Award Number: W81XWH-12-1-0606

TITLE: Stem Cell-Based Therapies for Epidermolysis Bullosa

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REPORT DATE: December 2015

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
December 2015	Final	30 Sep 2012 - 29 Sep 2015

4. TITLE AND SUBTITLE

Stem Cell-Based Therapies for Epidermolysis Bullosa

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0606

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Regents of the University of Colorado

1300 E. 17th Place, Building 500, Room W1126

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

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 entire funding period, we successfully accomplished most of the milestones toward the development of a proof-of-concept genome editing therapeutic strategy for JEB patients proposed as a main goal of this DOD application. We adapted integration-free reprogramming to both mouse and human somatic cells. We optimized iPSC differentiation protocols toward keratinocyte and mesenchymal lineages. We are currently completing gene editing of JEB iPSCs, fulfilling the final task for the successful accomplishment of the proposed study. Our developed iPSC-based stem cell therapy can be applicable not only to JEB patients but also to patients with other inherited skin diseases, as well as veterans with chronic wounds.

15. SUBJECT TERMS
Epidermolysis Bullosa (EB); Junctional EB, Induced Pluripotent Stem Cells (iPSC)

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
U	U	U	UU	44	19b. TELEPHONE NUMBER *(include area code)*

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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 scaring, 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 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 mouse studies outlined in our proposal up until this date. Despite this delay, we have achieved several important milestones of the proposed grant (see below).

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

We successfully generated IRG^{RFP}/*LAMA3*$^{fl/wt}$/K14-Cre.ER and showed that the JEB phenotype can be induced in these mice as indicated by the formation of blisters and the lack of laminin expression in the basement membrane (**Fig. 1**). Although we were unsuccessful with a mRNA-based reprogramming of mouse cells, we were able to successfully reprogram fibroblasts isolated from both WT and IRG^{RFP}/*LAMA3*$^{fl/wt}$/K14-Cre.ER mice using non-integrating episomal vectors (Okita, K., et al, 2011) as an alternative to mRNA-based reprogramming. Episomal-based reprogramming is recognized to be one of the most consistent methods of integration-free reprogramming. We obtained the episomal vectors from Addgene (www.addgene.com) and optimized transfection conditions for mouse fibroblasts. As a result, we can now consistently generate integration-free iPSCs from mouse fibroblasts and have already characterized iPSCs generated from both WT and IRG^{RFP}/*LAMA3*$^{fl/wt}$/K14-Cre.ER mice. These iPSCs express pluripotency markers and form teratomas when injected into immunocompromised mice (not shown). Therefore, as a result of this funding, we were able to successfully accomplish this sub-aim and generate iPSCs from both WT and IRG^{RFP}/*LAMA3*$^{fl/wt}$/K14-Cre.ER mice using a non-integrating approach.

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

We have successfully employed our protocol for the differentiation of mouse iPSCs into a keratinocyte lineage [2] and obtained keratinocytes from both WT and IRG^{RFP}/*LAMA3*$^{fl/wt}$/K14-Cre.ER iPSCs. Importantly, we show that our previously reported iPSC differentiation protocol [2] also works on integration-free iPSCs.

Keratinocytes derived from integration-free iPSCs (iPSC-KCs) expressed keratinocytes markers, keratin (K) 5, K14 and p63 (**Fig.2**).

We have also successfully grafted keratinocytes derived from WT iPSCs into congenic mice indicating that iPSC-KCs are unlikely to cause the immune response in recipients. Interestingly, unlike keratinocytes derived from WT, keratinocytes derived from IRG^{RFP}/*LAMA3*$^{fl/wt}$/K14-Cre.ER iPSCs treated with *Cre*-recombinase (which promotes the elimination of functional laminin) failed to engraft into congenic mice and also grew poorly *in vitro*. This suggests that functional laminin is required for successful engraftment of keratinocytes and the *LAMA3* mutation needs to be corrected before we can assess the histocompatibility of IRG^{RFP}/*LAMA3*$^{fl/fl}$/K14-Cre.ER iPSC-derived keratinocytes. The gene correction experiments are currently underway.

