Number of Figures: 7

***Correspondence:**

Gary Hin-Fai Yam, Ph.D.

Department of Ophthalmology, University of Pittsburgh, 203 Lothrop Street, Pittsburgh, Pennsylvania 15213, US.

Telephone: +1 412 647 3853

E-mail: yamg@pitt.edu / gary.yam@gmail.com

Abstract

Background: Corneal stromal stem cells (CSSC) reduce corneal inflammation, prevent fibrotic scarring, and regenerate transparent stromal tissue in injured corneas. These effects rely on factors produced by CSSC to block the fibrotic gene expression. This study investigated the mechanism of the scar-free regeneration effect.

Methods: Primary human CSSC (hCSSC) from donor corneal rims were cultivated to passage 3 and co-cultured with mouse macrophage RAW264.7 cells induced to M1 pro-inflammatory phenotype by treatment with interferon- γ and lipopolysaccharides, or to M2 anti-inflammatory phenotype by interleukin-4, in a Transwell system. The time-course expression of human transforming growth factor $\beta 3$ (hTGF $\beta 3$) and hTGF $\beta 1$ were examined by immunofluorescence and qPCR. TGF $\beta 3$ knockdown for >70% in hCSSC [hCSSC-TGF $\beta 3$ (si)] was achieved by small interfering RNA transfection. Naïve CSSC and hCSSC-TGF $\beta 3$ (si) were transplanted in a fibrin gel to mouse corneas, respectively, after wounding by stromal ablation. Corneal clarity and the expression of mouse inflammatory and fibrosis genes were examined.

Results: hTGF $\beta 3$ was upregulated by hCSSC when co-cultured with RAW cells under M1 condition. Transplantation of hCSSC to wounded mouse corneas showed significant upregulation of hTGF $\beta 3$ at days 1 and 3 post-injury, along with the reduced expression of mouse inflammatory genes (*CD80*, *C-X-C motif chemokine ligand 5*, *lipocalin 2*, *plasminogen activator urokinase receptor*, *pro-platelet basic protein*, and *secreted phosphoprotein 1*). By day 14, hCSSC treatment significantly reduced the expression of fibrotic and scar tissue genes (*fibronectin*, *hyaluronan synthase 2*, *Secreted protein acidic and cysteine rich*, *tenascin C*, *collagen 3a1* and α -smooth muscle actin), and the injured

corneas remained clear. However, hCSSC-TGF β 3(si) lost these anti-inflammatory and anti-scarring functions, and the wounded corneas showed intense scarring.

Conclusion: This study has demonstrated that the corneal regenerative effect of hCSSC is mediated by TGF β 3, inducing a scar-free tissue response.

Keywords: Cornea wound healing; corneal stromal stem cells; TGF β 3; inflammation; fibrosis

List of abbreviations

ARG1: arginase 1

Col3a1: collagen 3a1

FBS: fetal bovine serum

hCSSC: human corneal stromal stem cells

HuNu: human specific nuclear antigen

IL-4: interleukin-4

iNOS: inducible nitric oxide synthase

LPS: lipopolysaccharides

mRAW: mouse RAW264.7 macrophages

MSC: mesenchymal stem cells

TGF: transforming growth factor

TNC: tenascin C

TNF α : tumor necrosis factor- α

TSG-6: tumor necrosis factor-stimulated gene 6

α SMA: α -smooth muscle actin

siRNA: small interfering RNA

Background

Vision loss is a global burden. Corneal scarring due to trauma or infection is a major cause of blindness with about 10 million people being affected worldwide [1]. The conventional treatment for most existing corneal scars is corneal transplantation (penetrating or lamellar keratoplasty) [2]. Although this procedure is regarded as a highly developed and successful treatment, it is restricted by the global shortage of transplantable donor materials, allograft immune response, long-term graft survival and complications, and the need of surgical expertise [1]. There is a clear need for new approaches to prevent and treat corneal scarring. Corneal stromal stem cells (CSSC), identified in the anterior limbal stroma, represent the mesenchymal progenitors of stromal keratocytes, the dominant cell type inside the corneal stroma [3]. Under our optimized culture condition, primary human CSSC (hCSSC) at early passages exhibit stem cell features, including clonal growth and expression of stem cell markers (CD73, CD90, CD166, SSEA4, OCT4, ABCG2, Nestin and Pax6), as well as the ability to differentiate to keratocytes when induced by specific cytokines, and negligible expression of fibroblast-related genes, such as α -smooth muscle actin (α SMA) and tenascin C (TNC) [4-7]. Using different animal models, including lumican knockout mice and corneal stromal ablation, the applications of hCSSC prevented fibrotic tissue formation, reduced stromal haze, and restored the corneal stromal collagen fibril microstructure and corneal transparency [8-11]. The treated corneas regenerated organized collagenous extracellular matrix (ECM), similar to that in the native corneal stroma [12, 13]. These findings demonstrate the therapeutic potential of hCSSC in repairing stromal damages and regenerating the transparent cornea. The *ex vivo* propagation allows cells

from one donor cornea to be used for multiple recipients or for multiple treatments, thus obviating the shortage of donor materials [14, 15].

The underlying mechanisms of how CSSC reduce corneal fibrosis and scarring, as well as regenerate stromal tissue, have yet to be elucidated. When cultured in low mitogen, ascorbate-containing media, hCSSC expressed an array of genes characteristic of keratocytes [3]. When cultured on a substratum of parallelly aligned nanofibers, the cells deposited layers of collagen fibrils packed in a highly organized pattern, closely resembling the native stromal lamellae [15, 16]. After injection into mouse corneal stroma, the added cells remained viable for many months, and appeared as quiescent keratocytes, expressing typical genes of keratocytes and producing human ECM components [8, 9, 17]. These observations strongly indicate that CSSC differentiate by default into a keratocyte lineage and attain keratocyte functions. Moreover, injection of hCSSC into mouse corneal stroma did not elicit T-cell-mediated immune response, suggesting their ability to immune tolerance and block inflammatory signals [8, 11]. The production of TSG-6 (tumor necrosis factor-stimulated gene 6) in wounded corneas further demonstrated the ability of hCSSC to block neutrophil infiltration, thus reducing the myofibroblast differentiation and scar-associated gene expression, including collagen 3a1 (Col3a1), tenascin C (TNC) and α -smooth muscle actin (α SMA) [18]. Recently, we have shown that hCSSC induced corneal regeneration via an indirect cell-cell interaction, an effect consistent with the paracrine action of extracellular vesicles [19]. Such treatment effects disappeared when the vesicles were depleted of microRNAs, after specific Alix knockdown, suggesting that some microRNAs could have therapeutic effects on corneal fibrosis and stromal regeneration.

Whether CSSC promote tissue regeneration by other mechanisms, in particular the scar-free healing response to produce transparent corneas, is under investigation.

Wound healing is regulated by various growth factors, including epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, interleukins (e.g., IL-1, 2, 6, 8) and tumor necrosis factor- α (TNF α) [20-22]. In different organs, including the cornea, members of transforming growth factor β (TGF β) family are known as key regulators of fibrosis and scar formation; by way of mechanisms underlying the canonical TGF β /Smad signaling [23-25]. Target inhibition and modulation of TGF β signaling using small interfering RNAs (siRNA) or neutralizing antibodies to regulate fibrosis and modulate wound healing have been reported in different studies [26-28]. The TGF β family consists of three closely related isoforms (β 1, β 2 and β 3), with distinct roles in cell differentiation, tissue regeneration and embryonic development [29]. TGF β 1 and β 2 mediate tissue fibrosis and scar formation [30, 31], whereas TGF β 3 acts as an inhibitor to these events [32]. The anti-fibrotic and scar-free healing effect of TGF β 3 have been reported in skin, lung and kidney models [33].

In this study, we examined TGF β 3 expression in hCSSC under pro- and anti-inflammatory conditions, mimicking different scenarios of tissue injury and wound healing process. The role of TGF β 3 and its knockdown expression on the stromal regenerative functions of hCSSC were also studied using a mouse corneal wound model.

Material and Methods

Human corneal biopsies

Human corneal-scleral rims (n=4), from de-identified donors younger than 60 years old and approved for research use, were obtained from the Center for Organ Recovery and Education, Pittsburgh, PA, USA. Tissues used were less than 9 days post-enucleation and preserved in Optisol GS (Bausch & Lomb Inc., Rochester, NY, US). Research followed the tenets of the Declaration of Helsinki and was approved by the University of Pittsburgh Institutional Review Board (IRB) and Committee for Oversight of Research and Clinical Training Involving Decedents (CORID protocol #161).

