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14. ABSTRACT 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. During the second year of funding, we developed a novel integration-free protocol for the reprogramming of human primary fibroblasts and keratinocytes into clinically relevant iPSCs. The efficiency of our method surpasses all previously published reports and results in the generation of stable iPSC lines. The protocol was employed for the reprogramming of human JEB fibroblasts into iPSCs, which now allows us to address the possibility of gene correction via ZFNs in these human JEB iPSCs. Our developed iPSC generation protocol is applicable not only to JEB patients but also to patients with other inherited skin diseases, as well as veterans with chronic wounds. In addition to reprogramming, we have further optimized our method for the differentiation of iPSCs into keratinocytes, thus fulfilling major prerequisites for the successful accomplishment of the proposed study.					
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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 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 iPSCs 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 last funding period, we generated the $IRG^{RFP}/LAMA3^{fl/wt}/K14-Cre.ER$ mouse line; however, due to continued delays in getting our ACURO animal protocol (we finally received approval on April 25, 2014) we were not able to initiate the studies outlined in our proposal. Since getting approval in April, we have isolated several independent keratinocyte and fibroblast cell lines for the generation of iPSCs (see below).

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

Since we could not perform experiments with the $IRG^{RFP}/LAMA3^{fl/wt}/K14-Cre.ER$ mice, our attempts to generate iPSCs have been restricted to wildtype (WT) fibroblasts and keratinocytes. So far, we have been unsuccessful with the mRNA reprogramming method using human-specific reprogramming factors. While we could potentially use lentiviral vectors to introduce the reprogramming factors into mouse cells, such as hSTEMCCA-loxP lentivirus [2], 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.

During this reporting period, we prepared cDNA templates encoding mouse reprogramming factors for mRNA synthesis to exclude the possibility of factor incompatibility between mouse and human, synthesized reprogramming mRNA molecules encoding mouse Oct4, c-Myc, Klf4, Sox2 and Lin28, and initiated several attempts to reprogram our mouse cell lines with mRNA encoding mouse-specific factors. During these attempts, we modified several conditions for plating and transfecting

mouse cells since human-specific conditions fail to reprogram mouse lines. While our recently modified conditions look promising and give us partially reprogrammed colonies, we are still unable to generate stable, fully reprogrammed iPSC lines from mouse cells with the mRNA approach. Thus, we will continue to optimize the conditions for the mRNA reprogramming of mouse fibroblasts and keratinocytes.

In addition to the mRNA reprogramming, during this reporting period, we attempted to employ several alternative integration-free reprogramming approaches to produce genome unmodified mouse iPSCs. We first employed the Sendai virus reprogramming protocol [3]. Unfortunately, this protocol failed to generate mouse iPSCs. We have also attempted to adapt a recently published method using small-molecule compounds [4]. We are currently reprogramming WT mouse fibroblasts employing the following cocktail published by Hou et al [4]: VPA, CHIR99021, RepSox, Tranylcypromine, DXNep and Forskolin. We are also initiating a reprogramming with episomal vectors [5], which is recognized to be one of the most consistent methods of integration-free reprogramming. We have already obtained the episomal vectors from Addgene (www.addgene.com) and are ready to initiate the reprogramming with this approach. Although episomal vectors have the ability to integrate into the genome, such integration is an extremely rare event. In addition, since we proposed to utilize the exome sequencing of generated iPSC lines (Aim. 2), we will be able to detect and exclude those rare iPSC clones where episomal vectors managed to integrate.

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. The IRG^{RFP}/LAMA3^{fl/wt}/K14-Cre.ER mouse line is currently being shipped to Dr. Tolar's group.

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 iPSCs were reprogrammed from a mixed population of fibroblasts that may have contained somatic mutations prior to reprogramming. In addition, most of the iPSCs 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 iPSCs, we propose the following:

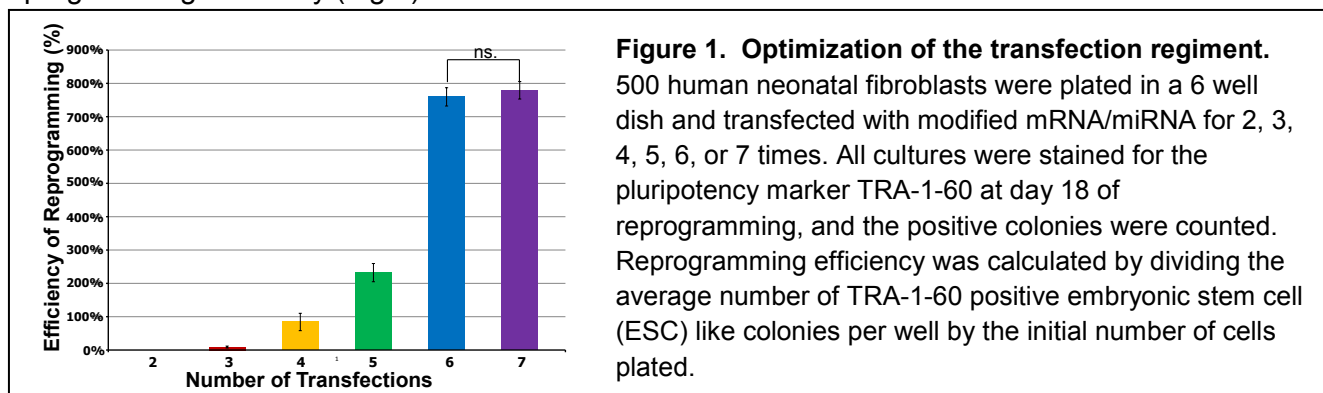
Task 2.1. *Establish 10 independent clones of keratinocytes harvested from skin biopsies obtained from JEB patients.*

We attempted to establish monoclonal lines from human JEB keratinocytes with laminin-322 deficiency. Unfortunately, human JEB keratinocytes grow very slowly as a single cell culture. To achieve reprogramming, somatic cells have to divide at the high rate, and the slow growth rate of monoclonal JEB keratinocytes may impede their ability to undergo efficient reprogramming. As an alternative approach, we are currently establishing monoclonal lines from fibroblasts isolated from the same JEB patient. Since the laminin mutation is present in all somatic cells of JEB patients, dermal fibroblasts serve as a good alternative to keratinocytes.

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

During the previous reporting period, we adapted the mRNA-based reprogramming approach [6] to human keratinocytes and successfully derived several iPSC clones. These results suggest that the mRNA-based reprogramming protocol can be employed for the reprogramming of JEB patient-specific keratinocytes. Since there is still a concern that JEB keratinocytes may be hard to reprogram, we have also optimized the mRNA-base reprogramming for human fibroblasts, including JEB fibroblasts. Surprisingly, we found that the incorporation of reprogramming mimic miRNAs [7] in combination with a feeder-free system significantly increases the efficiency of a synthetic mRNA-based reprogramming approach. As mentioned in our original application, there are still many obstacles that limit the broad application of iPSCs in the clinic and research settings, such as: (1) the relatively low efficiency and high cost of reprogramming protocols, including clinically relevant integration-free approaches; (2) substantial time required to establish a cell line from a patient's biopsy before iPSC generation can be initiated; (3) difficulties in reprogramming a low number of somatic cells with integration-free approaches. Our novel combinatorial mRNA/miRNA reprogramming approach addresses all of these obstacles, and can be applicable not only to the research proposed in this application, but also opens up new horizons to studying the biology of reprogramming.

