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Award Number: W81XWH-12-1-0606

TITLE: Stem-cell Based Therapies for Epidermolysis Bullosa

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REPORT DATE: October 2013

TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;  
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| REPORT DOCUMENTATION PAGE                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |             |                          |                            | Form Approved<br>OMB No. 0704-0188                        |                                           |
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| 1. REPORT DATE<br>October 2013                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |             | 2. REPORT TYPE<br>Annual |                            | 3. DATES COVERED<br>30 September 2012 – 29 September 2013 |                                           |
| 4. TITLE AND SUBTITLE<br><br>Stem-cell Based Therapies for Epidermolysis Bullosa                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |             |                          |                            | 5a. CONTRACT NUMBER                                       |                                           |
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| 6. AUTHOR(S)<br><br>Dennis Roop, Ph.D.<br><br>E-Mail: dennis.roop@ucdenver.edu                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |             |                          |                            | 5d. PROJECT NUMBER                                        |                                           |
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| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br><br>University of Colorado<br>Aurora, CO 80045-2505                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |             |                          |                            | 8. PERFORMING ORGANIZATION REPORT NUMBER                  |                                           |
| 9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)<br>U.S. Army Medical Research and Materiel Command<br>Fort Detrick, Maryland 21702-5012                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |             |                          |                            | 10. SPONSOR/MONITOR'S ACRONYM(S)                          |                                           |
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| 12. DISTRIBUTION / AVAILABILITY STATEMENT<br>Approved for Public Release; Distribution Unlimited                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |             |                          |                            |                                                           |                                           |
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| 14. ABSTRACT<br><br>This application addresses the FY11 PRMRP Topic Area, Epidermolysis Bullosa, and proposes to develop stem-cell based therapies for junctional epidermolysis bullosa (JEB), which is one of the most severe forms of epidermolysis bullosa (EB), a group of rare inherited skin blistering diseases. To accomplish this goal, we are proposing to develop stem-cell based therapies for EB using autologous induced pluripotent stem cells (iPSCs) derived from skin cells harvested from the same EB patient. We hypothesize that using a combination of genetically corrected patient-specific iPSC-derived keratinocyte stem cells for skin grafting in combination with iPSC-derived hematopoietic and mesenchymal stem cells for transplantation will be effective in correcting both lesions within the skin as well as in mucosal epithelia. During the first year of funding, we were able to successfully cross IRG mice with JEB mice to obtain IRGRFP/LAMA3fl/wt/K14-Cre.ER mice, which can now be used for the immunological studies proposed in the initial application. We also generated iPSCs from these mice using hSTEMCCA-loxP lentivirus to address the possibility of gene correction via ZFNs in these cells. In addition, we accomplished a major breakthrough by adapting a mRNA reprogramming protocol for the generation of iPSCs from human keratinocytes. This reprogramming protocol can now be employed to generate iPSCs not only from JEB patients but also from patients with other inherited skin diseases, as well as veterans with chronic wounds. |             |                          |                            |                                                           |                                           |
| 15. SUBJECT TERMS<br>Epidermolysis Bullosa (EB), Junctional EB (JEB); Induced Pluripotent Stem Cells (iPSCs).                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |             |                          |                            |                                                           |                                           |
| 16. SECURITY CLASSIFICATION OF:                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |             |                          | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES                                       | 19a. NAME OF RESPONSIBLE PERSON           |
| a. REPORT                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | b. ABSTRACT | c. THIS PAGE             |                            |                                                           | USAMRMC                                   |
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## Introduction