Human corneal stromal stem cell isolation and culture

Corneal rims, after the removal of corneal epithelium and endothelium, were dissected to isolate the anterior limbal stroma (1-2 mm wide, 0.5 mm deep), which was then cut into small segments for digestion (16 hours at 37°C) with collagenase A (0.5 mg/ml, Sigma-Aldrich, St Louis, MO, US), as previously described [10]. The single cell suspension was passed through a cell strainer (70 µm pore size, Corning, NY, US) and cells were seeded on culture surface coated with FNC mix (AthenaES, Baltimore, MD, US) with stem cell growth medium (JM-H) containing 2% pooled human serum (Innovative Res, Novi, MI, US) [34]. At 70% confluence, primary cells were collected after brief digestion with TrypLE Express (Thermo Fisher, Waltham, MA, US) and plated at 10 cells/cm² for clonal cell growth in subsequent passages. CSSC from a single donor cornea were expanded to P3 for use in experiments. Cultured cells expressed markers specific for mesenchymal stem cells (MSC) (including CD73, CD90 and CD166), and adult stem cells (including ABCG2, Nestin, Pax6, NGFR) by flow cytometry and RT-PCR [7, 10].

TGFβ3 knockdown in human CSSC by siRNA transfection

Human CSSC were washed twice to remove dead cells and debris. In fresh culture medium, they were added with a mixture of Viromer Blue transfection reagent for Transfection of Adherent Cells (OriGene, Rockville, MD, US), Viromer Blue buffer and siRNA specific for hTGFβ3 (42 nM; Ambion; antisense sequence: UCA GAG UGU ACA GUC CCA) or scrambled sequences (Silencer™ Select Negative Control #2, Ambion), according to the manufacturer's protocol. After 24 hours, the cells were placed in a Transwell system and co-cultured with RAW at M1 induction (method in next section) for 44 hours. Total protein samples were collected for hTGFβ3 and hTGFβ1 expression by western blotting.

***In vitro* inflammation assay**

In a Transwell system, mouse macrophage RAW264.7 cells (American Type Cell Collection, ATCC, Manassas, VA, US) were plated at 10^4 cells/cm² in DMEM/F-12 (Thermo Fisher) with 10% fetal bovine serum (FBS, Thermo Fisher) in the lower chamber. They were induced to M1 phenotype by treatment with interferon-γ (IFN-γ, 30 ng/ml; BioLegend, San Diego, CA, US) and lipopolysaccharides (LPS, 100 ng/ml; Sigma-Aldrich) or to M2 phenotype by interleukin-4 (IL-4, 20 ng/ml; BioLegend). hCSSC were seeded at 2×10^4 cells/cm² in the upper chamber and cultured to near confluence before assembly with the lower chamber having RAW cells for co-culture. At regular intervals up to 72 hours, RAW cells were collected for the expression of M0, M1 and M2 phenotype markers, while hCSSC were assayed for hTGFβ1 and hTGFβ3 expression.

Quantitative reverse transcription-polymerase chain reaction (qPCR)

Cells were lysed in RLT reagent (Qiagen, Germantown, MD, US) and total RNA was extracted using RNeasy Miniprep (Qiagen) followed by RNase-free DNase digestion (New England Biolabs, Ipswich, MA, US) according to the manufacturer's instructions. 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 candidate gene expression with specific TaqMan probes using universal master mix (Thermo Fisher) or specific target primers (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 triplicates. The relative RNA abundance was assayed after normalization with housekeeping 18S or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes and compared to M0 condition. The fold changes of each gene were calculated by $2^{-\Delta\Delta CT}$ and expressed as mean $\pm$ standard deviation (SD).

Western blotting

Cells were lysed in a radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher) added with CompleteTM protease inhibitor cocktail (Roche, Indianapolis, IN, US) and phenylmethylsulfonylfluoride (PMSF, 1 mM, Sigma-Aldrich). 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) and transferred to PVDF-FL membrane (BioRad). After blocking with 5% non-fat milk, the membrane was incubated with antibodies against TGF β 3 (Abcam), TGF β 1 (Abcam) and housekeeping α -tubulin (Sigma-Aldrich), followed

by species-specific secondary IRDye-labeled antibodies (LI-COR Biosciences, Lincoln, NE, US). Staining signals were detected and digitized using Odyssey scanner (LI-COR). The specific band density of TGF β 3 was measured and normalized to that of α -tubulin for relative expression in each sample. Experiments were conducted in duplicates.

Immunocytochemistry

Cells cultured on sterile glass coverslips were fixed in 2% neutral-buffered paraformaldehyde (EM Sciences, Hatfield, MA, US) for 20 min at room temperature. The rinsed samples were permeabilized and blocked in buffer containing 3% normal goat serum (Thermo Fisher) and 0.3% Triton X-100 (Tx, Sigma-Aldrich) for 45 min, followed by incubation with rabbit polyclonal antibody against human TGF β 3 (Abcam) or host species-matched isotype-specific immunoglobulin (Ig) overnight at 4°C. After washes using PBS with 0.1% BSA and 0.1% Tween-20, the samples were stained with anti-rabbit Ig conjugated with AlexaFluor 488 (Thermo Fisher) and anti-human nuclear antigen-PE (phycoerythrin) conjugate (Abcam) for an hour at room temperature in the dark. After washing, the coverslips were mounted with Fluoromount-G[®] containing 4'6-diamidino-2-phenylindole (DAPI) (Thermo Fisher) and viewed under confocal microscopy (FluoView 1000, Olympus). Experiments were performed in triplicates.

Murine corneal stromal wound model and CSSC treatment

The animal experiments were conducted in strict accordance with the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH, Bethesda, MD, US) and The Association for Research in Vision and Ophthalmology statement for

the use of animals in ophthalmic and vision research. The protocol was approved by the Institutional Animal Care and Use Committee of The University of Pittsburgh (ISO00012511). C57/BL6 mice (n=20 per experiment, total n=60 for triplicate runs) Charles River Lab, Wilmington, MA, US), 7–8 weeks of age, were housed in an AALAC-approved ABSL2 facility, and provided an unrestricted standard diet. They were anesthetized by intraperitoneal ketamine (50 mg/kg) and xylazine (5 mg/kg), and both eyes received topical proparacaine hydrochloride (0.5%, Alcaine®, Alcon, Fort Worth, TX, US). After saline rinses, the central corneal epithelium (2 mm diameter) was debrided using Algerbrush II (Accutome Inc, Malvern, PA, US) and surgical blade #15. The basement membrane and anterior stromal tissue was further removed by a second application of AlgerBrush [17]. The eyes were rinsed and briefly dried before cell treatment. Immediately after wounding, hCSSC in 10^4 cells/ μ l human fibrinogen (70 μ g/ml, Sigma-Aldrich) were mixed with 0.5 μ l thrombin (100 U/ml; Sigma-Aldrich) and applied to the wound site. After gelation, a second round of fibrinogen and thrombin was applied to the gel surface. The eyes received topical gentamicin (0.3%, Genoptic®, USP, Rockville, MD, US) and ketoprofen (3 mg/kg) for analgesia. We carefully designed the grouping of mouse eyes for injury and cell treatment so that each mouse had one functional eye for daily use and survival. In each set of experiment, 10 mice had one cornea injured, which served as sham control treated with medium without cells; and the other cornea remained normal. Another 10 mice had both corneas injured with one of them treated with hCSSC (reported to retain vision) [9, 10], and the other with hCSSC-TGF β 3(si). Experiments were done in triplicates.

Corneal scar intensity measurement

At day 14 post-wounding and cell treatment, the eyes were scanned with a Spectral domain Optical Coherent Topography (SDOCT) (Bioptigen, Durham, CA, US). Scanned images were analyzed in a masked fashion regarding the treatments. Image processing and analysis were conducted using ImageJ (NIH) and MetaMorph 7.7.3 (Molecular Devices Inc, San Jose, CA, US) [10]. For scar intensity quantification, pixel intensity were obtained from the corneal region with ImageJ and the average background intensity was subtracted. Control eyes were used to set the lower threshold and scar intensity of each mouse and the overall mean and SD are displayed graphically.

Mouse corneal tissue collection for RNA expression analysis

At post-wounding and treatment days 1, 3 and 14, mice were sacrificed. After enucleation, corneas were immediately excised on ice. Four to six corneas per group were pooled in ice-cold RLT lysis buffer (Qiagen) and disrupted with a MagNA Lyser Green Beads kit (Roche) at 6,000 rpm for 50 sec, in a MagNA Lyser Instrument (Roche), for 6 cycles, each with intermittent cooling. The lysate was passed through QIAshredder column and total RNA was isolated using RNeasy Miniprep (Qiagen) following manufacturer's instructions. Total RNA (0.5 µg) was transcribed to cDNA by SuperScript III (Thermo Fisher). Gene expression was assayed by qPCR with specific primers or TaqMan probes as listed in Table 1. Experiment was done in triplicates.