As a result of optimizing our protocol, we were able to attain an unprecedented efficiency of human cell reprogramming starting from as little as a single human cell. Using human primary neonatal fibroblasts, we generated ~1632 Tra-1-60 (a pluripotency marker) positive iPSC colonies from 200 starting cells within 2.5 weeks of reprogramming with only 7 transfections. A minimum of 3 transfections was required to obtain a few iPSC colonies, and 6-7 transfections to achieve the maximum reprogramming efficiency (Fig.1).



In our regiment, reprogramming efficiency was calculated by dividing the average number of resulting Tra-1-60 positive embryonic stem cell (ESC) like colonies per well on day 18 of reprogramming by the initial number of cells plated. Importantly, under our regiment, low density cultures show the increased efficiency of reprogramming (Table 1 and Fig. 2), probably due to a higher rate of cell division. The increased cell cycle progression has been previously shown to improve the reprogramming of somatic cells into iPSCs. With our protocol, we were also able to reprogram ~54% - 77% of individually plated human neonatal fibroblasts that survived through the remainder of the reprogramming protocol (Table 2); with many reprogrammed cells producing multiple Tra-1-60 positive iPSC colonies (Fig. 3 and 4). When our protocol was employed for the reprogramming of adult fibroblasts derived from a 50 year old healthy individual, the resulting efficiency of iPSC generation was ~36%. To address the applicability of our protocol in aging research and for the generation of iPSCs from the elderly veterans, the same adult fibroblast line was serially passaged until more than 91% of cells exhibited a senescent phenotype and then reprogrammed into iPSCs. The reprogramming of this senescent line took only 2.5 weeks and resulted in an efficiency of ~0.33%, which is significantly

higher than previously reported for the lentiviral approach [8]. The efficiency of iPSC generation from a JEB line (from a 60 year old patient) was ~10%.

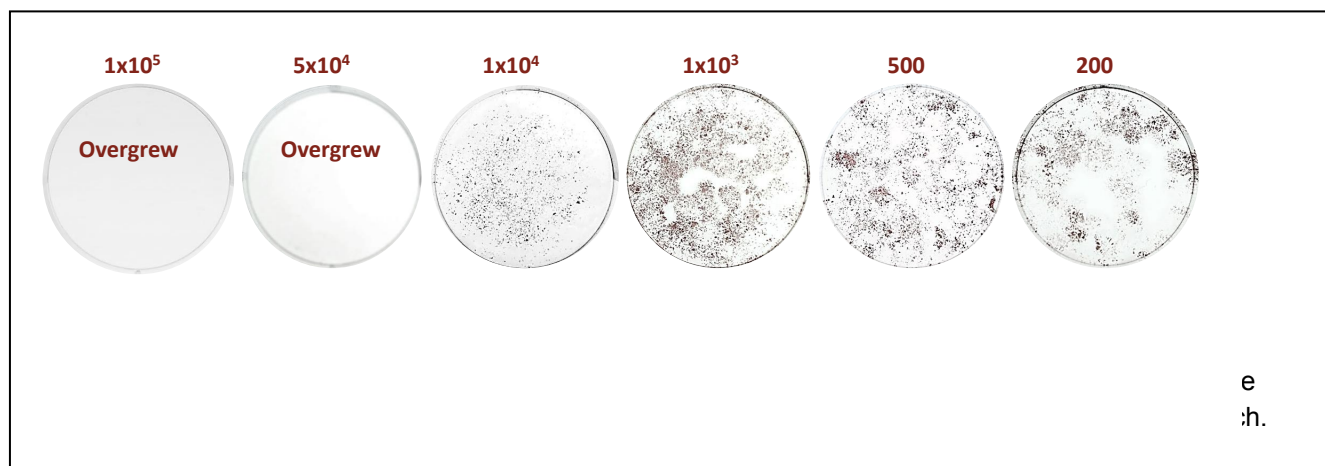


Table 1. Lower initial plating density results in the increased reprogramming efficiency in our combinatorial mRNA/miRNA approach.

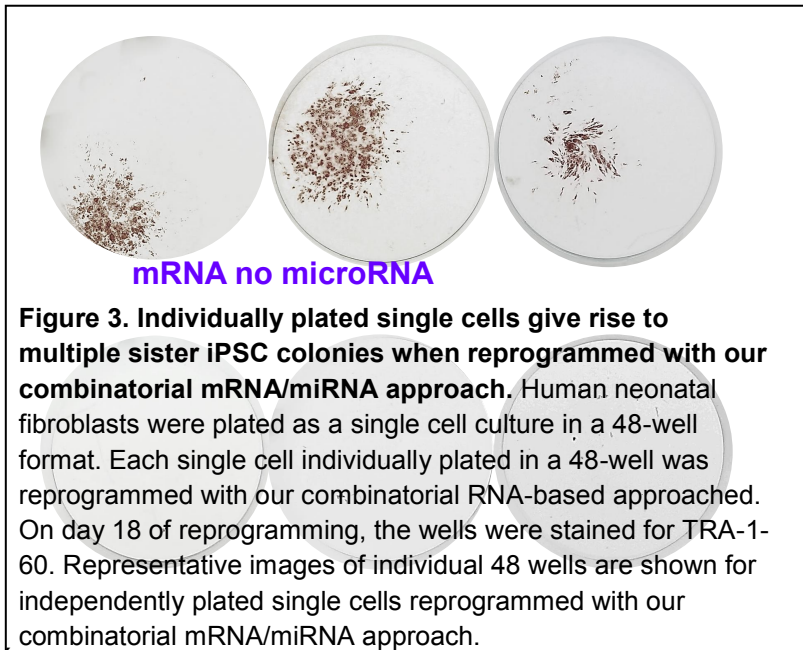
Cells Plated/well (6-well format)	Colonies/well	Efficiency
100,000	0.33 ± 0.58	0,0003% ± 0.0006%
50,000	4.7 ± 6.43	0.0093% ± 0.0129%
10,000	1012.7 ± 164.35	10.13% ± 1.64%
1000	4053 ± 122.11	405.30% ± 12.2%
500	3896 ± 131.14	779.20% ± 26.23%
200	1632.7 ± 439.05	816.40% ± 219.50%

Table 2. Our combinatorial RNA-based approach allows for the highly efficient reprogramming of individually plated single cells