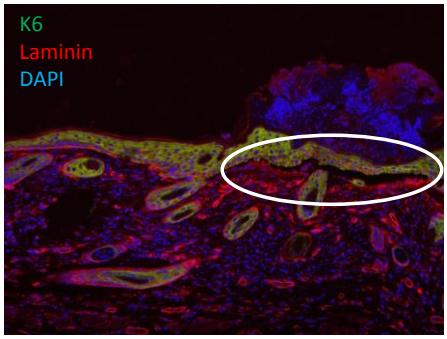

Fig.1. Inducible knockout of the LAMA3 gene exhibits symptoms similar to those seen in JEB patients. IRG^{RFP}/*LAMA3*$^{fl/wt}$/K14-Cre.ER mice were treated systemically with tamoxifen to induce the ablation of the LAMA3 gene. This treatment results in blister formation (encircled) similar to that seen in JEB patients and elimination of laminin (red) from the basement membrane. The image was taken with a 10x objective.

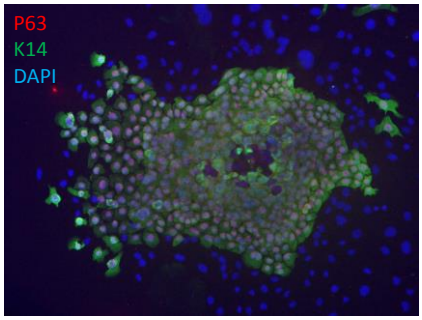

Fig 2. Differentiation of IRG^{RFP}/*LAMA3*$^{fl/wt}$/K14-Cre.ER iPSCs into a keratinocyte lineage. K14 (green) and p63 (red) expression was assessed on Day 18 of the protocol validating a successful differentiation of iPSCs into keratinocytes. The image was taken with a 10x objective.

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

These experiments are being performed by Dr. Tolar's group (see his report).

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:

<u>Task 2.1</u>. *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. Our attempt to create monoclonal lines from JEB keratinocytes resulted in the generation of only a few monoclonal lines, which could barely proliferate in culture. To achieve reprogramming, somatic cells have to divide at a high rate, and the slow growth rate of monoclonal JEB keratinocytes may impede their ability to undergo efficient reprogramming. As an alternative approach, we have established 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. Human JEB keratinocytes and fibroblasts for this experiment were obtained from our collaborators Drs. Pasmooij and Jonkman (Center from Blistering Diseases, Groningen, Netherlands) prior to the start of this project.

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

We adapted the mRNA-based reprogramming approach [3] to human WT keratinocytes (obtained from Life Technologies) 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. The mRNA-based reprogramming approach has been reported only for fibroblasts [3, 4] 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 and showed a normal karyotype (not shown). These results suggest that the mRNA-based reprogramming protocol can be employed for the reprogramming of JEB patient-specific keratinocytes.

Fig.3 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).

However, as noted in Task 2.1, we experienced difficulties in generating monoclonal lines of JEB keratinocytes. Therefore, we shifted our focus to the reprogramming of JEB fibroblasts. We optimized culturing conditions for an mRNA based reprogramming approach and incorporated reprogramming mimic miRNAs [5] into the reprogramming cocktail. As a result, we significantly improved the efficiency of the synthetic mRNA-based reprogramming approach and achieved reprogramming at the single cell level. 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 (obtained from ATCC), 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.4**).

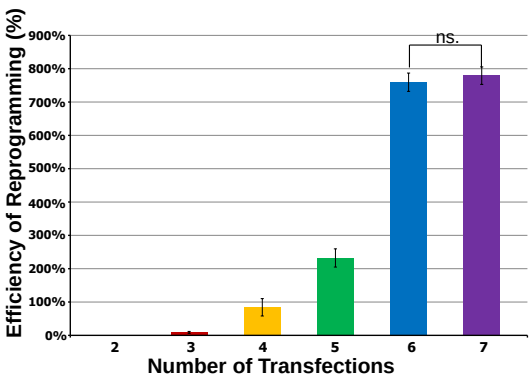

Fig. 4. Optimization of the transfection regiment. 500 human neonatal fibroblasts were plated in a 6 well dish and transfected with modified mRNA/miRNA for 2, 3, 4, 5, 6, or 7 times. All cultures were stained for the pluripotency marker TRA-1-60 at day 18 of reprogramming, and the positive colonies were counted. Reprogramming efficiency was calculated by dividing the average number of TRA-1-60 positive embryonic stem cell (ESC) like colonies per well by the initial number of cells plated.

