Corneal trauma and chemical burns lead to corneal scarring, producing a long-term reduction in vision, sometimes 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.
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1. **INTRODUCTION:**

Corneal trauma and chemical burns lead to corneal scarring, producing a long-term reduction in vision, sometimes 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. This project will develop a therapeutic device (ReCoBand) containing live stem cells that can be applied to the cornea, in a field hospital to eyes with trauma or burns to prevent long-term scarring or blindness. The first aim of this project will discover genes in the CSSC that correlate with their ability to block scarring. We will develop a rapid, simple test that can serve as a quality control for each CSSC cell line to assure it will work to block scarring. In the second phase of the project we will devise a thin collagen sheet which CSSC cells embedded in to act as a regenerative corneal bandage (ReCoBand). These bandages can be held in place on the cornea by a soft contact lens. We will optimize means of storing the ReCoBand frozen so they will be available to doctors in field hospitals. In the third phase of the project we will investigate the timing for the start of therapy to determine how soon does the bandage need to be put in place after an injury. At the end of this three-year project we will have defined the components required to create a novel treatment system that prevents corneal scarring that can move directly into an application to the FDA for a new ‘biologic device’. Because the stem cells in this device do not actually enter the body, this device will be considered safer than procedures involving transplantation of stem cell and thus may move to clinical trials in a timely manner. If successful the ReCoBand may save vision of many and reduce the need for corneal transplantation.

2. **KEYWORDS:**

Vision, Blindness, Cornea, Scarring, Stem Cells, Therapy, Immunomodulation

3. **ACCOMPLISHMENTS:**

**Major goals and objectives of the project:**

- Task 1. Develop a metric for analysis of stem cell regenerative potential
- Task 2. Demonstrate the roles of specific genes in stromal regeneration in vivo
- Task 3. Assess plastic compressed collagen as a stem cell delivery vehicle
- Task 4. Assess cell sheets as delivery vehicle for stem cells.
- Task 5. Determine type of injury and timing for which corneal stem cell-based therapy is most effective.

**Milestones:**

Task 1. Milestone (12 months): Isolation of a library of CSSC lines with identified gene expression phenotypes. **Achieved**


Task 3. Milestone (24 months): Assessment of plastic compressed collagen as stem cell delivery vehicle.


Task 5. Milestone(s) (36 months): Understanding the time frame and injury type for which stem cell therapy will be useful vehicle.
What was accomplished under these goals?

Corneal Stromal Stem Cells (CSSC) are obtained from biopsies of corneal tissue. The quality and potency of individual stem cell lines varies greatly from one individual to another. A major hurdle for developing a standardized stem cell therapeutic reagent is determining potency of the cells. Our approach to determining this was collection of a library of 24 cell lines from different individual donors and comparison of them as to their gene expression, and biological properties. The goal is to define a set of genes that allow rapid screening of any cell line to determine its potential for therapeutic use in treating corneal damage.

The major activity of the first year of the project was to prepare and characterize a library of stem cell lines. This initially involved obtaining appropriate permissions and establishing connections with surgeons in the area. Donated corneal tissue is used for transplant and the remaining corneal rim serves as the source for stem cells. The tissue is de-identified and permission was obtained by the eye bank for research use. The list of cell lines and age of donors is present in Table 1. (Note: Table and illustrations included in the Appendix.

After obtaining the library of cell lines, the major goal of the project is to identify expressed genes that can be used to identify cell with high levels of regenerative potential. Our proposed approach was to examine the lines for well-known stem cell properties and identify genes associated with the ‘best’ stem cell lines. Stem cells are known to (a) grow clonally, (b) to differentiate to specialized tissues (in this case cornea), and (c) to suppress immune responses. There are also (d) a number of genes expressed in stem cells and not somatic cells. It is well known that stem cells lose their potential as they age in culture. Initially we expanded a CSSC line through 25 population doublings to provide “good” and “bad” cells from the same line (Fig 1A). We found that, as expected, the clonogenicity of the cells was markedly reduced as the cells age (Fig 1B). Surprisingly, expression of genes identified as ‘stem cell’ genes was generally not affected (Fig 1C) except for a few including PAX6, a gene we originally used to identify these cells. To our great surprise, we found that the potential for these cells to differentiate to corneal stromal cells as evidenced by expression of the keratocan gene (Fig 1D) or secretion of corneal keratan sulfate proteoglycan (Fig 1E) was not reduced as cells age.