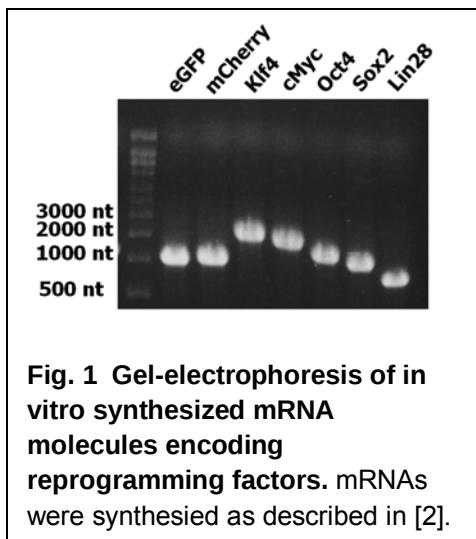
This application addresses the FY11 PRMRP Topic Area, Epidermolysis Bullosa, and proposes to develop stem-cell based therapies for junctional epidermolysis bullosa (JEB), which is one of the most severe forms of epidermolysis bullosa (EB), a group of rare inherited skin blistering diseases. JEB sentences those afflicted to a life of severe pain and disability due to constant blistering and scarring, and in some cases, early death. These diseases are devastating and despite all efforts, current therapy for EB is primarily limited to wound care. Therefore, there is a desperate need for the development of a safe stem cell-based approach for EB which would provide a permanent corrective therapy. To accomplish this goal, we are proposing to develop stem-cell based therapies for EB using autologous induced pluripotent stem cells (iPSC) derived from skin cells harvested from the same EB patient. We hypothesize that using a combination of genetically corrected patient-specific iPSC-derived keratinocyte stem cells for skin grafting in combination with iPSC-derived hematopoietic and mesenchymal stem cells for transplantation will be effective in correcting both lesions within the skin as well as in mucosal epithelia.

## Body

**Aim 1: To determine the histocompatibility of iPSC-derived keratinocytes and mesenchymal cells.** The assumption has been made that patient-specific iPSC could be used to generate an unlimited supply of adult stem cells that could then be returned to the same patient as an autograft without the risk of rejection. However, the possibility remains that the reprogramming process may alter the expression of histocompatibility antigens that would result in immune rejection. To rigorously examine the histocompatibility of iPSC-derived keratinocytes and mesenchymal cells, we proposed to use a mRNA-based reprogramming protocol to generate iPSC from keratinocytes isolated from the inducible JEB mouse model ( $LAMA3^{fl/wt}/K14-Cre.ER$ ) obtained from Dr. Monique Aumailley. To follow iPSC-derived keratinocytes and mesenchymal cells when they are grafted/transplanted into syngeneic JEB mice, we proposed to mate these mice, which are congenic on a C57BL/6J background, with IRG transgenic mice, a double-fluorescent, Cre-reporter strain which expresses red fluorescence protein (RFP) prior to Cre recombinase exposure, and green fluorescence protein (GFP) following *cre*-mediated recombination [1]. During the initial funding period, we successfully crossed IRG mice (purchased from The Jackson Laboratory) with JEB mice and obtained  $IRG^{RFP}/LAMA3^{fl/wt}/K14-Cre.ER$  mice, which we can now use for the proposed studies.

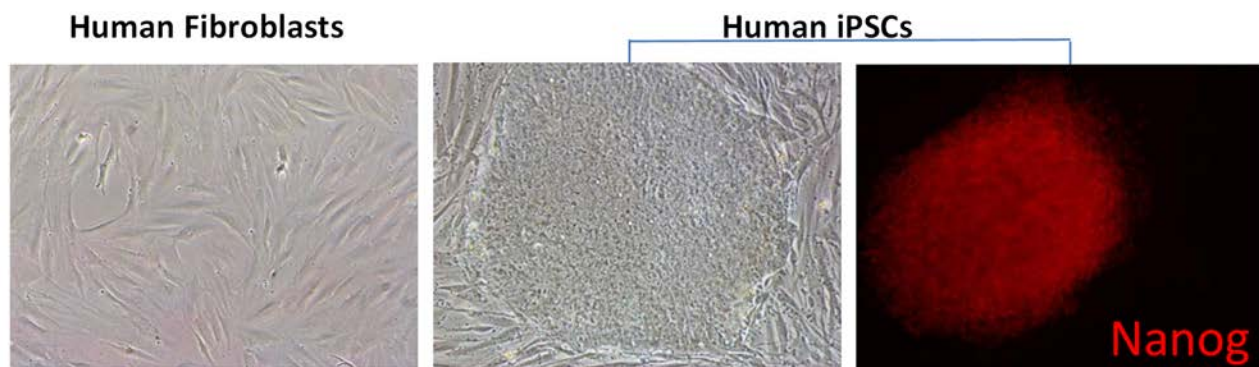
**Task 1.1.** *Generate iPSC from keratinocytes isolated from the JEB mouse model using the mRNA reprogramming method.*