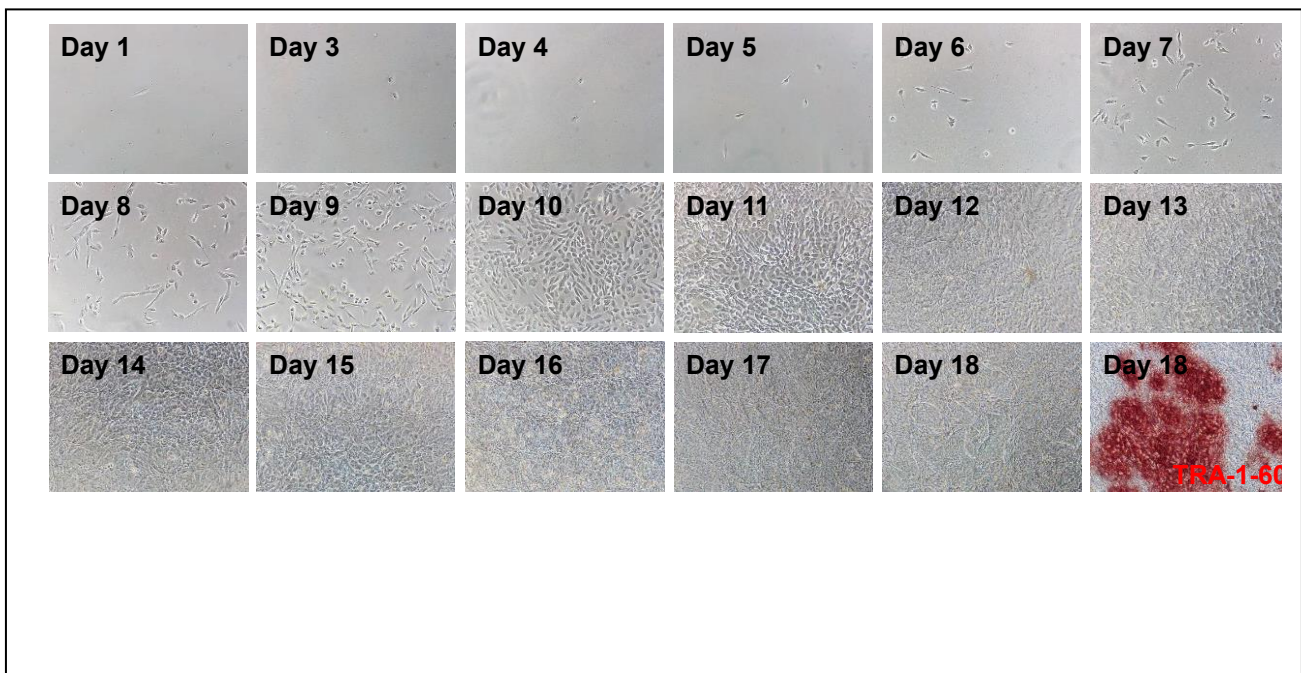
Primary Neonatal Cell Line	Wells with an individually plated single cell	Wells with dividing cells throughout reprogramming	Wells with TRA-1-60 positive colonies	<u>Efficiency:</u> Wells with TRA-1-60 positive colonies/wells with dividing cells
N1	50	37	20	54.1%
N2	86	77	60	77.9%

The established iPSC lines generated from neonatal, adult and senescent human fibroblasts, JEB fibroblasts, and human keratinocytes were stable, exhibited normal karyotypes and have been successfully maintained for at least 14 passages. The pluripotency of the generated iPSCs was

confirmed by gene expression analysis using nCounter® Analysis from Nanostring Technologies (Fig. 5) and RNA-Seq (Fig.6), and by the differentiation into cell types of all three germ layers both *in vitro* (Fig. 7) and *in vivo* (Fig. 8). Thus, our protocol allows for the reprogramming of a variety of somatic human cells, including human keratinocytes, with kinetics and the efficiency which surpass all previously published reports. The approach is cost effective, provides an opportunity to shorten the time between the biopsy and the generation of stable high-quality iPSC lines, and allows for the production of iPSCs from individually plated cells in a feeder-free system.



A
We are currently preparing a manuscript for publication.



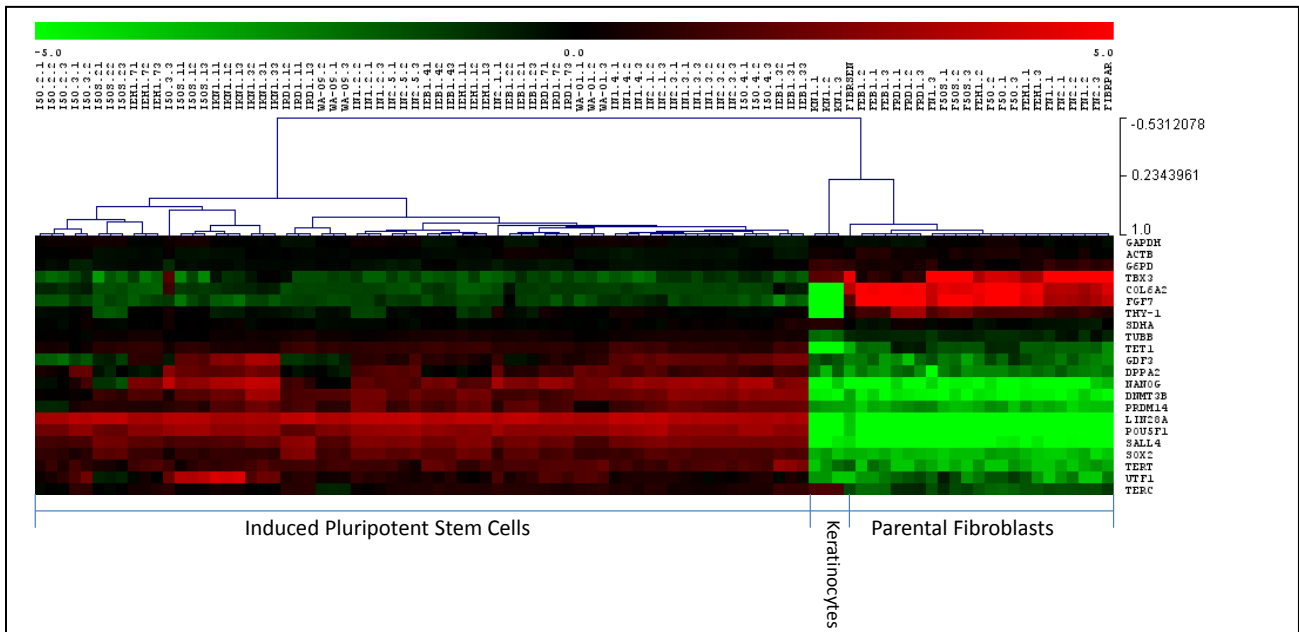


Figure 5. Molecular characterization of iPSCs generated with our RNA-based approach. The combined heatmap and unsupervised hierarchical clustering of the expression of a set of selected genes (shown on the right) using nCounter® Analysis System from Nanostring Technologies is shown. Our generated iPSCs exhibit the robust expression of pluripotency genes and cluster separately from keratinocytes and parental fibroblasts. iPSCs named “IKN1” are derived from human keratinocytes (KN1). All generated iPSC lines were maintained for at least 14 passages before they were analyzed.