As a second approach we identified four genes that are reduced as cells age (PAX6, TSG6, GRP, CHI3L1) and we screened the expression of these genes in low passage of our cell line library. As expected the cell lines showed variable expression of each of these genes (Fig 2). Again we were surprised to find that there was no consistency among the expression levels for any individual cell. Furthermore, cells with high and low expression of TSG6 showed no consistency in clonogenic or differentiation assays nor were other stem cells genes expressed with any predictable pattern (not shown).

These results, although not providing a direct route to our goal, illustrate a novel and very important point about stem cells. Phenotypic properties associated with these cells are not necessarily linked. If we want to understand which of these lines will prevent corneal inflammation and scarring, testing for some other stem cell property will not inform us. It is not feasible from a time and budget standpoint to test all 24 lines in in vivo wound healing assays. Considerable evidence supports that idea that preventing
corneal scarring results from the potential of stem cells to suppress the host immune response. Consequently we are developing in vitro protocols that assay immune suppression by these cells. In Fig 3, we found that proliferation of T-cells in vitro after stimulation of the CD3 receptor was blocked by the presence of CSSC in a 1:10 ratio with the T-Cells. Interestingly aged (passage 6) CSSC had little effect on T-cell proliferation. Also in the PBMC cultures, (Fig 4) presence of CSSC generated a population of activated monocytes expressing TGFβ. Such cells are considered anti-inflammatory, alternately activated macrophages associated with regeneration. We have also developed an assay to examine the effect of stem cells on neutrophil migration (not shown). We believe screening of our cell library with these three assays will be a better predictor of in vivo regenerative potential than the more typical assays of differentiation, clonal growth and stem cell gene expression.

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

Although this is not specifically a training grant, Dr. Syed-Picard served for 8 months on this project as a research associate and obtained valuable skills in cell techniques and will serve as author on the in process manuscript.

How were the results disseminated to communities of interest?

A manuscript describing the data in the figures is in preparation. The results were also presented at several meetings including Association for Research in Vision and Ophthalmology, Denver May 2015.

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

All 24 cell lines will be screened using the three immune-suppression assays described above. Cells with strong and with weak ability to modify immune response will further be tested our wound healing model in vivo. After confirmation of their relative effectiveness, global gene expression patterns from 6 lines will be obtained by RNA-seq, as outlined in the initial proposal. This advances the project toward accomplishing Milestone of Task 2.

At the same time, we will begin experiments embedding the stem cells in compressed plastic collagen to place on wounded corneas. These experiment are a component of Task 3.

4. IMPACT:

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

Work with adult stem cells focuses largely on the potential of these cells. Cells are often used without purification and characterized by expression of a set of cell surface proteins found on many mesenchymal cells. Little effort has gone into understanding how potent a particular line of cells is, and if cells from different individuals vary. Our work is on track to illustrate 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.
What was the impact on other disciplines?  Nothing to Report
What was the impact on technology transfer?  Nothing to Report
What was the impact on society beyond science and technology? Nothing to Report

5.  CHANGES/PROBLEMS:

Changes in approach and reasons for change.
The original plan was to examine gene patterns from cells during aging in culture. As discussed above, we have found that although these cells have a reduced ability to prevent T-cell proliferation, the change in stem cell genes as cells age does not correlate consistently with gene expression patterns in the library of early passage cells. We feel that a more robust approach will be to screen all of the cell lines for their immunosuppressive properties. Adding these assays represent a minor modification in the experimental approach to Task 2 but does not alter the goals or the timeline of the project. It is expected that little or no change in the budget of the project as well.

Actual or anticipated problems or delays and actions or plans to resolve them.
The initial delay in defining cell lines with high regenerative potential will push back the RNAseq analysis of these lines by 1-2 months. This analysis was originally planned for CY15Q4, and will likely be carried out in CY16Q1 but this change will not impact the overall time course of the project.

Changes that have a significant impact on expenditures. Nothing to Report
Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents. Nothing to Report

6.  PRODUCTS

Publications, conference papers, and presentations
Oral Presentation at ARVO  (Association for Research in Vision and Ophthalmology)
Denver May 4, 2015
Abstract in Appendix

Technologies or techniques- Nothing to Report
Inventions, patent applications, and/or licenses- Nothing to Report
Other Products - Nothing to Report

7.  PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS
What individuals have worked on the project?

Name: James Funderburgh  
Project Role: PI  
Univ. Pitt. ID 37069  
Nearest person month worked: 2.4  
Contribution to Project: JLF designed the project and is directing it.