While we were crossing IRB and JEB mice, we synthesized modified mRNA molecules encoding Oct4, Sox2, Klf4, Myc and Lin28 using cDNA templates obtained from Addgene ([www.addgene.com](http://www.addgene.com)), as previously described [2, 3] ( Fig.1). We used templates that encode human factors to reprogram mouse cells into iPSCs



since human reprogramming factors have been shown to be capable of reprogramming mouse fibroblasts into iPSCs with the same efficiency as mouse factors [4]. Unfortunately, our initial attempts to generate iPSCs from mouse wildtype (WT) fibroblasts have been unsuccessful using mRNA molecules encoding human factors. On the other hand, the same factors were capable of reprogramming human fibroblasts into iPSCs with high efficiency (Fig.2). We are currently preparing cDNA templates encoding mouse reprogramming factors for mRNA synthesis to exclude the possibility of factor incompatibility between mouse and human. As an alternative approach, we are also adapting Sendai virus [5] and episomal [6] approaches for the reprogramming of mouse cells into iPSCs. While we could potentially use lentiviral vectors to introduce the reprogramming factors into mouse cells, such as hSTEMCCA-loxP lentivirus [4], the use of viral vectors to deliver reprogramming factors could introduce genetic alterations into iPSC that would affect the histocompatibility of keratinocyte, hematopoietic and mesenchymal cells derived from these iPSCs. Thus, we are

specifically interested in adapting an integration-free approach for the reprogramming of mouse cells.



**Fig.2 Generating iPSCs from human fibroblasts using an optimized mRNA reprogramming approach.** iPSCs were generated from human fibroblasts using an optimized modified mRNA-based reprogramming approach. Middle panel shows the iPSC colony morphology, and right panel documents that these iPSCs express the pluripotency factor Nanog (red).

**Task 1.2.** Perform skin grafts with iPSC-derived  $IRG^{GFP}/LAMA3^{+/-}$  keratinocytes onto congenic  $IRG^{RFP}/LAMA3^{fl/wt}/K14-Cre.ER$  recipients.

This task will be initiated once we have completed Task 1.1.

**Task 1.3.** Transplant iPSC-derived  $IRG^{GFP}/LAMA3^{+/-}$  mesenchymal cells into congenic  $IRG^{RFP}/LAMA3^{fl/wt}/K14-Cre.ER$  recipients.

This task will be initiated by Dr. Tolar's group upon completion of the Task 1.1.

**Aim 2: To determine the genetic stability of human iPSC generated from keratinocytes obtained from JEB patient biopsies.** A recent report cautions that the process of reprogramming into iPSCs may introduce somatic mutations into the genome. Upon closely examining this paper, most of the iPSC were reprogrammed from a mixed population of fibroblasts that may have contained somatic mutations prior to reprogramming. In addition, most of the iPSC were generated using retroviral vectors to deliver the reprogramming factors. To avoid the concern about heterogeneity in the starting population and rigorously determine the genetic stability of human iPSC, we propose the following:

**Task 2.1.** Establish 10 independent clones of keratinocytes harvested from skin biopsies obtained from JEB patients.

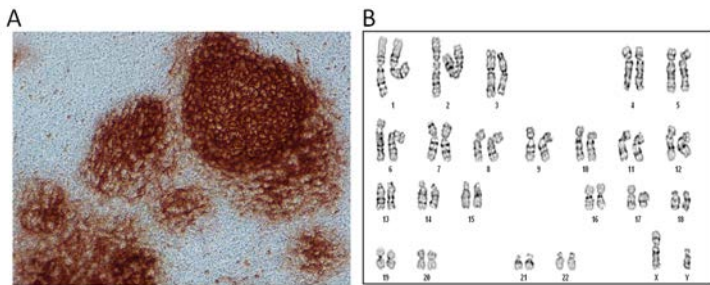
The initiation of this task was delayed due to unexpected difficulties in obtaining IRB approval for this proposed aim. However, after obtaining IRB approval, we were able to obtain keratinocytes from 3 different JEB patients and are currently expanding these lines to initiate the task of obtaining clonal lines.