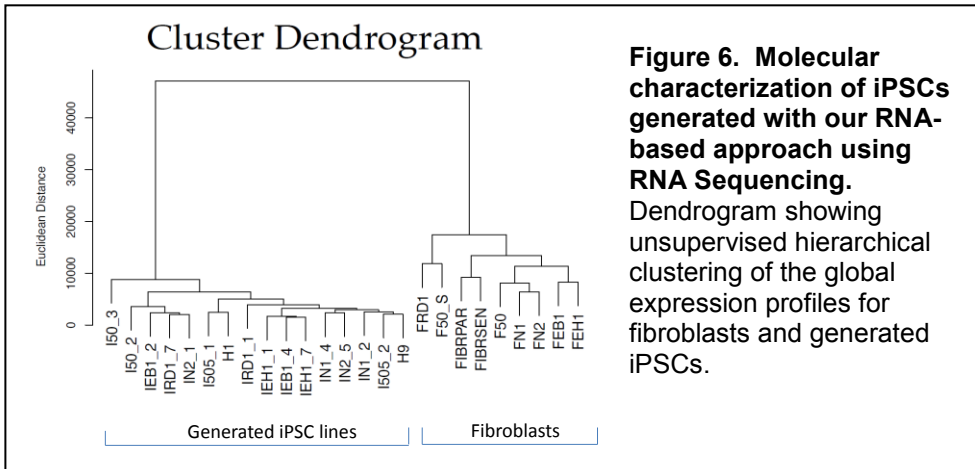
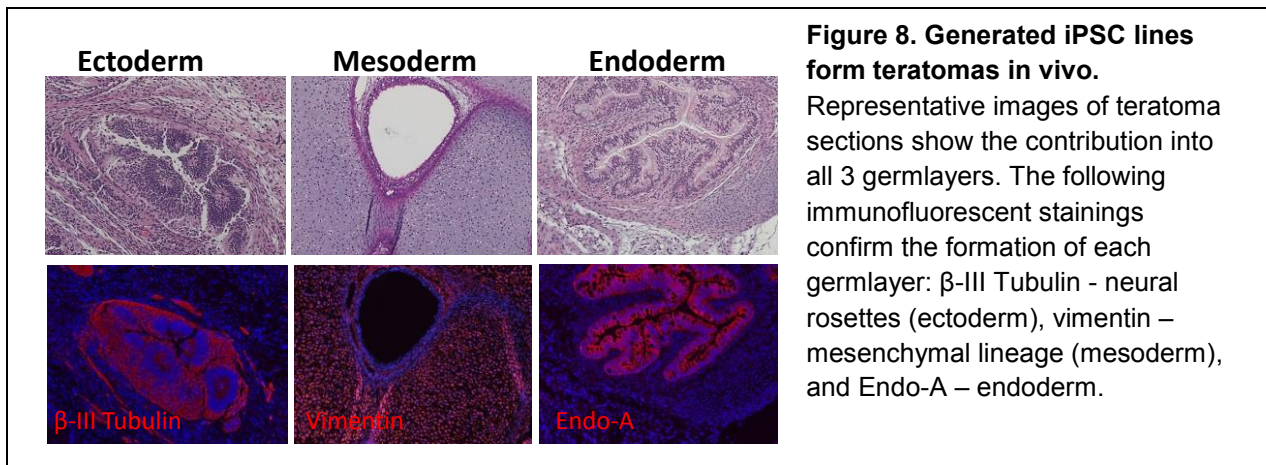
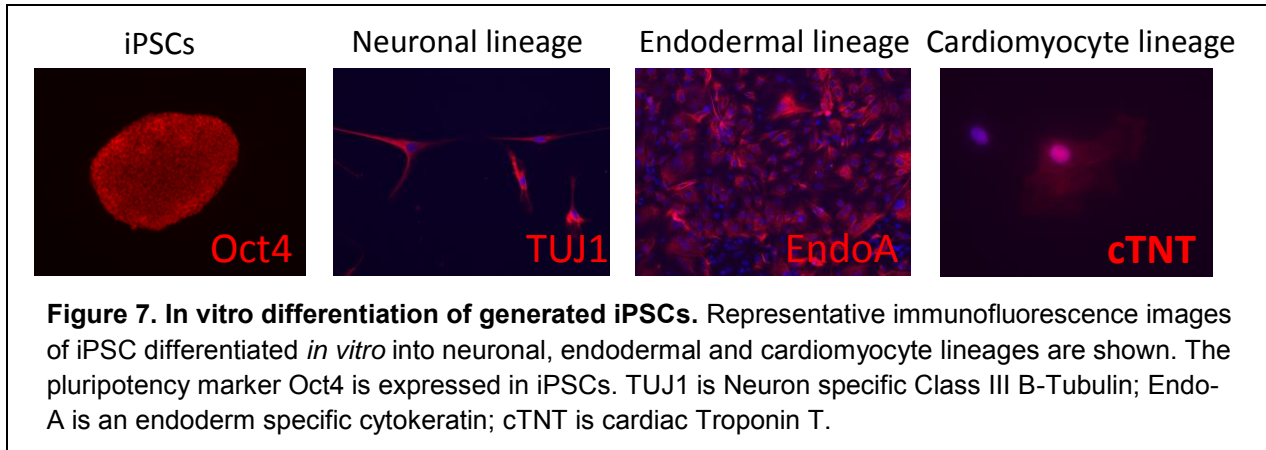


Figure 6. Molecular characterization of iPSCs generated with our RNA-based approach using RNA Sequencing. Dendrogram showing unsupervised hierarchical clustering of the global expression profiles for fibroblasts and generated iPSCs.



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.

We have collected samples from all our generated iPSCs for subsequent exome sequencing.

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.

During this reporting period, we have optimized our protocol for the differentiation of human iPSCs generated with a RNA-based approach into keratinocytes. These optimizations has been published during this reporting period (see appendix for the copy of the published manuscript: Kogut, I., Roop, D. R., and G. Bilousova. Differentiation of human induced pluripotent stem cells into a keratinocyte lineage. *Methods Mol Biol.* 2014;1195:1-12. doi: 10.1007/7651_2013_64; PMID: 24510784)

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 Lin⁻/PDGFR α ⁺ 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 Lin⁻/PDGFR α ⁺ BM cells into injured epithelia. To confirm these observations, we propose the following:

Task 3.1. *To determine whether mouse iPSC-derived Lin⁻/PDGFR α ⁺ cells will home into injured epithelia.*

To be completed by Dr. Tolar.

Task 3.2. *To determine whether human iPSC-derived Lin⁻/PDGFR α ⁺ 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 iPSCs. 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.*

In collaboration with Sigma, we are currently designing an optimum binding site for ZFNs to correct the genetic defect in mouse JEB iPSCs. Upon generation of integration-free mouse JEB iPSCs described in the Task 1.1, we will perform a gene targeting experiment with designed ZFNs.

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.*

In collaboration with Sigma, we are currently designing an optimum binding site for ZFNs to correct the genetic defect in human JEB iPSCs.

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

- Produced mRNAs encoding mouse reprogramming factors for the generation of mouse iPSCs;
- Adapted a RNA-based reprogramming protocol for the generation of human iPSCs from keratinocytes and fully characterized the generated keratinocyte-derived iPSCs;
- Developed a novel RNA-based reprogramming technique, which results in the highly efficient generation of integration-free clinically relevant human iPSCs. The developed protocol allows for the production of iPSCs from individually plated cells in a feeder-free system, which will be applicable for comprehensive studies of reprogramming mechanisms on a single cell level (manuscript is in preparation);
- Generated iPSCs from human JEB fibroblasts with Laminin-332 deficiency;
- Optimized the protocol for the differentiation of human iPSCs generated with a RNA-based reprogramming approach into keratinocytes.

Reportable Outcomes

- Published a manuscript: Kogut, I., Roop, D. R., and G. Bilousova. Differentiation of human induced pluripotent stem cells into a keratinocyte lineage. *Methods Mol Biol.* 2014;1195:1-12. doi: 10.1007/7651_2013_64; PMID: 24510784 (see appendix for the copy).

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 EB, 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 the clinical trial. We were able to accomplish several important goals during the current year of funding. Specifically, we expanded our colony of IRG^{RFP}/LAMA3^{fl/wt}/K14-Cre.ER mice and generated several keratinocyte and fibroblast lines from these mice for the subsequent generation of iPSCs. We are also adapting several integration-free protocols for the generation of mouse iPSCs, which will allow us to address the immunogenicity of mouse iPSC-derived cell lines upon transplantation into congenic recipient mice. We also adapted a RNA-based reprogramming protocol for the generation of iPSC from human keratinocyte line and fully characterize these keratinocyte-derived iPSCs for pluripotency with RNA-Seq and functional tests. In addition, we developed an integration-free clinically relevant RNA-based reprogramming protocol, which results in the extremely high efficiency of iPSC production. Such a high efficiency has never been reported before and has been impossible to achieve with conventional reprogramming protocols. This protocol opens many new potential areas of research and provides an easy reprogramming method for clinical applications. We are currently preparing a manuscript and expect to submit it for publication within 3 months. We have also optimized the protocol for the differentiation of iPSCs into keratinocytes and published it in *Methods of Molecular Biology*. The steps accomplished during this report period are critical to address the next steps of our proposal and eventually develop a genome editing therapeutic strategy for JEB patients.