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. 5), 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. 6 and 7). When our protocol was employed for the reprogramming of adult fibroblasts derived from a 50 year old healthy individual (ATCC), 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 [6]. The efficiency of iPSC generation from a JEB line (obtained from Drs. Pasmooij and Jonkman) was ~10%.

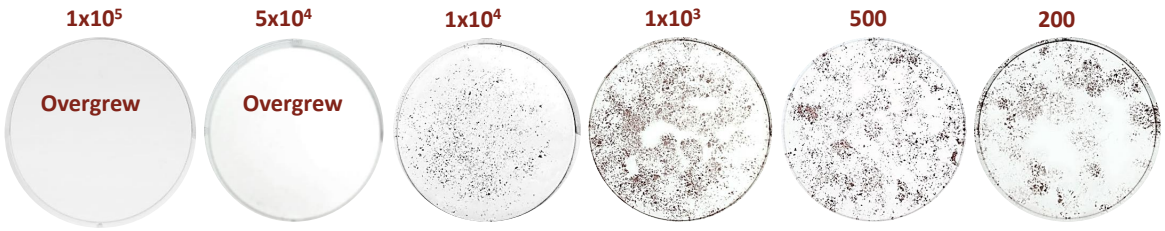

Fig. 5. Lower initial plating density results in the increased reprogramming efficiency in our combinatorial mRNA/miRNA approach. Representative images of wells (6-well format) stained for the pluripotency marker TRA-1-60 on day 18 of reprogramming with our combinatorial RNA-based approach. The initial plating densities of human primary fibroblasts are indicated above each well.

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	Mature microRNAs	Wells with an individually plated single cell	Wells with dividing cells throughout reprogramming	Wells with TRA-1-60 positive colonies	**Efficiency:** Wells with TRA-1-60 positive colonies/wells with dividing cells
N1	**+**	157	144	106	**73.6%**
	_	134	116	0	**0%**
N2	**+**	141	130	101	**77.7%**
	_	107	98	8	**8.2%**
N5	**+**	110	108	98	**90.7%**
	_	111	110	16	**14.5%**

Fig. 6. Individually plated single cells give rise to multiple sister iPSC colonies when reprogrammed with our combinatorial mRNA/miRNA approach. Human neonatal fibroblasts were plated as a single cell culture in a 48-well format. Each single cell individually plated in a 48-well was reprogrammed with our combinatorial RNA-based approach. On day 18 of reprogramming, the wells were stained for TRA-1-60. Representative images of individual 48 wells are shown for independently plated single cells reprogrammed with our combinatorial mRNA/miRNA approach.

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. 8) and RNA-Seq (Fig.9), and by the differentiation into cell types of all three germ layers both *in vitro* (Fig. 10) and *in vivo* (Fig. 11). 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. We are currently preparing a manuscript for publication.