Statistical analysis

All data were presented as mean $\pm$ SD. Mean value was compared by two-tailed t tests or ANOVA with a post hoc Bonferroni test using GraphPad Prism 7. Corneal scar intensities

were compared using non-parametric Mann-Whitney U test. $P < 0.05$ was considered statistically significant.

Results

Human CSSC expressed TGF β 3 in M1 pro-inflammatory phase of mouse macrophages

The functional response and stability of mouse RAW264.7 macrophages (RAW) was examined by cytokine stimulation to M1 (pro-inflammatory) and M2 (anti-inflammatory) conditions. RAW propagated at M0 phase (Fig. 1a) were treated with LPS and IFN γ to M1 phenotype and showed a consistent upregulation of mouse iNOS (inducible nitric oxide synthase) at various time intervals (Fig. 1b). The M1 macrophages were differentiated, appeared spindle-shape and adhered to the culture surface (Fig. 1a). When treated with IL4, RAW were maintained as small, rounded morphology and had upregulated mouse ARG1 (arginase 1) expression (Fig. 1a and b), indicative of the M2 phenotype. Similar expression patterns were reported in different studies [35, 36]. The co-culture of hCSSC with RAW at the M1 stage demonstrated an inflammation-associated cellular response with an elevated expression of TSG-6 (Fig. 1b). In this pro-inflammatory condition, hTGF β 3 was significantly upregulated (Fig. 1c), starting at 24 hours of co-culture and continuing to increase until the end of experiment (84 hours). This induction was absent in M0 and M2 phases of macrophages. On the other hand, there was no significant changes of hTGF β 1 expression in hCSSC under M0, M1, or M2 conditions.

Immunostaining showed that TGF β 3 was expressed in hCSSC when co-cultured with RAW cells in M1 phase (Fig. 2a). Since the anti-TGF β 3 antibody appeared labeled in

mouse RAW cells (both nuclear and cytoplasmic) (Fig. 2d), the specificity in hCSSC was distinguished by the co-labelling of HuNu (human specific nuclear antigen). In HuNu-labelled hCSSC, TGF β 3 expression was evident under the M1 pro-inflammatory condition. TGF β 3 signals in hCSSC were located inside the cytoplasm as a diffuse punctate pattern with some labelling close to the plasma membrane (Fig. 2a). When co-cultured with RAW cells at M2 and M0 phases, hCSSC negligibly expressed TGF β 3 (Fig. 2b and c).

M1 macrophages induced hCSSC to produce TGF β 3 via non-contact cell-to-cell communication

In a Transwell system, hCSSC and RAW cells were placed in separate chambers. In the presence of RAW cells treated with LPS+IFN γ to induce M1 pro-inflammatory condition, the expression of hTGF β 3 in hCSSC was detected after 24 hours and was significantly upregulated by 8.2 ± 0.1 folds at 48 hours, compared to the untreated control (Fig. 3a). Without RAW co-culture, hCSSC under M1 induction did not show any hTGF β 3 changes. When comparing this Transwell co-culture of hCSSC with RAW cells under M0, M1 or M2 conditions for 36 hours, we observed a significant upregulation of hTGF β 3 in hCSSC in the presence of M1 RAW cells, but not with M0 and M2 cells (Fig. 3b). Again, TGF β 1 expression was not altered under different phases (Fig. 3b).

Suppression of hTGF β 3 expression in hCSSC under M1 condition by siRNA

In the Transwell setting, (i) naïve hCSSC, (ii) hCSSC transfected with hTGF β 3 siRNA [hCSSC- TGF β 3(si)], or (iii) hCSSC transfected with scrambled sequences were co-cultured with RAW cells under M1 condition. After 48 hours, hCSSC-TGF β 3(si) showed

reduced hTGF β 3 expression, by 70-84% of that in naïve hCSSC (Fig. 4a). This was not found for hCSSC transfected with scrambled sequences. The expression of hTGF β 1 was not affected by hTGF β 3 knockdown (Fig. 4b).

hTGF β 3 was upregulated in mouse stromal wounds after hCSSC treatment

Our *in vitro* models showed hTGF β 3 upregulation in hCSSC under a pro-inflammatory condition. We further explored this association with an *in vivo* model of corneal injury and wound healing. Human CSSC were applied in a fibrin gel to mouse corneal stromal ablation wounds. At day 1 and 3 after wounding and cell treatment, hTGF β 3 was significantly upregulated, when compared to hCSSC before transplantation ($P < 0.05$, One-way ANOVA) (Fig. 5). The expression of hTGF β 3 on day 1 was increased by 63 ± 8 folds (Fig. 5a) and to a high of 256 ± 63 folds at day 3 (Fig. 5b). This change was negligible with hCSSC-TGF β 3(si) treatment. In contrast, the expression of hTGF β 1 had no change on day 1 post-wounding and treatment (Fig. 5a) and had only mild elevation at day 3 (4 ± 1.8 folds, compared to non-transplanted hCSSC; Fig. 5b). In terms of hTGF β 3/ β 1 ratio, hCSSC treatment for 1 and 3 days showed similar range of values (Fig. 5a and b). The mean ratio at day 1 was 45 and on day 3 was 65 (n=5 mice per group with injury only and injury with hCSSC treatment, respectively).

Both anti-inflammatory and anti-fibrotic effects of hCSSC treatment on mouse corneal stromal wounds were reduced by TGF β 3 knockdown

In our previous analysis using nanoString™ for Mouse Inflammation and Mouse Pan-Cancer Panels, a number of mouse inflammatory genes were affected by hCSSC treatment

[19]. We assayed the RNA expression of early inflammatory genes, including *CD80*, *C-X-C motif chemokine ligand 5 (CXCL5)*, *lipocalin 2 (Lcn2)*, *plasminogen activator urokinase receptor (PLAUR)*, *pro-platelet basic protein (Pbbp)* and *secreted phosphoprotein 1 (Spp1)*. These genes were upregulated at day 1 after wounding when compared to normal corneas, and the elevated expression was significantly suppressed by hCSSC treatment (Fig. 6). This anti-inflammatory effect was abolished by the treatment with hCSSC-TGF β 3(si) ($P<0.05$, one-way ANOVA), with levels of gene expression similar to that of the wounded corneas without treatment.

At day 14 post-wounding and cell treatment, the scar-reducing effect by hCSSC was abolished when the treatment used hCSSC-TGF β 3(si) (Fig. 7a). On brightfield illumination of the corneas, the mean scar intensity (n=6 corneas per group) was significantly greater after treatment with hCSSC-TGF β 3(si) when compared to naïve hCSSC ($P=0.008$, Mann-Whitney U test; Fig. 7b). Expression of mouse fibrosis genes (*Col3a1* and α SMA) was significantly suppressed by naïve hCSSC treatment ($P<0.05$, paired Student's t-test) but remained elevated with hCSSC-TGF β 3(si) (Fig. 7c). This was similarly observed for other fibrotic genes, including *fibronectin (FN1)*, *hyaluronan synthase 2 (HAS2)*, *Secreted protein acidic and cysteine rich (SPARC)* and *tenascin C (TNC)* (Fig. 7d).

Discussion

In this study, we describe a novel underlying mechanism of hCSSC regeneration of corneal stroma after corneal injury. Specifically, our data demonstrate that: (i) TGF β 3 expression by hCSSC is upregulated in an inflammatory milieu *in vitro*; (ii) hTGF β 3 is expressed after

hCSSC treatment to the stromal wound of mouse corneas and (iii) the specific knockdown of TGFβ3 attenuates these anti-inflammatory and anti-fibrotic activities of hCSSC *in vivo*. These findings have deepened our understanding on the therapeutic regulation of CSSC to suppress corneal haze and scarring, and to regenerate stromal tissue.