**Task 2.2** Generate 5 independent iPSC lines from each clonal keratinocyte line derived from each JEB patient using the mRNA reprogramming method.

The mRNA-based reprogramming approach has been reported only for fibroblasts [2, 3] and not for keratinocytes. For this reason, we attempted to apply the mRNA-based reprogramming approach on WT human keratinocytes before employing JEB clonal keratinocytes lines. The modified mRNA molecules encoding the reprogramming factors have been synthesized as described under the Task 1.1. Within 2 weeks of initiating mRNA transfections, we obtained a substantial number of embryonic stem cell (ESC)- like colonies, which were mechanically picked and expanded (Fig.3). The obtained iPSC-colonies expressed pluripotency markers (Fig.4A) and showed a normal karyotype (Fig. 4B). These results suggest that the mRNA-based reprogramming protocol can be employed for the reprogramming of JEB patient-specific keratinocytes.



**Fig.2 Generating iPSCs from human keratinocytes using an optimized mRNA reprogramming approach.** iPSCs were generated from human keratinocytes (left panel, Day 0) using a mRNA-based transfection protocol for reprogramming. At day 10 post-initiation of mRNA transfections, the first iPSC-like colonies start to emerge (middle panel). These colonies have been picked mechanically and expanded to obtain clonal iPSC lines (right panel).



**Fig.4 Characterization of iPSCs derived from human keratinocytes.** Panel A documents the expression of the pluripotency marker Tra 1-60 in iPSCs derived from human keratinocytes. These iPSCs also show normal G-banding karyotype, as demonstrated in a representative image in B.

**Task 2.3.** Perform total exome sequencing on each keratinocyte line before generating iPSC.

This task will be initiated upon the completion of the Task 2.1 and before the generation of iPSCs from JEB keratinocytes.

**Task 2.4.** Perform total exome sequencing on each iPSC line derived from each clonal line of keratinocytes.

This task has not been initiated.

**Task 2.5.** Generate 5 independent keratinocyte cell lines from one of the sequenced iPSC lines from each patient and perform total exome sequencing on these lines.

This task has not been initiated.

**Task 2.6.** Generate 5 independent mesenchymal cell lines from one of the sequenced iPSC lines from each patient and perform total exome sequencing on these lines.

This task has not been initiated.

**Aim 3. To develop methods to increase the homing of iPSC-derived  $\text{Lin}^-/\text{PDGFR}\alpha^+$  cells into injured epithelia.** A recent report suggests that it may be possible to mobilize BM-derived cells into the circulation by systemically administering recombinant HMGB1, which results in increased homing of  $\text{Lin}^-/\text{PDGFR}\alpha^+$  BM cells into injured epithelia. To confirm these observations, we propose the following:

**Task 3.1.** To determine whether mouse iPSC-derived  $\text{Lin}^-/\text{PDGFR}\alpha^+$  cells will home into injured epithelia.

To be completed by Dr. Tolar.

**Task 3.2.** *To determine whether human iPSC-derived Lin<sup>-</sup>/PDGFR $\alpha$ <sup>+</sup> cells will home into injured epithelia.*

To be completed by Dr. Tolar.