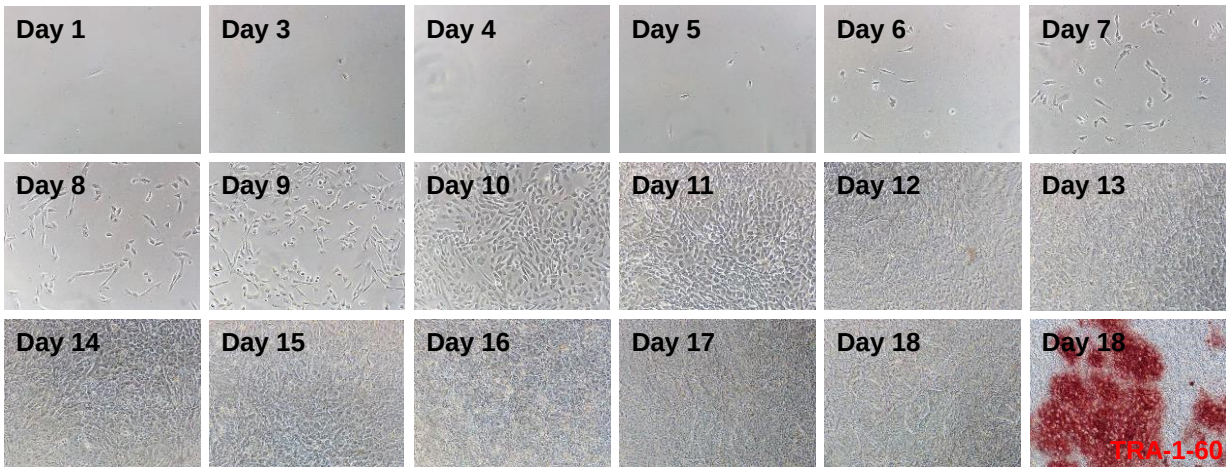

Fig. 7. The reprogramming of an individually plated single cell. The reprogramming of individually plated single cells was monitored and the images were taken daily.The representative images of the single cell reprogramming are shown, and the days the images were taken are indicated. On day 18 of reprogramming, the wells were staining with Tra-1-60 to confirm the generation of iPSC colonies.

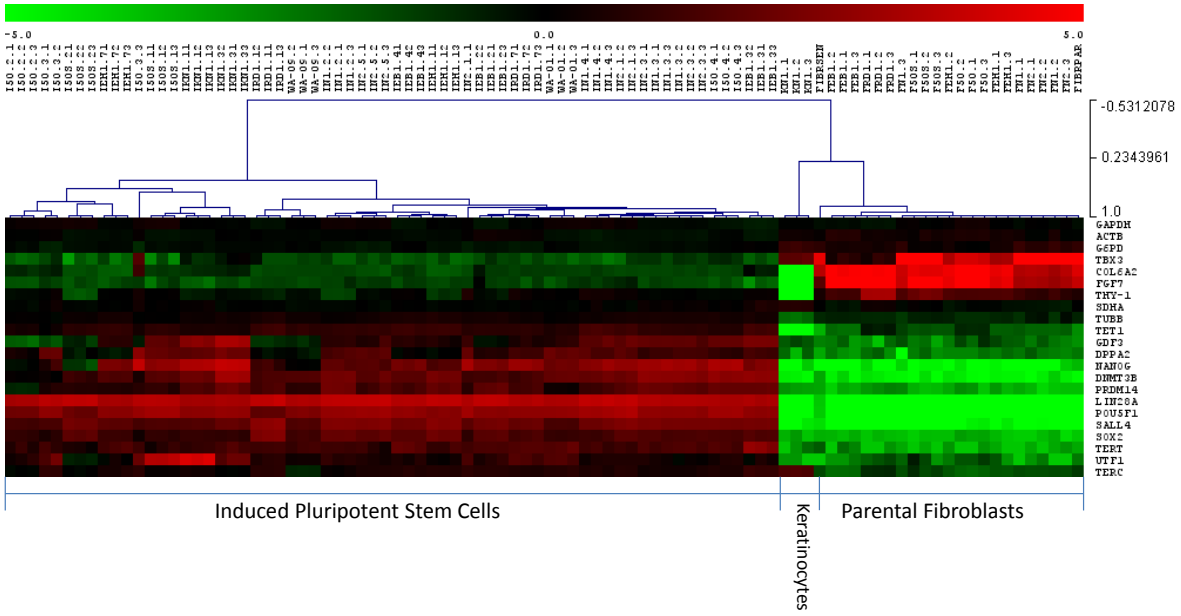

Fig. 8. Molecular characterization of iPSCs generated with our RNA-based approach. The combined heatmap and unsupervised hierarchical clustering of the expression of a set of selected genes (shown on the right) using nCounter® Analysis System from Nanostring Technologies is shown. Our generated iPSCs exhibit the robust expression of pluripotency genes and cluster separately from keratinocytes and parental fibroblasts. iPSCs named “IKN1” are derived from human keratinocytes (KN1). F – stands for human dermal fibroblast; FN – neonatal fibroblasts, F50 – fibroblasts from healthy 50 year old patient, F50S- senescent line derived from F50, FEH1 – fibroblasts from patient with EHK disease (EHK stands for epidermolytic hyperkeratosis), FEB1 – fibroblasts from patient with EBS disease (EBS stands for epidermolysis bullosa simplex), FRD1 – fibroblasts from a patient with RDEB disease (RDEB stands for recessive dystrophic epidermolysis bullosa).