TGFβ3 was expressed by hCSSC in an inflammatory milieu *in vitro* and *in vivo*. We set up a co-culture system of hCSSC interacting with murine macrophages (RAW264.7 cells) having M0, M1 and M2 phenotypes, mimicking the uncommitted, pro-inflammatory and anti-inflammatory scenarios, respectively, during injury and wound healing process. Human TGFβ3 was significantly upregulated in hCSSC in the presence of M1-type macrophage (pro-inflammatory). The induction was detected at around 24 hours of co-culture and escalated thereafter. Similar results were detected when hCSSC were co-cultured with M1 macrophage in a Transwell system, suggesting paracrine action of diffusible molecules from the activated macrophages. Since negligible TGFβ3 changes were detected when hCSSC alone was treated with IFNγ and LPS, the cytokine induction is suggested to be specifically associated to the presence of M1 macrophages, and possibly the secreted molecules from macrophages. The production of IL-1β and IL-6 from activated macrophages has been shown to upregulate TGFβ3 expression in chondrocytes [37, 38]. Here, we show that CSSC might adopt a similar response to the activated macrophages. Further studies will identify the molecular mechanisms underlying CSSC responses in this wound-associated pro-inflammatory condition.

Using an *in vivo* mouse corneal wounding model, hTGFβ3 expression was also significantly upregulated after hCSSC treatment; there was an increase from day 1 to 3 post-treatment (the mean change of 63 folds at day 1 increased to 256 folds at day 3). At a

later time point (day 14), the treated corneas were devoid of haze formation and remained clear, similar to our previous studies [9, 18]. This therapeutic effect was suspended with hCSSC treated to TGF β 3 knockdown. As such, the anti-inflammatory and anti-fibrotic functions were lost, and the injured corneas developed intense scarring. The timing of TGF β 3 production by hCSSC in the early wound healing phase was similar to that of TSG-6 expression, which was also detectable after 1 day of hCSSC treatment on fresh stromal wounds [18]. Corneal injury-induced inflammation is initiated with the infiltration of neutrophils, followed by macrophages. CSSC, displaying the characteristics of MSC, could serve as a guardian against the excessive inflammatory responses through multiple mechanisms e.g., the expression of IL-1 receptor antagonist blocking IL-1 inflammatory signaling, TSG-6 production to reduce NF- κ B signaling in the resident macrophages while producing prostaglandin E2 to induce IL-10 expression by macrophages, as well as the expression of sodium dismutase 3 to reduce radical oxygen species in suppressing innate immunity [39]. Among them, the blocking of neutrophil infiltration is crucial at the early wound stage to control inflammation; however, this action might not be effective for anti-fibrosis as hCSSC producing TSG-6 still exhibited fibrotic gene expression [18]. Though our work has demonstrated that CSSC functions through TGF β 3 signaling to control fibrosis development, whether any coordinated or synergistic action by TGF β 3 and TSG-6 in controlling inflammation and wound healing awaits further investigation. In the present corneal wounding study, only female mice were used due to them being less aggressive after wounding and during social housing, to give reliable results. In fact, both sexes have been shown to exhibit similar responses of corneal stromal wound healing, scar

formation and stem cell treatment outcomes in our continuous studies (manuscript submitted).

In the human genome, there are 33 functional genes encoding TGF β family polypeptides [40]. Besides the three TGF β isoforms (β 1, β 2 and β 3), the family sequences consist of bone morphogenic proteins (BMPs), “growth and differentiation factors” (GDFs), activins, inhibins and nodal proteins [41]. The TGF β isoforms regulate a variety of cellular processes in both development and adults through gene regulation via Smad- and non-Smad-dependent pathways [42]. In the context of Smad-dependent signaling, which is more extensively studied, the mature and dimeric TGF β ligands signal through the binding to the cell surface receptor complexes that combine two type I and two type II receptors (T β RI and II), resulting in conformational changes at the ligand-receptor interface and receptor activation [43, 44]. Smad proteins then convey signals from the receptors into nucleus to modulate target gene expression (review in [42]). TGF β isoforms have pleiotropic functions in controlling physiological phenomena during embryonic development, tissue differentiation and specialization, inflammation and immunity [29]. A broad action profile of TGF β is also found on the proliferation and differentiation of MSC, production of ECM substances and the chemotaxis effect on various cell types involved in wound healing and the associated inflammatory responses [40].

Among the isoforms, TGF β 1 and β 2 is documented to promote fibrosis, fibroblast transition from keratocytes and myofibroblast differentiation, and to induce fibrosis and scar formation after wounding [45]. TGF β 3, on the other hand, is shown to be anti-fibrotic in skin, lung and kidney models [46-48]. In wounded human corneas, an increase in TGF β 3 levels relative to TGF- β 1/ β 2 isoforms was associated with a scar-free phenotype [49, 50].

The presence of TGF β 3 in long-term culture of human stromal fibroblasts resulted in non-fibrotic characteristics and the deposited ECM resembled native stromal tissue. When compared to the treatment with TGF β 1 or β 2, the expression of Col3 and α SMA was suppressed by β 3, while Col1 synthesis was unaltered. In another study, TGF β 3 reduced the fibrotic gene expression of β 1-treated fibroblasts, indicating a possible “rescue” effect in addition to the preventive action on fibrosis development [32]. In a tendon injury model, the addition of TGF β 3 to tenocytes modulated Smad signaling by downregulating Smad3 and its phosphorylation, which suppressed the nuclear translocation of Smad4 and reduced fibrosis-related gene expression [51]. It also upregulated Smad7 to interact with T β R1, which in turn prevented its phosphorylation and activation of receptor-regulated Smad2/3, and thus inhibiting TGF β /Smad signaling.

Why TGF β isoforms give rise to different tissue responses is still a mystery. The TGF β 1, β 2 and β 3 have high sequence homology and share most of their cell surface receptors. After ligand binding, the receptor phosphorylation is important in downstream activities [44]. Additional control can be exerted by ubiquitin and related molecules. T β Rs are marked for polyubiquitination by E3 ligases, recruited via Smad7 to T β R1 in complex, for proteasomal degradation, and hence resulting in different signaling responses [52, 53]. Whether TGF β 3 binding to T β R complex stimulates the degradation of a particular receptor type which lead to changes in gene expression is yet to be determined. Moreover, most ligand presentations to their receptors are regulated by co-receptors, which are transmembrane proteins or proteins anchored in the membrane by glycoposphoinositol. They also mediate signaling by other cytokine receptors, giving a broad integration of signal transduction that impacts diverse cellular and pathophysiological conditions. The

active TGF β 1 and β 3 peptides bind to the T β RII/I complex with high affinity, while β 2 engages with this receptor in the presence of co-receptor T β RIII, a cell surface β -glycan [54]. Whether the crosstalk and differential regulation of receptor activities contribute to the different cytokine actions are important topics to be further elucidated.

Conclusion: hCSSC deliver anti-inflammatory and anti-fibrotic effects via TGF β 3 production, supporting the role of CSSC as a therapeutic agent for corneal repair and regeneration. Without any known harmful effects, exogenous TGF β 3 can be applied to the wound site to stimulate scar-free tissue response; however, CSSC can continuously produce TGF β 3 at physiologically relevant levels. Therefore, the use of native cells is promising in the regeneration of unscarred corneal stroma to restore vision for corneal diseases.

Declarations

1. Ethics approval and consent to participate: This study followed the tenets set forth by the Declaration of Helsinki and was approved by the University of Pittsburgh's Institutional Review Board and Committee for Oversight of Research and Clinical Training Involving Decedents (CORID protocol #161). The animal experiments were conducted with protocol approved by the Institutional Animal Care and Use Committee, The University of Pittsburgh (ISO00012511).

2. Consent for publication: Not applicable.

3. Data Availability Statement: Data are available from the corresponding author upon reasonable request.

4. Competing interests: No financial and non-financial competing interest exist for any author.

5. Funding: This work was supported by the Department of Defense Grant W81WH-19-1-0778 (JLF, YD), NIH Grants RO1 EY016415 (JLF) and P30 EY008098 (JLF), Stein Innovator Award from Research to Prevent Blindness (JLF), Eye and Ear Foundation of Pittsburgh, and Louis J Fox Centre for Vision Restoration.

6. Author contributions: LW, JLF: conception and design; LW, IK, MG, TY, RB, MLF: collection and assembly of data; LW, JLF, YD, GY: data analysis; JLF, YD: financial support; LW, JLF, YD, GY: manuscript writing. All authors approved the manuscript.

7. Acknowledgement: We thank K. Davoli in histological processing of corneal samples and K. Lathrop, PhD, for microscopy training.