**Aim 4. To develop an efficient and safe method for the genetic correction of the defective gene in JEB-specific iPSC.** Two recent reports have shown that zinc finger nucleases (ZFNs) can dramatically increase the efficiency of homologous recombination in iPSC. To confirm these observations and eliminate concerns about off target events, we propose the following:

**Task 4.1.** *Generate iPSC from the mouse model of JEB, correct the genetic defect using ZFN-mediated homologous recombination and confirm the absence of off target events using total exome sequencing.*

As described in the accomplishments under the Task 1.1, our initial attempts to generate iPSCs from mouse wildtype (WT) fibroblasts have been unsuccessful using the integration-free mRNA reprogramming method. Although the presence of reprogramming transgenes in iPSCs may have a significant impact for the immunological studies (Aim 1. of this project), the transgene should not affect the outcomes of the genetic correction experiment described in this Aim due to its [transgene's] silencing during the reprogramming. Therefore, while adapting integration-free protocols for the generation of mouse iPSCs, which are necessary to accomplish Aim 1., we generated iPSCs from the mouse model of JEB using hSTEMCCA-loxP lentivirus [4]. These mouse iPSCs expressed pluripotency markers and formed teratomas when injected into immunocompromised mice. In collaboration with Sigma, we are currently designing an optimum binding site for ZFNs to correct the genetic defect in mouse JEB iPSCs.

**Task 4.2** *Derive keratinocyte cells from genetically corrected mouse JEB iPSC and determine their ability to repair blistered areas in the JEB mouse model.*

This task will be initiated upon completion of the Task 4.1.

**Task 4.3** *Derive mesenchymal cells from genetically corrected mouse JEB iPSC and determine their ability to repair blistered areas in the JEB mouse model using the systemic delivery of HMGB1 as developed in Aim 3.*

To be completed by Dr. Tolar.

**Task 4.4** *Using JEB patient-specific iPSC generated in Aim 2, correct the genetic defect using ZFN-mediated homologous recombination and confirm the absence of off target events using total exome sequencing.*

This task has not been initiated.

**Task 4.5** *Derive keratinocyte cells from genetically corrected patient-specific JEB iPSC and determine their ability to regenerate a stable functioning skin in long-term graft assays using immunocompromised NSG mice.*

This task has not been initiated.

**Task 4.6** *Derive mesenchymal cells from genetically corrected patient-specific JEB iPSC and determine their ability to stably engraft long-term into the BM of immunocompromised NSG mice.*

This task has not been initiated.

## Key Research Accomplishments

- Successfully crossed IRG mice (purchased from The Jackson Laboratory) with JEB mice and obtained IRG<sup>RFP</sup>/LAMA3<sup>fl/wt</sup>/K14-Cre.ER mice, which can now be used for the proposed studies.
- Generated iPSCs from the mouse model of JEB using hSTEMCCA-loxP lentivirus;
- Obtained keratinocyte lines from 3 different JEB patients;
- Adapted a mRNA-based reprogramming protocol for the generation of human iPSCs from keratinocytes.

## Reportable Outcomes

The study has been funded for less than a year. Therefore, this time is not sufficient to produce reportable outcomes.

## Conclusion

Epidermolysis bullosa (EB) represents a group of rare currently incurable inherited skin blistering diseases. This application addresses the development of stem-cell based therapies for one of the most severe forms of EBs, Junctional EB (JEB). To accomplish the main goal of the study, we proposed to develop a genome editing strategy for JEB patient-specific iPSCs using ZFN-induced homologous recombination, which is then followed by the differentiation of genetically corrected iPSCs into keratinocytes and mesenchymal cells suitable for autologous transplantation. We proposed to employ both the mouse model for JEB to address the immunogenicity of iPSCs-based therapy, as well as actual human samples to move the study closer to a clinical trial. Although we had a delay in the release of the funds from the DOD due to an unexpected delay in the approval of our IRB protocol, we were able to accomplish several important goals during the first year of funding. Specifically, we obtained IRG<sup>RFP</sup>/LAMA3<sup>fl/wt</sup>/K14-Cre.ER mice by crossing IRG mice with inducible JEB mice and generated iPSCs from these mice using hSTEMCCA-loxP lentivirus. This allows us to address the immunogenicity of mouse iPSC-derived cell lines upon transplantation into congenic recipient mice. We also adapted a mRNA-based reprogramming protocol for the generation of iPSC from human keratinocyte lines, which has never been reported before. These accomplishments are critical to address next steps of our proposal and eventually develop a genome editing therapeutic strategy for JEB patients.

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## Appendices

None at this time.