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Differentiation of Human Induced Pluripotent Stem Cells into a Keratinocyte Lineage

Igor Kogut, Dennis R. Roop, and Ganna Bilousova

Abstract

Direct reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) provides an opportunity to develop novel personalized treatment options for numerous diseases and to advance current approaches for cell-based drug discoveries and disease modeling. The ability to differentiate iPSCs into relevant cell types is an important prerequisite for the successful development of iPSC-based treatment and modeling strategies. Here, we describe a protocol for the efficient differentiation of human iPSCs into functional keratinocytes. The protocol employs treating iPSCs with retinoic acid and bone-morphogenetic protein-4 to induce differentiation toward a keratinocyte lineage, which is then followed by the growth of differentiated iPSCs on collagen type I- and collagen type IV-coated dishes to enrich for iPSC-derived keratinocytes.

Keywords: Induced pluripotent stem cells, iPSCs, Differentiation, Keratinocytes, Retinoic acid, Bone-morphogenetic protein-4

Abbreviations

ColI	Type I collagen
ColIV	Type IV collagen
ESC	Embryonic stem cell
iPSC	Induced pluripotent stem cell
Krt14	Keratin 14
RA	Retinoic acid
BMP4	Bone-morphogenetic protein-4

1 Introduction

The discovery that the ectopic expression of selected transcription factors can reprogram somatic cells into embryonic stem cell (ESC)-like cells, termed induced pluripotent stem cells (iPSCs), has opened up a new era in research and therapy (1–5). The iPSC technology addresses many obstacles associated with the use of ESCs, including ethical concerns, and allows for the generation of

patient-specific pluripotent stem cells, which can be genetically corrected, differentiated into adult lineages, and returned to the same patient as an autograft (6–9). In addition to genetic disorders, the iPSC technology can be applicable to tissue regeneration, basic science research of human development, and disease modeling. However, before iPSC-based approaches are successfully implemented into the clinic, efficient protocols for the differentiation of iPSCs into relevant cell types need to be developed.

In this chapter, we describe a protocol for the efficient differentiation of human iPSCs into keratinocytes, which may potentially be applicable for cell transplantation in the clinic and for modeling inherited skin diseases, such as the epidermolysis bullosa (EB) subtypes and congenital ichthyoses (10–12). The protocol has been adapted from our previously published work (13) on the differentiation of mouse iPSCs into keratinocytes as well as studies published by other groups on the differentiation of human ESCs and iPSCs into epithelial and keratinocyte lineages (14, 15) with modifications. The resulting iPSC-derived keratinocyte-like cells express the markers specific to authentic basal layer keratinocytes, such as keratin 14 (Krt14) and keratin 5 (Krt5), and are able to reconstitute a normal stratified epidermis when grafted onto an immunodeficient mouse. The protocol requires the seeding of iPSCs onto Geltrex (Gibco) and collagen type I (ColI)-coated dishes followed by the combined treatment with retinoic acid (RA) to induce iPSC differentiation into an ectodermal fate (16) and with bone-morphogenetic protein-4 (BMP4) to block the commitment toward a neural fate (17). In addition, we discovered that growth on collagen type IV (ColIV)- and ColI-coated dishes, which mimics the environment of the basal layer of the skin, improves the efficiency of differentiation to a keratinocyte fate. To enrich for keratinocyte stem cells that are positive for Krt14, a keratin marker confirming commitment of the ectoderm to a keratinocyte fate, we exploit the ability of Krt14-positive cells to rapidly attach to ColI/ColIV-coated surfaces (18).

The methodology for iPSC differentiation toward a keratinocyte lineage relies primarily on the ability to maintain long-term human keratinocyte cultures. Therefore, before initiating this iPSC differentiation protocol, we recommend establishing the growth conditions for culturing normal human keratinocytes that allow for their maintenance in culture for at least 6–10 passages. We found that commercially available CnT-07 medium or EpiLife medium supplemented with EpiLife Defined Growth Supplement (EDGS) promotes more efficient expansion of human keratinocytes seeded onto ColI-coated dishes. The growth of differentiated iPSC-derived cultures under keratinocyte cell culture conditions following the rapid attachment to ColI/ColIV-coated plates allows for the efficient enrichment for Krt14-positive keratinocytes up to 80–90 % (13, 15).

2 Materials

2.1 Coating Tissue Culture Dishes with Geltrex and Coll

1. Collagen, type I: 3 mg/mL solution (Advanced BioMatrix).
2. Geltrex hESC-qualified Reduced Growth Factor Basement Membrane Matrix (Gibco).
3. Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (Gibco).
4. 60 mm tissue culture (TC) dishes.

2.2 Plating iPSCs for Differentiation

1. N2B27 medium: Combine DMEM/F12 and Neurobasal medium (Gibco) in a 1:1 ratio and supplement with 0.1 mM nonessential amino acids, 1 mM glutamine, 55 μ M 2-mercaptoethanol (2-ME), N2 supplement (100 \times) (Life Technologies), B27 supplement (50 \times) (Life Technologies), 50 μ g/mL ascorbic acid, 0.05 % bovine serum albumin (BSA), 50 U/mL penicillin–streptomycin, 100 ng/mL basic FGF (Life Technologies), and 10 μ g/mL Y27632 (Sigma-Aldrich).
2. Dispase (BD).

2.3 Differentiation of iPSCs with RA and BMP4

1. 1 mM stock solution of all-trans RA (Sigma-Aldrich) reconstituted in dimethyl sulfoxide (DMSO).
2. 25 μ g/ μ L stock solution of human BMP4 (R&D Systems) reconstituted in sterile 4 mM HCl containing 0.1 % BSA.
3. Defined keratinocyte serum-free medium (DKSFM) (Gibco) supplemented with 50 U/mL penicillin–streptomycin. DKSFM is sold as a kit containing DKSFM basal medium and DKSFM growth supplement.
4. 1 \times PBS.
5. CnT-07 epidermal keratinocyte medium (CELLnTEC) containing 50 U/mL penicillin–streptomycin. CnT-07 is sold as a kit containing CnT basal medium and supplements A, B, and C.

2.4 Rapid Attachment and Culturing of iPSC-Derived Keratinocytes

1. 100 mm tissue culture dish.
2. Collagen, type IV, powder (Sigma-Aldrich).
3. 0.25 % Glacial acetic acid.
4. Collagen, type I, 3 mg/ml solution (Advanced BioMatrix).
5. CnT-07 (*see* Section 2.3).
6. Accutase (Gibco).
7. 1 \times PBS without Ca²⁺ and Mg²⁺.

2.5 Equipment

1. Biological safety cabinet.
2. 37 °C water bath.
3. 37 °C/5 % CO₂ humidified tissue culture incubator.
4. Centrifuge (room temperature).

3 Methods

3.1 Coating Tissue Culture Dishes with Geltrex and ColI

The procedure is to be performed in a biological safety cabinet using aseptic techniques. Similar to Matrigel, Geltrex matrix solidifies rapidly at room temperature (RT). Therefore; it is recommended to aliquot each new batch of the matrix upon arrival and use pre-chilled pipet tips, racks, and tubes while working with the reagent. We recommend making 50, 100, and 200 μL aliquots and to store them at $-80\text{ }^{\circ}\text{C}$. Use Geltrex at 1:100 dilutions. While the maintenance of feeder-free iPSC cultures requires only Geltrex as a surface coating agent, for iPSC differentiation, the combination of Geltrex and ColI is more efficient to induce the commitment toward a keratinocyte lineage (*see Note 1*). The coating procedure below is described for a 60 mm tissue culture dish. If a larger dish is to be used, adjust the volume of the coating solution accordingly.