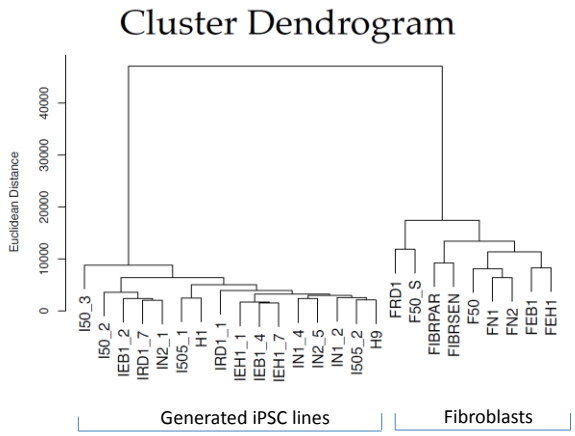

Fig. 9. Molecular characterization of iPSCs generated with our RNA-based approach using RNA Sequencing. Dendrogram showing unsupervised hierarchical clustering of the global expression profiles for fibroblasts and generated iPSCs.

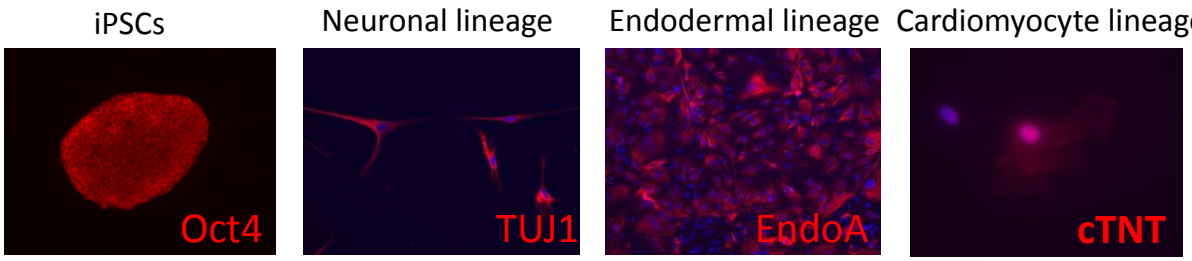

Fig. 10. In vitro differentiation of generated iPSCs. Representative immunofluorescence images of iPSC differentiated *in vitro* into neuronal, endodermal and cardiomyocyte lineages are shown. The pluripotency marker Oct4 is expressed in iPSCs. TUJ1 is Neuron specific Class III B-Tubulin; Endo-A is an endoderm specific cytokeratin; cTNT is cardiac Troponin T.

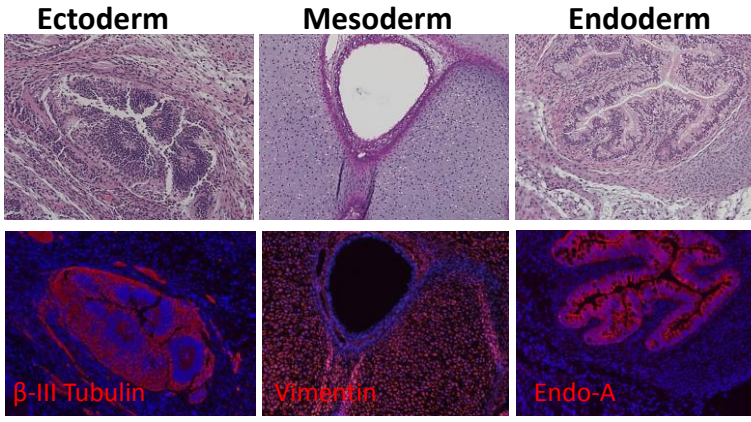

Fig. 11. Generated iPSC lines form teratomas in vivo. Representative images of teratoma sections show the contribution into all 3 germlayers. The following immunofluorescent staining confirm the formation of each germlayer: β-III Tubulin - neural rosettes (ectoderm), vimentin – mesenchymal lineage (mesoderm), and Endo-A – endoderm.

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

We are currently preparing samples from JEB patients for exome sequencing. Due to the difficulties in reprogramming JEB keratinocytes described in Tasks 2.1 and 2.3, we are focusing on JEB fibroblasts.