Name: Yiqin Du  
Project Role: Co-investigator  
Univ. Pitt. ID 82941  
Nearest person month worked: 3  
Contribution to Project: Dr. Du examined tissue for quality, carries out dissection, oversees tissue culture of the stem cells.

Name: Martha Funderburgh  
Project Role: Research Specialist  
Univ. Pitt. ID 37455  
Nearest person month worked: 3  
Contribution to Project: Ms. Funderburgh is in charge of record-keeping for human tissues, oversees regulatory approval of human and animal studies. She helps in dissection is involved in passaging and cryopreserving cells.

Name: Moira Geary  
Project Role: Animal Technician  
Univ. Pitt. ID 120308  
Nearest person month worked: 3  
Contribution to Project: Ms. Geary maintained the mouse breeding colony so that animals will be available for experiments planned for next quarter. These duties are essential even when animal work is not ongoing.

Name: Fatima Syed-Picard  
Project Role: Research Associate  
Univ. Pitt. ID 118336  
Nearest person month worked: 0.8  
Contribution to Project: Dr. Syed-Picard has been consulting the PI and Co-I in design of the cell-based constructs to be used in the second year of the project. She is currently carrying out preliminary experiments optimizing different approaches that will be used.

Name: Mary M. Mann  
Project Role: Research Specialist  
Univ. Pitt. ID 38967  
Nearest person month worked: 4  
Contribution to Project: Ms. Mann maintains laboratory solutions, prepares culture media and has carried out cell counts, passaging, and clonogenicity analyses of the current cell lines.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?  Nothing to report

What other organizations were involved as partners?  Nothing to report
8. SPECIAL REPORTING REQUIREMENTS: QUAD CHARTS:

CY15Q3 Quad Chart in Appendix

9. APPENDICES:
   1. Figures
   2. ARVO Abstract
   3. Quad chart
Table 1.  
Database of Corneal Stroma Stem Cell (CSSC) lines.

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Figure 1. Aging of CSSC Does not inhibit Differentiation Potential. (A) CSSC underwent 25 population doublings over a 20 day period before replicative senescence. (B) During passage, potential for clonal growth was lost. (C) Comparing Passage 7 with passage 2 CSSC, mRNA expression of OCT4 and PAX6 was reduced, whereas that of SOX2, SIX2, ABCG2, and CDH2 were increased. (D) In differentiation conditions, upregulation of keratocan mRNA was highest in p5-p6 cells in spite of their reduced stem cell markers. (E) Under differentiation conditions, induction of keratan sulfate proteoglycan (KSPG) secretion was greater in p5-p6 cells.
Stem cells vary in expression of “stem cell” genes. Expression of three genes (CHI3L1, GRP, PAX6, TSG6), identified as decreased in passaged cells, were compared by quantitative PCR in 20 CSSC lines (passage 1 or 2). Values shown are Ct value relative to 18S RNA. Lines expressing high levels (black bracket) or low levels (red brackets) of TSG6 showed little correlation with expression levels of other genes.
**Fig 3. CSSC inhibit T-cell proliferation.** A Human peripheral blood mononuclear cells are labeled with the dye CDFA. When stimulated with Anti-CD3/CD28 beads T-cells in the PBMC population proliferate generating cells with reduced staining which can be quantified by flow cytometry to obtain a proliferation index. B. Proliferation of PBMC T-cells is reduced or eliminated in the presence of increasing numbers of passage 2 (p2) CSSC cells (blue). Passage 6 (aged) CSSC are not effective in blocking proliferation (orange).
Fig 4. CSSC increase population of Monocytes Expressing TGFβ. **A.** Co-culture of CSSC with human blood peripheral mononuclear cells results in appearance of cells expressing CD14 and TGFβ (circle). A regenerative macrophage phenotype **B.** If the cultures are not stimulated with Anti-CD3/CD28 Beads, few TGF/CD14 cells are seen, but in the presence of CD3/CD28, increasing numbers of CSSC increase the abundance of the TGFβ/CD14 macrophages.
Stem cells from corneal stroma suppress T-cell activation via cell-cell interactions