8. Authors' information: Not applicable.

References

1. Gain P, Julienne R, He Z, Aldossary M, Acquart S, Congnasse F, et al., Global survey of corneal transplantation and eye banking. *JAMA Ophthalmol*. 2016;134(2):167-73.
2. Tan DT, Dart JK, Holland EJ, Kinoshita S. Corneal transplantation. *Lancet*. 2012;379(9827):1749-61.
3. Du Y, Funderburgh ML, Mann MM, Sandalraj N, Funderburgh JL. Multipotent stem cells in human corneal stroma. *Stem Cells*. 2005;23(9):1266-75.
4. Funderburgh ML, Du Y, Mann MM, SundarRaj N, Funderburgh JL. PAX6 expression identifies progenitor cells for corneal keratocytes. *FASEB J*. 2005;19(10):1371-3.
5. Branch MJ, Hashmani K, Dhillon P, Jones DR, Dua HS, Hopkinson A. Mesenchymal stem cells in the human corneal limbal stroma. *Invest Ophthalmol Vis Sci*. 2012;53(9):5109-16.
6. Kureshi AK, Funderburgh JL, Daniels JT. Human corneal stromal stem cells exhibit survival capacity following isolation from stored organ-culture corneas. *Invest Ophthalmol Vis Sci*. 2014;55(11):7583-8.
7. Funderburgh JL, Funderburgh ML, Du Y. Stem cells in the limbal stroma. *Ocul Surf*. 2016;14(2):113-20.
8. Du Y, Sandalraj N, Funderburgh ML, Harvey SA, Birk DE, Funderburgh JL. Secretion and organization of a cornea-like tissue in vitro by stem cells from human corneal stroma. *Invest Ophthalmol Vis Sci*. 2007;48:5038-45.
9. Du Y, Carlson EC, Funderburgh ML, Birk DE, Pearlman E, Guo N, et al. Stem cell therapy restores transparency to defective murine corneas. *Stem Cells*. 2009;27(7):1635-42.
10. Basu S, Hertsenberh AJ, Funderburgh ML, Burrow MK, Mann MM, Du Y, et al. Human limbal biopsy-derived stromal stem cells prevent corneal scarring. *Sci Transl Med*. 2014;6(266):266ra172.
11. Ghoubay D, Bordeir M, Grieve K, Martos R, Bocheux R, Nguyen TM, et al. Corneal stromal stem cells restore transparency after N₂ injury in mice. *Stem Cells Transl Med*. 2020;9:917-35.
12. Wu J, Du Y, Mann MM, Yang E, Funderburgh JL, Wagner WR. Bioengineering organized, multilamellar human corneal stromal tissue by growth factor supplementation on highly aligned synthetic substrates. *Tissue Eng Part A*. 2013;19(17-18):2063-75.
13. Wu J, Du Y, Mann MM, Funderburgh JL, Wagner WR. Corneal stromal stem cells versus corneal fibroblasts in generating structurally appropriate corneal stromal tissue. *Exp Eye Res*. 2014;120:71-81.
14. Lagali N. Corneal stromal regeneration: current status and future therapeutic potential. *Curr Eye Res*. 2020;45(3):278-90.

15. Wu J, Du Y, Walkins SC, Funderburgh JL, Wagner WR. The engineering of organized human corneal tissue through the spatial guidance of corneal stromal stem cells. *Biomaterials*. 2012;33(5):1343-52.
16. Ghezzi CE, Marelli B, Omenetto FG, Funderburgh JL, Kapan DL. 3D functional corneal stromal tissue equivalent based on corneal stromal stem cells and multi-layered silk film architecture. *PLoS One*. 2017;12(1):e0169504.
17. Syed-Picard FN, Du Y, Hertsenber AJ, Palchesko R, Funderburgh ML, Feinberg AW, et al. Scaffold-free tissue engineering of functional corneal stromal tissue. *J Tissue Eng Regen Med*. 2018;12(1):59-69.
18. Hertsenber AJ, Shojaati G, Funderburgh ML, Mann MM, Du Y, Funderburgh JL. Corneal stromal stem cells reduce corneal scarring by mediating neutrophil infiltration after wounding. *PLoS One*. 2017;12(3):e0171712.
19. Shojaati G, Khandakar I, Funderburgh ML, Mann MM, Basu R, Stolz DB, et al., Mesenchymal stem cells reduce corneal fibrosis and inflammation via extracellular vesicle-mediated delivery of miRNA. *Stem Cells Transl Med*. 2019;8(11):1192-201.
20. Behm B, Babilas P, Landthaler M, Schremi S. Cytokines, chemokines and growth factors in wound healing. *J Eur Acad Dermatol Venereol*. 2012;26(7):812-20.
21. Doersch KM, DelloStritto DJ, Newell-Rogers MK. The contribution of interleukin-2 to effective wound healing. *Exp Biol Med (Maywood)*. 2017;242(4):384-96.
22. Yamakawa S, Hayashida K. Advances in surgical applications of growth factors for wound healing. *Burns Trauma*. 2019;7:10.
23. Karamichos D, Guo ZQ, Hutcheon AE, Zieske JD. Human corneal fibrosis: an in vitro model. *Invest Ophthalmol Vis Sci*. 2010;51(3):1382-8.
24. Meng XM, Nikolic-Paterson DJ, Lan HY. TGF-beta: the master regulator of fibrosis. *Nat Rev Nephrol*. 2016;12(6):325-38.
25. Walton KL, Johnson KE, Harrison CA. Targeting TGF-beta mediated SMAD signaling for the prevention of fibrosis. *Front Pharmacol*. 2017;8:461.
26. Ling H, Roux E, Hempel D, Tao J, Smith M, Lonning S, et al. Transforming growth factor- β neutralization ameliorates pre-existing hepatic fibrosis and reduces cholangiocarcinoma in thioacetamide-treated rats. *PLoS One*. 2013;8(1):e54499.
27. Wang YW, Liou NH, Cherng JH, Chang SJ, Ma KH, Fu E, et al. siRNA-targeting transforming growth factor-beta I receptor reduces wound scarring and extracellular matrix deposition of scar tissue. *J Invest Dermatol*. 2014;134(7):2016-25.
28. Yoshizawa H, Morishita Y, Watanabe M, Ishibashi K, Muto S, Kusano E, et al. TGF- β_1 -siRNA delivery with nanoparticles inhibits peritoneal fibrosis. *Gene Ther*. 2015;22(4):333-40.
29. Wojdasiewicz P, Poniatowski LA, Szukiewicz D. The role of inflammatory and anti-inflammatory cytokines in the pathogenesis of osteoarthritis. *Mediators Inflamm*. 2014;2014:561459.

30. Saika S, Yamanaka O, Okada Y, Tanaka S, Miyamoto T, Sumioka T, et al. TGF- β in fibroproliferative diseases in the eye. *Front Biosci (Schol Ed)*. 2009;1:376-90.
31. Gilbert RWD, Vickaryous MK, Vilorio-Petit AM. Signalling by transforming growth factor β isoforms in wound healing and tissue regeneration. *J Dev Biol*. 2016;4(2):21.
32. Karamichos D, Hutcheon AE, Zieske JD. Transforming growth factor- β 3 regulates assembly of a non-fibrotic matrix in a 3D corneal model. *J Tissue Eng Regen Med*. 2011;5(8):e228-38.
33. Ferguson MW, O'Kane S. Scar-free healing: from embryonic mechanisms to adult therapeutic intervention. *Philos Trans R Soc Lond B Biol Sci*. 2004;359(1445):839-50.
34. Jiang Y, Vaessen B, Lenvik T, Blackstad M, Reyes M, Verfaillie CM. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. *Exp Hematol*. 2002;30(8):896-904.
35. Bardi GT, Smith MA, Hood JL. Melanoma exosomes promote mixed M₁ and M₂ macrophage polarization. *Cytokine*. 2018;105:63-72.
36. Taciak B, Bialasek M, Braniewska A, Sas Z, Sawicka P, Kiraga L, et al. Evaluation of phenotypic and functional stability of RAW 264.7 cell line through serial passages. *PLoS One*. 2018;13(6):e0198943.
37. Madej MP, Topfer E, Boraschi D, Italiani P. Different regulation of interleukin-1 production and activity in monocytes and macrophages: innate memory as an endogenous mechanism of IL-1 inhibition. *Front Pharmacol*. 2017;8:335.
38. Gubernatorova EO, Gorshikova EA, Namakanova OA, Zvartsev RV, Hidalgo J, Drutskaya MS, et al. Non-redundant functions of IL-6 produced by macrophages and dendritic cells in allergic airway inflammation. *Front Immunol*. 2018;9:2718.
39. Prockop DJ, Oh JY. Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. *Mol Ther*. 2012;20(1):14-20.
40. Morikawa M, Derynck R, Miyazono K. TGF- β and the TGF- β family: context-dependent roles in cell and tissue physiology. *Cold Spring Harb Perspect Biol*. 2016;8(5):a021873.
41. Wakefield LM, Hill CS. Beyond TGF β : roles of other TGF- β superfamily members in cancer. *Nat Rev Cancer*. 2013;13(5):328-41.
42. Derynck R, Budi EH. Specificity, versatility, and control of TGF- β family signaling. *Sci Signal*. 2019;12(570):eaav5183.
43. Cheifetz S, Wathebee JA, Tsang ML, Anderson JK, Mole JE, Lucas R, et al. The transforming growth factor- β system, a complex pattern of cross-reactive ligands and receptors. *Cell*. 1987;48(3):409-15.
44. Chaikuad A, Bullock AN. Structural basis of intracellular TGF- β signaling: receptors and smads. *Cold Spring Harb Perspect Biol*. 2016;8(11):a022111.