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. We are completing the characterization of JEB iPSC clones to ensure that only the clones with normal karyotype and an adequate differentiation capacity into a keratinocyte lineage are included into exome sequencing experiments.

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 funding period, we have optimized our protocol for the differentiation of human iPSCs generated with a RNA-based approach into keratinocytes. These optimizations have been published (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) . Exome sequencing will be performed after Task 2.4, which is currently underway. However, many of iPSC lines have already been differentiated into keratinocytes.

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

See Dr. Tolar's report.

Aim 3. To develop methods to increase the homing of iPSC-derived Lin$^-$/PDGFRα$^+$ 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$^-$/PDGFRα$^+$ BM cells into injured epithelia. To confirm these observations, we propose the following:

Task 3.1. *To determine whether mouse iPSC-derived Lin$^-$/PDGFRα$^+$ cells will home into injured epithelia.*

See Dr. Tolar's report.

Task 3.2. *To determine whether human iPSC-derived Lin$^-$/PDGFRα$^+$ cells will home into injured epithelia.*

See Dr. Tolar's report.

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 have designed an optimum binding site for ZFNs to correct the genetic defect in mouse JEB iPSCs. We are currently testing these ZFNs using our IRG^{RFP}/*LAMA3*$^{fl/wt}$/K14-Cre.ER 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.*

See Dr. Tolar's report.

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. However, all currently available gene modification strategies are limited by the extremely low efficiency and as a result, depend on the use of integrating antibiotic-resistance markers to select for those few gene edited cells [7, 8]. This antibiotic-resistance marker can be later excised leaving a "*scar*" of additional nucleotides in the genomic DNA. The presence of this genomic *"scar"* can prohibit the use of these cells in clinical applications as well as may potentially elicit an immune response to keratinocytes derived from corrected iPSCs. A more suitable clinically relevant gene targeting approach should rely on *"scarless"* (*footprint-free)* genome modifications without the use of selection markers. This approach is more challenging since the highest possible efficiency of currently available gene targeting techniques rarely exceeds 1-2% for human pluripotent cells [9], which is below a detection limit for conventional methods. One of the alternative approaches that can be employed for the detection of a low level of genome edited cells is the use of the BIO-RAD QX200™ Droplet Digital™ PCR System, which the Gates Center has recently purchased. This system detects as low as 0.1% of cells with desired genomic modifications [10]. As a result, we now have the necessary equipment and expertise to optimize the "*scarless*" gene editing approach by screening a variety of factors that improve homologous recombination in iPSCs. We are currently obtaining training to use this machine through BioRad.

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 will be initiated upon the completion of Task 4.4.

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.*

See Dr. Tolar's report.

Key Research Accomplishments

- Generated IRG^{RFP}/*LAMA3*$^{fl/wt}$/K14-Cre.ER mice as an important mouse model for the proposed studies;
- Successfully generated iPSCs from both WT and IRG^{RFP}/*LAMA3*$^{fl/wt}$/K14-Cre.ER mice using a non-integrating approach;
- Differentiated WT and IRG^{RFP}/*LAMA3*$^{fl/wt}$/K14-Cre.ER iPSCs into keratinocyte and performed grafting experiments. The grafting experiments indicated that the mutation in laminin needs to be corrected before the iPSC-derived cells can be successfully engrafted *in vivo.* However, the successful engraftment of WT iPSC-KCs into congenic mice suggests that immune rejection of iPSC-KCs is an unlikely hurdle for future clinical applications of iPSC technology for the treatment of skin diseases;
- Adapted a RNA-based reprogramming protocol for the generation of human iPSCs from keratinocytes and fully characterized the generated keratinocyte-derived iPSCs;
- Generated iPSCs from human JEB fibroblasts with Laminin-332 deficiency using an optimized RNA-based reprogramming technique. This optimized technique 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);
- Optimized the protocol for the differentiation of human iPSCs generated with a RNA-based reprogramming approach into keratinocytes and differentiation JEB iPSCs into keratinocytes;
- Performed grafting experiments of keratinocytes derived from JEB iPSCs;
- The genetic correction of mouse and human JEB iPSCs is currently being performed. To ensure that selection for footprint-free iPSC clones post-correction, we are currently adapting the BIO-RAD QX200™ Droplet Digital™ PCR System.