1. Remove a 50 μL aliquot of Geltrex from the $-80\text{ }^{\circ}\text{C}$ freezer, and place it on ice in the biological safety cabinet.
2. Add 5 mL of cold sterile DMEM/F12 to a 15 mL conical tube.
3. Use a 1 mL glass pipet, take 1 mL cold DMEM/F12 from the 15 mL conical tube prepared in step 2, and add to the frozen Geltrex. Gently pipet up and down to thaw and dissolve Geltrex. Transfer the dissolved Geltrex to the rest of DMEM/F12 in the 15 mL conical tube prepared in step 2. Pipet to mix diluted Geltrex.
4. Add 50 μL of 3 mg/mL ColI stock solution into diluted Geltrex from step 3. Pipet to mix diluted Geltrex with ColI. Add 4 mL of coating solution into 60 mm dish. Tap or swirl the plate to ensure that the entire surface is coated.
5. Incubate the dish with Geltrex/ColI coating solution at $37\text{ }^{\circ}\text{C}$ in the tissue culture incubator for at least 1 h.
6. Once the coating is complete, leave the coating solution in the dish and proceed with the plating of iPSCs as described in the next subsection (*see Section 3.2*). Alternatively, aspirate the coating solution and add 2 mL of fresh DMEM/F12 into the-coated dish to prevent it from drying before plating the cells.

3.2 Plating iPSCs for Differentiation

Prepare one 60 mm tissue culture dish of feeder-free iPSCs grown to $\sim 70\%$ of confluency (*see Note 2*). Examine cells under a microscope to confirm the absence of contamination and the maintenance of their undifferentiated phenotype. If the cells are stressed or dying, they start to differentiate, presenting themselves as “cobblestone” areas with larger polymorphic cells, and should not be used for the differentiation toward keratinocytes. For iPSC differentiation toward keratinocytes, we recommend a 1:8 split ratio of iPSCs (*see Note 3*).

1. Prewarm N2B27 medium and Dispase in the 37 °C water bath.
2. Using the microscope, confirm that the colonies are ready for passaging. Gently aspirate medium from the dish. Add 2 mL of 1 × PBS, swirl the plate to wash the cells, and gently aspirate PBS.
3. Add 1 mL of Dispase and return the plate to the 37 °C tissue culture incubator for 3–5 min.
4. While the cells are being incubated with Dispase, gently aspirate the Geltrex/ColI coating solution (or DMEM/F12) from step 6 in the Geltrex/ColI coating procedure (*see* Section 3.1) and add 4 mL of complete N2B27 medium into the coated dish.
5. After 3–5-min incubation with Dispase, confirm that the cells are ready to be picked by looking for rolled or folded edges around the colonies.
6. Transfer the plate to the biological safety cabinet, and carefully aspirate Dispase. After the treatment with Dispase, the colonies are very loosely attached to the surface of the dish and may peel off if too much force is used (*see* Note 4).
7. Gently add 2 mL of plain DMEM/F12. Aspirate off the medium, and repeat the wash three times.
8. Add 2 mL of complete N2B27 into the dish, and gently scrape the colonies off the plate. Transfer the cells from the dish into a 15 mL conical tube, and add 6 mL of complete N2B27 to bring the total volume of cell suspension to 8 mL.
9. Gently mix the cell suspension to break large clumps of cells. Transfer 1 mL of the cell suspension to the coated dish prepared in step 3 of the current subsection. Discard or replate the leftover cells using the conditions established for a given laboratory (*see* Note 5).
10. Transfer the newly plated cells to the incubator, and gently shake the plate back and forth and side to side to distribute the cells evenly (*see* Note 6). Incubate the cells overnight in the 37 °C tissue culture incubator.

3.3 Differentiation of iPSCs with RA and BMP4

The differentiation and subculturing of iPSC-derived keratinocytes are to be performed in a biological safety cabinet using aseptic techniques. The protocol schematic is outlined in Fig. 1. Examine the new plate the day after passaging to confirm the successful attachment of iPSCs. If iPSCs start forming colonies (Fig. 2a), proceed with the differentiation protocol below (*see* Note 7).

1. Prewarm complete DKSFM (with antibiotics and DKSFM supplement) in the 37 °C water bath.
2. Add 5 mL of prewarmed DKSFM from the previous step to a 15 mL conical tube, add 5 µL of 1 mM RA to achieve 1 µM final working concentration and 5 µL of 25 µg/µL BMP4 to achieve 25 ng/mL final working concentration, and mix well.

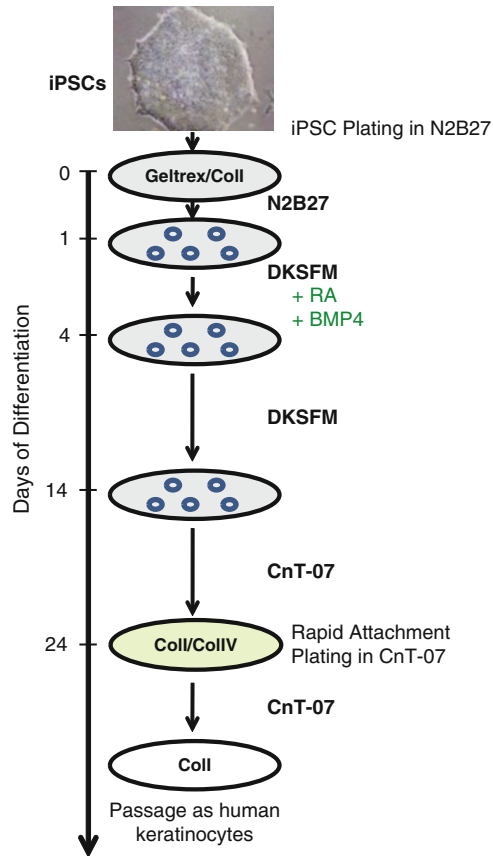


Fig. 1 Schematic representation of the protocol for the differentiation of human iPSCs into a keratinocyte lineage

3. Aspirate off N2B27 medium from the dish with plated iPSCs, wash once with 4 mL of $1 \times$ PBS, and add 4 mL of DKSFM containing $1 \mu\text{M}$ RA and $25 \text{ ng}/\mu\text{L}$ BMP4 from the step above. This is day 1 of differentiation procedure.
4. Transfer the cells to the incubator and incubate for 48 h.
5. Replace the medium with fresh DKSFM containing $1 \mu\text{M}$ RA and $25 \text{ ng}/\mu\text{L}$ BMP4 after 48 h of incubation. Transfer the cell to the incubator for another 48 h.
6. After the second round of 48-h induction (day 4 of differentiation), replace the medium with complete DKSFM without RA and BMP4. Incubate cells in the incubator for 10 days in complete DKSFM, changing medium every other day.
7. On day 14 of differentiation, prepare complete CnT-07 medium by adding antibiotics and provided supplements and prewarm the medium. By this day, the majority of the cells in the outgrown iPSC colony start exhibiting an epithelial-like phenotype (*see* Fig. 2b).