45. Biernacka A, Dobaczewski M, Frangogiannis NG. TGF- β signaling in fibrosis. *Growth Factors*. 2011;29(5):196-202.
46. Yu L, Border WA, Huang Y, Noble NA. TGF- β isoforms in renal fibrogenesis. *Kidney Int*. 2003;64(3):844-56.
47. Ask K, Bonnaiaud P, Maass K, Eickelberg O, Margetts PJ, Warburton D, et al. Progressive pulmonary fibrosis is mediated by TGF- β 1 but not TGF- β 3. *Int J Biochem Cell Biol*. 2008;40(3):484-95.
48. Han A, Bandyopadhyay B, Jayaprakash P, Lua I, Sahu D, Chen M, et al. The anti-motility signaling mechanism of TGF β 3 that controls cell traffic during skin wound healing. *Biol Open*. 2012;1(12):1169-77.
49. Guo X, Hutcheon AE, Zieske JD. Molecular insights on the effect of TGF- β 1/- β 3 in human corneal fibroblasts. *Exp Eye Res*. 2016;146:233-41.
50. Karamichos D, Hutcheon AE, Zieske JD. Reversal of fibrosis by TGF- β 3 in a 3D in vitro model. *Exp Eye Res*. 2014;124:31-6.
51. Jiang K, Chun G, Wang Z, Du Q, Wang A, Xiong Y. Effect of transforming growth factor- β 3 on the expression of Smad3 and Smad7 in tenocytes. *Mol Med Rep*. 2016;13(4):3567-73.
52. Kavsak P, Rasmussen RK, Causign CG, Bonni S, Zhu H, Thomsen GH. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF β receptor for degradation. *Mol Cell*. 2000;6(6):1365-75.
53. Ebisawa T, Fukuchi M, Murakami G, Chiba T, Tanaka K, Imamura T, et al. Smurf1 interacts with transforming growth factor- β type I receptor through Smad7 and induces receptor degradation. *J Biol Chem*. 2001;276(16):12477-80.
54. Heldin CH, Moustakas A. Signaling Receptors for TGF- β Family Members. *Cold Spring Harb Perspect Biol*. 2016;8(8):a022053.

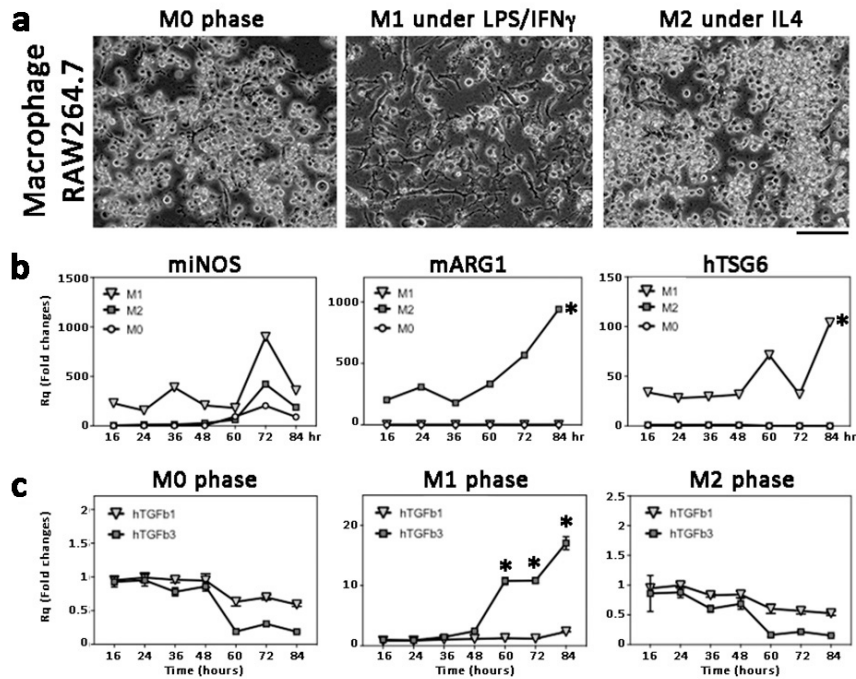


Figure 1. TGF β 3 upregulation in human CSSC under M1 pro-inflammatory condition. (a) Mouse macrophage RAW264.7 at M0 phase was treated with LPS and IFN γ for 48 hours to M1 pro-inflammatory phase. The cells were differentiated and became adherent with spindle shape appearance. When treated with IL4, they were induced to M2 proliferating phase mimicking anti-inflammatory condition. Scale bar: 80 μ m. (b) M1 and M2 marker expression. Mouse iNOS (miNOS) was upregulated in M1 phase and mouse ARG1 (mARG1) was induced under M2 condition. Co-culture of hCSSC with mouse RAW cells in different phases showed human TSG6 (hTSG6) upregulation under M1 phase. (c) Expression of hTGF β 1 and hTGF β 3 under co-culture of hCSSC with mouse RAW cells in different phases. Significant upregulation of hTGF β 3 in M1, but not in M0 and M2 conditions. Values represent the mean of triplicate experiments. * $P < 0.05$, paired Student's t-test.

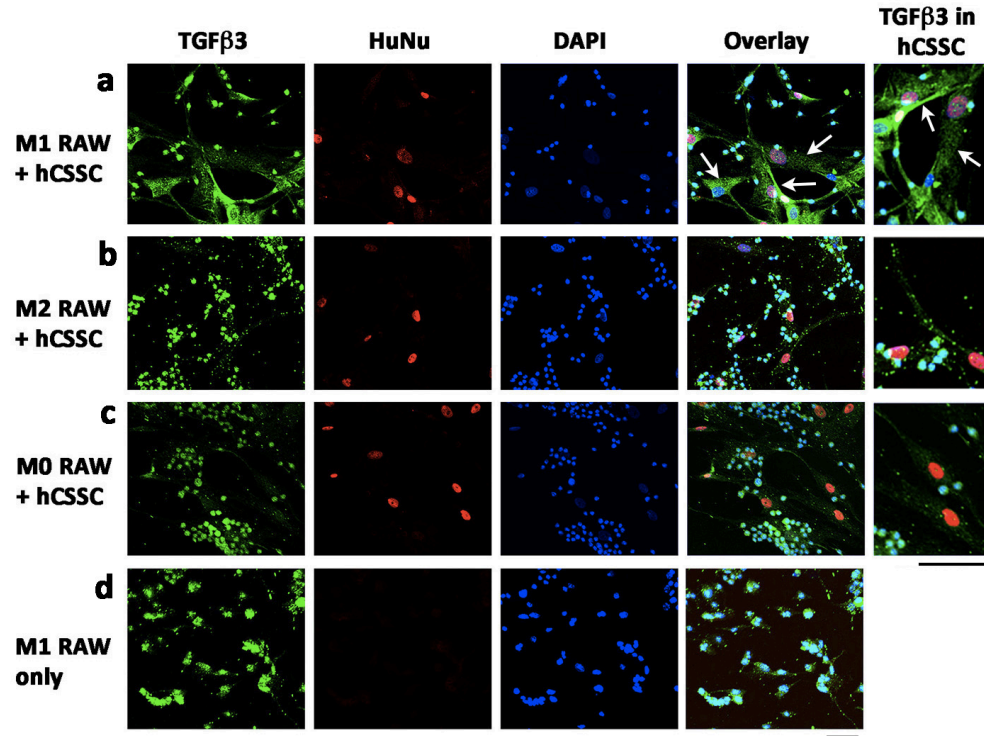


Fig 2. Immunostaining of TGFβ3 expression in hCSSC under co-cultured with mouse RAW cells. After 72 hours of co-culture with RAW cells in (a) M1, (b) M2 or (c) M0 condition, hCSSC were distinguished from mouse cells by human specific HuNu labelling (red). A cytoplasmic vesicular-like pattern of TGFβ3 expression (green) was detected in hCSSC (arrow) under M1 condition of RAW cells. The signal was much reduced when co-cultured with RAW cells under M2 and M0 conditions. The immunoreactivity of anti-TGFβ3 antibody was similar in RAW cells under M0, M1, M2 with CSSC co-culture and (d) single culture conditions. Experiment was performed in triplicates. Scale bar: 50 μm.