Abstract Number: 2073

Author Block: James L. Funderburgh¹, Martha L. Funderburgh¹, Mary M. Mann¹, Yiqin Du¹, Kyle C. McKenna¹, Andrew Hertsenberg¹

¹ Ophthalmology, Univ of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, United States

Disclosure Block: James L. Funderburgh, None; Martha L. Funderburgh, None; Mary M. Mann, None; Yiqin Du, None; Kyle C. McKenna, None; Andrew Hertsenberg, None

Purpose: Human corneal stromal stem cells introduced into mouse corneal stroma, remain viable for extended time periods, restoring transparency to corneas of lumican null mice and to wounded corneas of normal mice without immune rejection. Mesenchymal stem cells are also reported to suppress corneal allograft rejection. Primary human fibroblasts in mouse corneas, however, elicit a strong T-cell response demonstrating the specialized immune-modulatory property of stem cells. This study examined the mechanism by which human corneal stromal stem cells (hCSSC) block activation of T-cells, mediators of tissue rejection.

Methods: Splenocytes from OT-1 mice were activated by mouse peritoneal macrophages primed with ovalbumin peptides. Human peripheral blood mononuclear cells (PBMCs) labeled with CFSE were activated with concanavalin A (ConA) or with anti-CD3/CD28 beads in vitro for four days in the presence of hCSSC. Proliferation and expression of activated T-cell markers were examined by flow cytometry.

Results: T-cells from OT-1 mice responded to ovalbumin-primed antigen-presenting cells by proliferation and by expression of CD25 and CD69 antigens. The presence of hCSSC at a 1:100 ratio of hCSSC:splenocytes significantly reduced activation and 1:5 ratio eliminated activation. CD3 T-cells in PBMCs, activated with ConA or anti-CD3/CD28 divided up to 5 times in 4 days. PBMC:hCSSC at a ratio of 1:6 fully blocked this proliferation. Suppression of T-cell activation was enhanced by activation of hCSSC with IFN-gamma and TNF-alpha. Physical separation of hCSSC from PBMCs in transwells eliminated the ability to block T-cell activation. Results achieved statistical significance (p<0.05) as determined by t-test.

Conclusions: Our data support the idea that T-cell activation can be blocked by corneal stromal stem cells by direct cell-cell contact, possibly via interaction of the PD-L1 (CD274) cell-surface protein. This suppression mechanism appears to be distinct from the suppression of neutrophil activation mediated by stem cell secretion of TSG6. Adult corneal stromal stem cells, therefore appear to manage immune response in their environment via multiple mechanisms.

Layman Abstract (optional): Provide a 50-200 word description of your work that non-scientists can understand. Describe the big picture and the implications of your findings, not the study itself and the associated details.: Adult stem cells suppress inflammation and tissue rejection. This work examines the molecules involved in this process. It appears that stem cells employ at least two mechanisms in suppressing inflammatory responses.
Early Intervention Stem Cell-Based Therapy (EISCBT) for Corneal Burns and Trauma
Log Number: MR130197    Contract # W81XWH-14-1-0465

PI: James Funderburgh         Org: University of Pittsburgh         Award Amount: $992,782

### Aims

- Develop stable characterized stem cell lines to serve as a basis for EISCBT.
- Optimize a stabilized cell delivery system for corneal stromal stem cell EISCBT.
- Determine type of injury and timing for which EISCBT is most effective.

### Approach

This study will use animal models of corneal trauma and chemical burns to develop a biologic reagent that can be delivered to injured military personnel rapidly after suffering a corneal insult. The study will develop a human cell reagent and delivery system that can be rapidly translated to clinical trials.

### Timeline and Cost

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<td>Demonstration of function in vivo</td>
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</table>

| Estimated Budget (Total $K) | $80 | $321 | $334 | $257 |

Updated: (Oct 20, 2015)  

= Current Location in Timeline

### Goals/Milestones

- **CY15Q2**  Isolate and passage Stem Cells Lines from Human Cadaveric Corneal Tissue. Collection to continue until 24 lines established (green box).
- **CY15Q4**  Define gene markers that predict regenerative potential.
- **CY16Q3**  Identify and cryopreserve high RP cell lines.
- **CY16Q4**  Test delivery system and storage of ReCoBand
- **CY17Q3**  Demonstrate functionality of the system in two in vivo models.

Comments: These goals to be accomplished by the end of the designated Quarter.

Budget Expenditure to Date: Estimate $320K