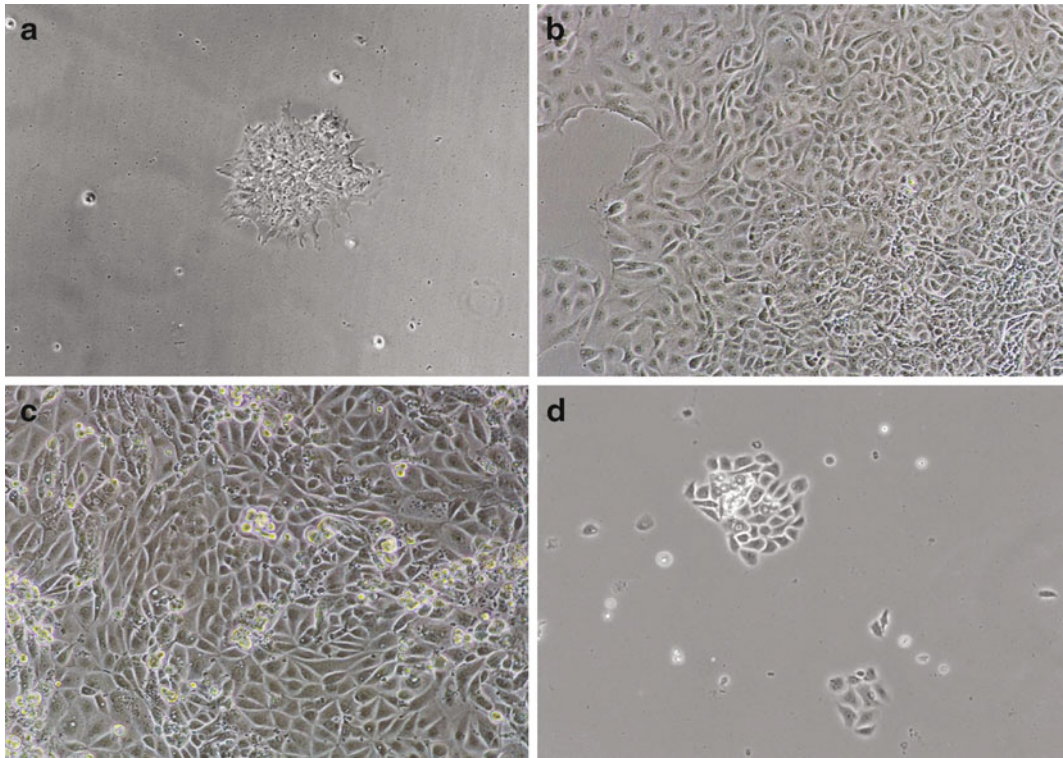


Fig. 2 The stages of iPSC differentiation during keratinocyte derivation. Human iPSCs generated with a modified mRNA-based approach from human neonatal fibroblasts were differentiated into keratinocytes using RA and BMP4. **(a)** Human iPSCs seeded at low density for differentiation on day 1 of differentiation. **(b)** An iPSC colony outgrown on a Geltrex/Coll-coated plate after the combined treatment with RA and BMP4 on day 14 of differentiation. **(c)** An iPSC colony outgrown on a Geltrex/Coll-coated plate on day 24 of differentiation before rapid attachment plating. **(d)** iPSC-derived keratinocytes at passage 1 post rapid attachment plating (day 29 of differentiation, day 5 post rapid attachment). All images were taken with 10 \times objectives

8. Aspirate off DKSEFM from differentiated cells and replace with 4 mL of complete CnT-07. Incubate the cells in the tissue culture incubator for another 10 days, changing complete CnT-07 every other day.

3.4 Rapid Attachment and Culturing of iPSC-Derived Keratinocytes

On day 24 of differentiation, many cells that migrate away from the outgrown iPSC colony will exhibit a keratinocyte-like phenotype (Fig. 2c) and start expressing p63, a master regulator required for the commitment of the ectoderm to a keratinocyte fate (19, 20), and Krt14 (*see Note 8*). By this day, the 60 mm dish used for iPSC differentiation is fully confluent and needs to be passaged. To enrich for iPSC-derived keratinocytes during passaging, we perform the rapid attachment of the differentiated iPSC culture to Coll/ColIV-coated plates. We recommend using up to four 100 mm Coll/ColIV-coated tissue culture dishes to

perform the rapid attachment procedure from one 60 mm dish containing differentiated iPSCs. If only one 100 mm dish is to be used, plate one-fourth of the differentiated iPSC culture for the rapid attachment procedure.

3.4.1 Coating Plates with ColI and ColIV

The procedure is to be performed in the biological safety cabinet using aseptic techniques.

1. Reconstitute ColIV powder to a concentration of 2 mg/mL in sterile 0.25 % glacial acetic acid. Dissolve for several hours at 2–8 °C, occasionally swirling. Make aliquots, and store them at –20 °C.
2. Thaw the aliquot of ColIV stock solution (2 mg/mL) very slowly by placing the vial in an ice bucket and keeping it at 4 °C for several hours.
3. Resuspend ColIV stock solution in the appropriate volume (5 mL per each 100 mm dish) of sterile 0.25 % glacial acetic acid to a final working concentration of 7 µg/mL. Add an appropriate volume of ColI stock solution to achieve a final working ColI concentration of 30 µg/mL. Coat the plates by using 5 mL of working solution to cover a 100 mm dish. Incubate the plates at room temperature in the biological safety cabinet for 1 h.
4. Aspirate the liquid from the coated plates, and rinse the dishes once with 5 mL of sterile 1× PBS and once with 5 mL of ddH₂O.
5. Air-dry the washed dishes in the biological safety cabinet. Use plates directly or seal them with Parafilm and store at 4 °C for up to 6 months. To use a previously stored ColIV-coated plate, allow the plate to warm up at room temperature in the biological safety cabinet for at least 1 h prior to plating cells.

3.4.2 Rapid Attachment of iPSC-Derived Keratinocytes

1. On day 24 of differentiation, prewarm complete CnT-07, Accutase, and ColI/ColIV-coated dish(es).
2. Wash the cells with 1× PBS, add 2 mL of Accutase, and incubate in the tissue culture incubator for 5 min (*see Note 9*). Confirm under the microscope that cells start detaching.
3. Add 3 mL of complete Cnt-07, pipet up and down to dislodge the cells, and collect the cell suspension into a 15 mL conical tube. Spin the cells down at 260 × *g* for 5 min, and aspirate the supernatant. Resuspend the pellet in 10 mL of complete Cnt-07 medium, repeat the spin at 260 × *g* for 5 min, and aspirate the supernatant.
4. Resuspend the pellet in 4 mL of complete CnT-07 and pipet up and down to break cell clumps into single cells.

5. Add 9 mL of complete CnT-07 medium into each ColI/ColIV-coated dish, and transfer 1 mL of cell suspension from step 4 above into each ColI/ColIV-coated dish. Allow the cells to attach to the coated dish at room temperature for 15–30 min (*see Note 10*).
6. Carefully aspirate the medium with the floating cells (these are undifferentiated or partially differentiated iPSCs). Do not disturb the attached cells (these are iPSC-derived Krt14-positive cells). Add 10 mL of fresh complete CnT-07 medium into the plate with the attached cells. Let the cells expand in the 37 °C tissue culture incubator, changing the medium every other day. Passage cells as needed (*see Note 11*) with Accutase in CnT-07 or EpiLife (with EDGS supplement) on ColI-coated dishes. After passage 2 or 3 and following the rapid attachment step, the culture should consist of ~90 % of Krt14-positive cells exhibiting a keratinocyte-like phenotype (*see Fig. 2d*). The keratinocyte-like phenotype of the obtained culture can be verified by the standard immunofluorescence analyses for Krt14 expression and by the ability to reconstitute a normal stratified epidermis in organotypic cultures.