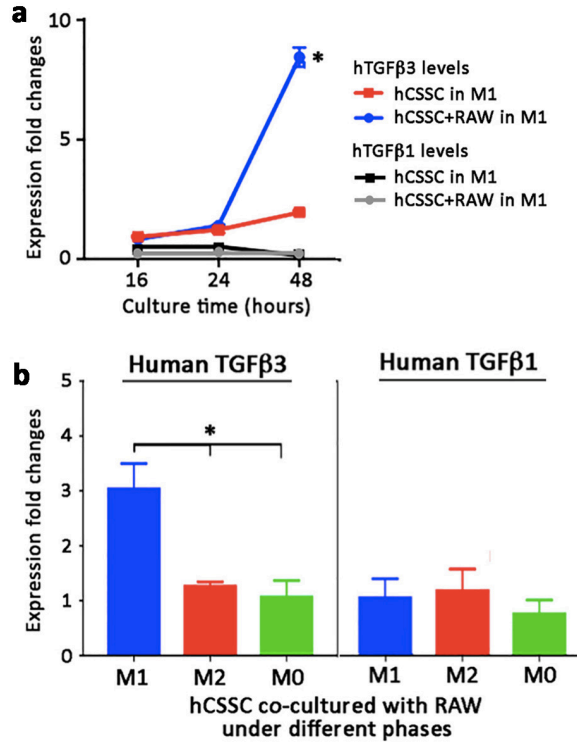


Fig 3. M1-type macrophages induced TGFβ3 expression in hCSSC via paracrine action. (a) In a Transwell system, the co-culture of hCSSC and mRAW cells at M1 condition in separate chambers for 48 hours showed a significant hTGFβ3 upregulation from hCSSC. The induction was detectable after 24 hours. For hCSSC alone under M1 induction, there were no clear changes of hTGFβ3 expression. The expression of hTGFβ1 was not altered in hCSSC alone or in co-culture with mRAW cells after M1 induction. (b) hTGFβ3 upregulation was significantly greater when hCSSC were co-cultured with mRAW cells under M1 but not under M2 and M0 conditions. hTGFβ1 expression remained unchanged under different conditions. Mean and SD are calculated from triplicate experiments. * P<0.05, paired Student's t-test.

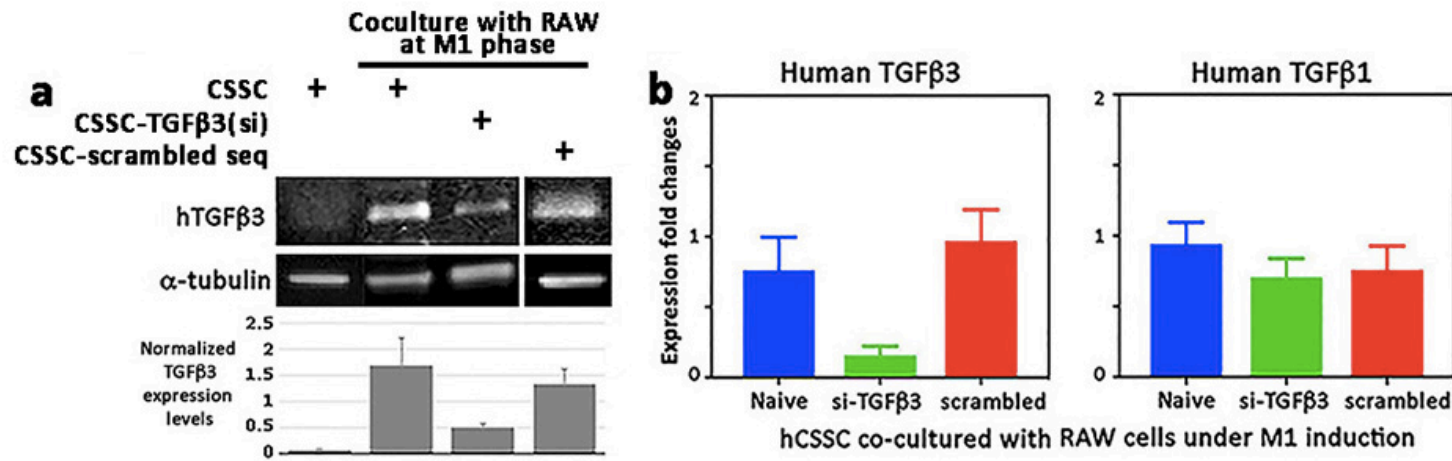


Fig 4. TGFβ3 knockdown in human CSSC. (a) Human CSSC were transfected with hTGFβ3 siRNA or scrambled sequences. After co-culture with RAW cells under M1 phase in a Transwell system for 48 hours, naïve hCSSC and hCSSC transfected with scrambled sequences showed similar TGFβ3 upregulation, whereas cells with TGFβ3 siRNA transfection showed reduced expression (decreased by 70-84%) of hTGFβ3. Similar results were obtained in two separate experiments. (b) The reduced hTGFβ3 expression was specific for si-TGFβ3 transfection, while there was no changes for scrambled sequences. The expression of hTGFβ1 remained unchanged with TGFβ3 knockdown in CSSC. Mean and SD are calculated from triplicate experiments.

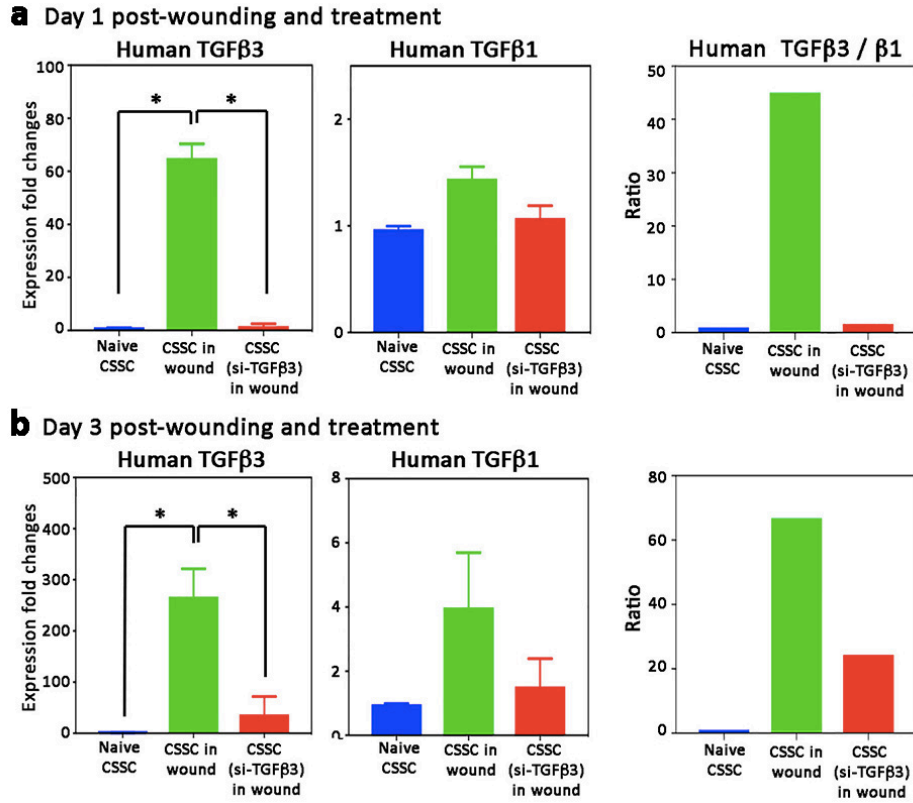


Fig 5. TGFβ3 was upregulated after hCSSC treatment to injured mouse corneas. After stromal ablation wounding, hCSSC or hCSSC-TGFβ3(si), suspended in fibrin gel, were applied to the fresh wounds. At 2 different time points, (a) 1 day and (b) 3 days, the expression of hTGFβ3 was significantly induced in corneas treated with hCSSC but not with hCSSC-TGFβ3(si). On the other hand, the expression of hTGFβ1 was not altered at either time points. The ratio of hTGFβ3/β1 was maintained between day 1 and day 3 post-treatment. Mean and SD represent data from triplicate experiments. * $P < 0.05$, paired Student's t-test.