4 Notes

1. We initially used growth factor reduced BD Matrigel to plate iPSCs for differentiation. However, the combination of Geltrex and ColI gives a higher yield of keratinocytes upon iPSC differentiation.
2. The provided protocol is optimized for iPSCs generated with an integration-free modified mRNA-based reprogramming approach (21, 22). We maintain iPSCs on either mitomycin C-inactivated human neonatal fibroblasts or Geltrex matrix in N2B27 medium (23) under low O₂ conditions (5 %). While iPSCs are maintained under low-oxygen conditions, the differentiation toward a keratinocyte lineage is performed under atmospheric O₂ (~20 %) in the regular tissue culture incubator. To avoid spontaneous differentiation, the iPSC culture should only be grown to a subconfluent state. Healthy undifferentiated human iPSCs usually form round tight colonies with clear margins (Fig. 2a). Avoid using partially differentiated iPSCs for keratinocyte derivation. Although the provided protocol has been shown to produce functional keratinocytes from human iPSCs generated by an integrating lentivirus approach, there is always a possibility that the partial reactivation of exogenous factors, especially c-Myc and Klf4, may influence the differentiation of these lentivirally derived iPSCs into keratinocytes, and the protocol may require optimizations for this type of iPSCs.

3. While we recommend a 1:4 or a 1:5 split ratio for the maintenance of iPSCs, for their differentiation, iPSCs need to be seeded as small clumps at very low density to allow for enough surface area for the sufficient expansion of differentiating cells. The colonies should be evenly dispersed in the dish. To achieve this, gently shake the dish from side to side and front to back during passaging.
4. If iPSC colonies peel off while being incubated with Dispase, collect Dispase with detached iPSC colonies into a 15 mL conical tube, add plain DMEM/F12 into the dish, gently scrape the remaining colonies off, and transfer the colonies from the dish into the 15 mL conical tube with Dispase and the rest of iPSCs. Spin the cells down at $75 \times g$ for 10 min, and aspirate the supernatant. Gently resuspend the iPSC pellet in plain DMEM/F12, spin the cells down at $75 \times g$ for 10 min, and repeat the wash two times. Proceed with step 9 of Section 3.2.
5. While we regularly use N2B27 medium for the maintenance of human iPSCs, other media can also be used.
6. Do not swirl the dish since the cells tend to cluster in the middle when the dish is being swirled.
7. If the colonies start to differentiate spontaneously, discard the dish and repeat the replating of iPSCs using a fresh iPSC culture.
8. We are able to obtain a maximum of 25–30 % of K14-positive cells in the entire culture before the rapid attachment step. The efficiency of differentiation usually varies from 5 to 30 % among experiments and among iPSC lines.
9. We do not recommend using trypsin at this stage of the protocol.
10. If only a few cells attach, incubate the plate for up to an hour in the 37 °C tissue culture incubator. Alternatively, skip the rapid attachment during the first passage. Instead, split the entire plate of differentiated iPSCs onto four fresh ColI-coated dishes in complete CnT-07. Let the cells reach 60–70 % confluency, and then perform the rapid attachment plating as described in Section 3.4.
11. It may take up to 2 weeks to expand the culture of iPSC-derived keratinocytes post rapid attachment plating. Do not allow the cells to overgrow, since this will induce premature differentiation. Ideally, the cells should be subcultured onto a fresh ColI-coated dish once they reach 60 % confluency. We recommend using Accutase instead of trypsin for keratinocyte passaging.

Acknowledgements

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Stem cell Based Therapies for Epidermolysis Bullosa

Log number PR110793
W81XWH-12-1-0606



PI: Roop, Dennis R.

Org: Regents of the University of Colorado

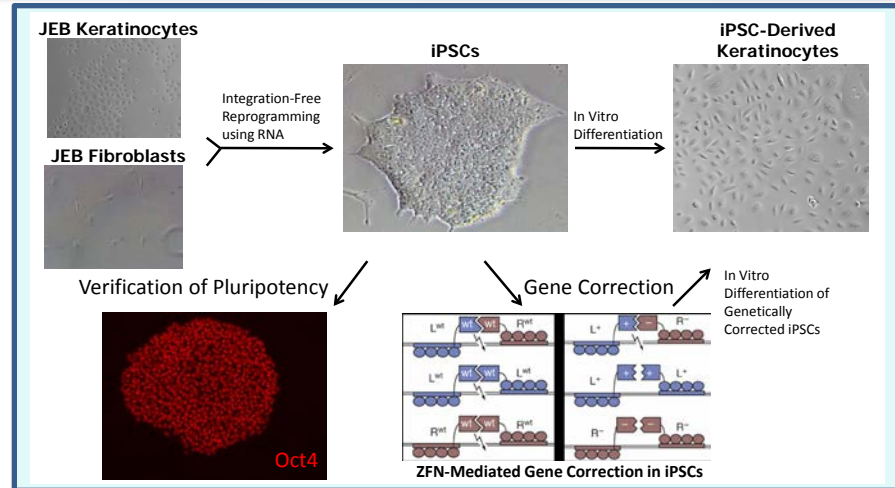
Award Amount: \$552,470

Study/Product Aim(s)

- To determine the histocompatibility of iPSC-derived keratinocytes and mesenchymal cells.
- To determine the genetic stability of human iPSC generated from keratinocytes obtained from JEB patient biopsies.
- To develop methods to increase the homing of iPSC-derived Lin⁻/PDGFR α ⁺ cells into injured epithelia.
- To develop an efficient and safe method for the genetic correction of the defective gene in JEB-specific iPSC.

Approach

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.



Accomplishment: We developed a very efficient integration-free approach to produce clinically relevant iPSCs and generated iPSCs from human JEB fibroblasts. We have also improved our protocol for the differentiation of iPSCs into keratinocytes.

Timeline and Cost

Activities	CY	12	13	14
1. To determine the histocompatibility of iPSC-derived keratinocytes and mesenchymal cells.				
2. To determine the genetic stability of human iPSC generated from keratinocytes obtained from JEB patient biopsies.				
3. To develop methods to increase the homing of iPSC-derived Lin ⁻ /PDGFR α ⁺ cells into injured epithelia (Tolar's group)				
4. To develop an efficient and safe method for the genetic correction of the defective gene in JEB-specific iPSC.				
Estimated Budget (\$K)	\$552,470	184718	184137	183615

Goals/Milestones (Example)

CY12 Goal – Generation and validation of mouse and human iPSCs

- Optimization of iPSC generation from human keratinocytes;
- Generate integration-free mouse JEB iPSCs, assess histocompatibility;
- Generation of ZFNs to genetically correct a mouse JEB cells.

CY13 Goals – Genetic stability and differentiation of human JEB iPSCs

- Generation and characterization of JEB iPSCs;
- Differentiation of generated iPSCs into keratinocytes;
- Generation of ZFNs to genetically correct human JEB iPSCs.

CY14 Goals – Characterization of genetically corrected JEB iPSCs

- Correct JEB iPSCs using ZFNs;
- Differentiate corrected iPSCs into keratinocytes and mesenchymal cells.

Comments/Challenges/Issues/Concerns

- Due to a delay in getting approval of our ACURO animal protocol, Aim 1 (generation and characterization of mouse JEB iPSCs) could not be initiated until after April 25, 2014.

Budget Expenditure to Date

Projected Expenditure: \$368,855

Actual Expenditure: \$368,885

Updated: 08/11/2014