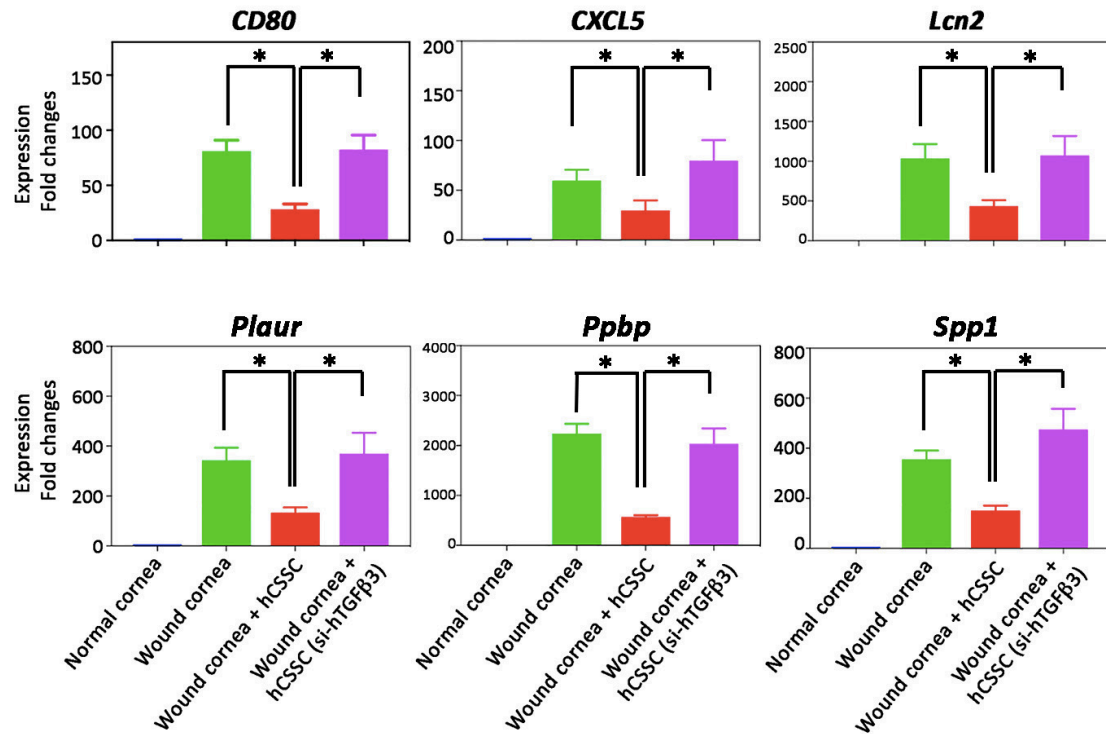


Fig 6. The anti-inflammatory effect of hCSSC on mouse stromal wound model was attenuated by TGFβ3 knockdown. After cell treatment for 1 day, mouse specific early inflammatory genes (*CD80*, *CXCL5*, *Lcn2*, *PLAUR*, *Ppbp* and *Spp1*) were upregulated in the wounded corneas, as compared to naïve corneas. Gene expression was suppressed by hCSSC treatment, representing an anti-inflammatory effect. Such a response was attenuated when hCSSC-TGFβ3(si) were applied. Mean and SD represent data from triplicate experiments. * $P < 0.05$, paired Student's t-test.

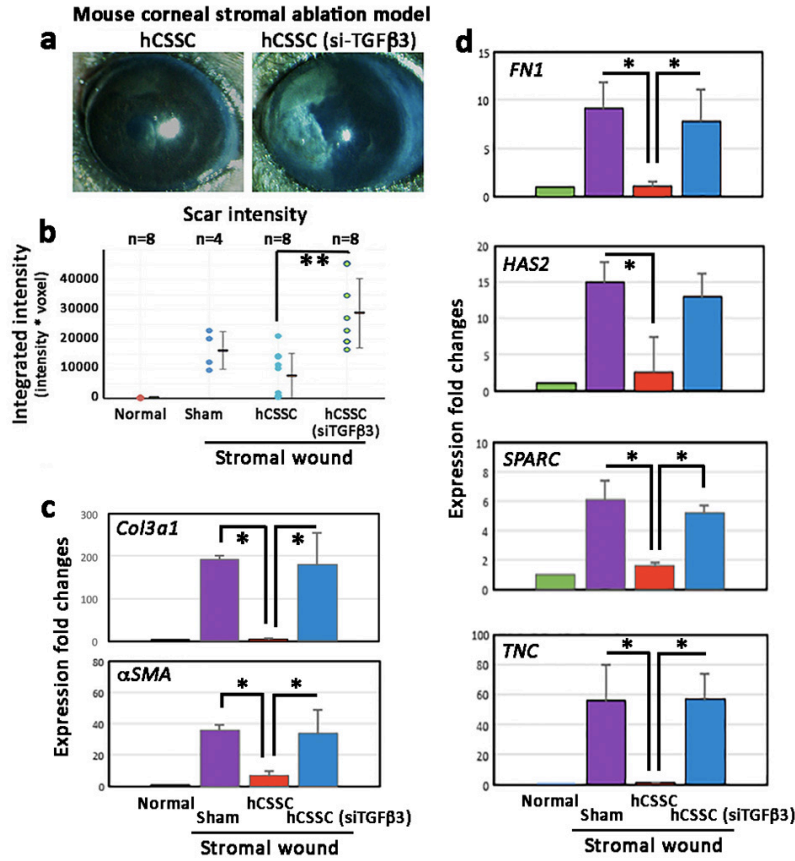


Fig 7. The anti-fibrotic effect of hCSSC on mouse stromal wound model was reduced by TGFβ3 knockdown. Fresh wounds were treated with hCSSC or hCSSC-TGFβ3(si). (a) After 14 days, intense scar was formed in corneas treated with hCSSC-TGFβ3(si), but not with naïve CSSC. (b) Scar intensity measurement showed that corneas treated with hCSSC-TGFβ3(si) had significantly greater scar intensities than those with naïve hCSSC. The levels were similar as in the sham control after wounding. (c) Mouse scar tissue genes (*Col3a1* and α SMA) were suppressed after treatment of naïve hCSSC but remained elevated for hCSSC-TGFβ3(si) treatment. (d) Other mouse fibrotic genes (*FN1*, *HAS2*, *SPARC* and *TNC*) were also elevated in corneas with hCSSC-TGFβ3(si) treatment, but not with naïve hCSSC. Mean and SD are calculated from triplicate experiments. (b) $**P < 0.05$, Mann-Whitney U test. (c, d) $*P < 0.05$, paired Student's t-test.

Table 1. Expression primers.

	Gene	GeneBank Accession No.	Sequences
Oligonucleotide primers			
1	hTGFβ1	NM_000660.7	F: GACTGCGGATCTCTGTGTCATT R: CAGTAGTGTTCCCCACTGGTC
2	hTGFβ3	NM_003239.4	F: CTTCCAGATCCTTCGGCCAG R: ATCAAAGGACAGCCACTCGG
3	hTSG6	NM_007115.4	F: AAGGATGGGGATTCAAGGAT R: GCTTGTATTTGCCAGACCGT
4	mARG1	NM_031508.1	F: GATTATCGGAGCGCCTTTCT R: CCACACTGACTCTTCCATTCTT
5	mCol3a1	NM_009930.2	F: CGTAAGCACTGGTGGACAGA R: CGGCTGGAAAGAAGTCTGAG
6	mFN1	NM010233.2	F: TACTCGAGCCCTGAGGATGG R: GCAAGGCAACCACACTGACT
7	mHAS2	BC080281	F: CCAAGGTTCTGCTTCCTCAC R: CTCTCCATACGGGGAGAGTC
8	miNOS	NM_001313922.1	F: TCACCTTCGAGGGCAGCCGA R: TCCGTGGCAAAGCGAGCCAG
9	mSPARC	XM_030245730.1	F: ATGAGGGCCTGGATCTTCTT R: CACGGTTTCCTCCTCCACTA
10	mSMA	NM_009696.3	F: TGTGCTGGACTCTGGAGATG R: GAAGGAATAGCCACGCTCAG
11	mTNC	NM_035737.2	F: GACTGCCCTGGGAAGTGTA R: CATAGCCTTCGAAGCACACA
TaqMan assay IDs			
7	hTGFβ1	NM_000660.7	Hs99999918_m1
8	hTGFβ3	NM_003239.4	Hs04398989_m1
9	hGAPDH	NM_001256799.3	Hs01120607_g1
10	mCD80	NM_009855.2	Mm00711660_m1
11	mLcn2	NM_032517.1	Mm01324470_m1
12	mPLAU	NM_032899.1	Mm01149438_m1
13	mPpbp	NM_023785.3	Mm00470163_m1
14	mSpp1	NM_001204203	Mm00436767_m1
15	mG6pdx	NM_032034.3	Mm00656735_g1

Note: h - human; m - mouse