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 1 for the copy).

- Published a manuscript: Bilousova, G., and D.R. Roop. (2014). Induced Pluripotent Stem Cells in Dermatology: Potentials, Advances, and Limitations. Cold Spring Harb Perspect Med. Nov 3;4(11). pii: a015164. doi: 10.1101/cshperspect.a015164. Review (see appendix 1 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 a clinical trial. We were able to successfully accomplish most of the goals proposed in the original application. Specifically, we generated and expanded our colony of IRG^{RFP}/*LAMA3*$^{fl/wt}$/K14-Cre.ER mice; established several keratinocyte and fibroblast lines from these mice and generated iPSCs from these cells using a non-integrating episomal approach. We obtain preliminary data indicating that iPSC-KCs are unlikely to cause an immune response in recipients. We also adapted a RNA-based reprogramming protocol for the generation of

iPSC from a human keratinocyte line and fully characterize these keratinocyte-derived iPSCs for pluripotency with RNA-Seq and functional tests. We have also optimized a mRNA-based reprogramming protocol, which now results in the extremely high efficiency of iPSC production from human fibroblasts, including JEB fibroblasts. We are currently preparing a manuscript and expect to submit it for publication within 2-3 months. We have also optimized the protocol for the differentiation of iPSCs into keratinocytes and published it in Methods of Molecular Biology. We are now close to the completion of the gene editing of human JEB iPSCs, which is a final step toward the development of a proof-of-concept genome editing therapeutic strategy for JEB patients, which was proposed as a main goal of this DOD application.

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Methods in Molecular Biology (2014) 1195: 1–12
DOI 10.1007/7651_2013_64

Published online: 8 February 2014

Differentiation of Human Induced Pluripotent Stem Cells into a Keratinocyte Lineage

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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 μM 2-mercaptoethanol (2-ME), N2 supplement (100×) (Life Technologies), B27 supplement (50×) (Life Technologies), 50 μg/mL ascorbic acid, 0.05 % bovine serum albumin (BSA), 50 U/mL penicillin–streptomycin, 100 ng/mL basic FGF (Life Technologies), and 10 μg/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 μg/μL 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× 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× PBS without Ca^{2+} and Mg^{2+}.

2.5 Equipment

1. Biological safety cabinet.
2. 37 °C water bath.
3. 37 °C/5 % CO_2 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 °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 °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 °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 ~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.

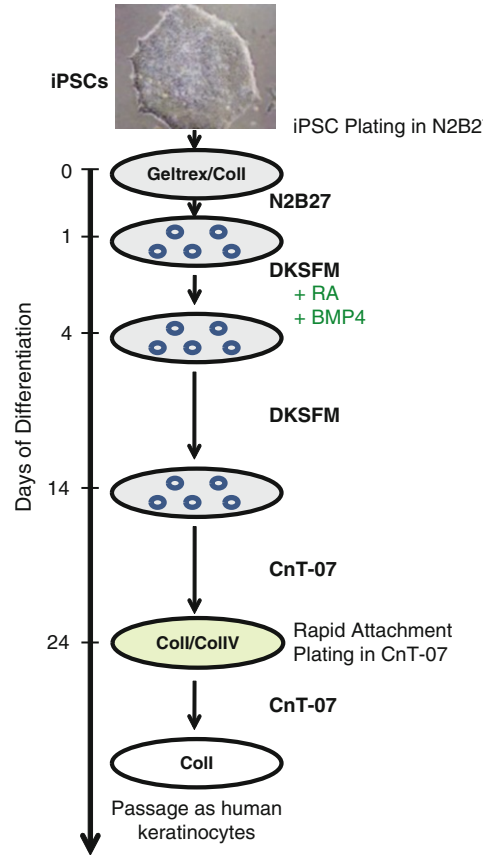

Fig. 1 Schematic representation of the protocol for the differentiation of human iPSCs into a keratinocyte lineage

3. Aspirate off N2B27 medium from the dish with plated iPSCs, wash once with 4 mL of 1× PBS, and add 4 mL of DKSFM containing 1 μM RA and 25 ng/μL 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 μM RA and 25 ng/μL 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).

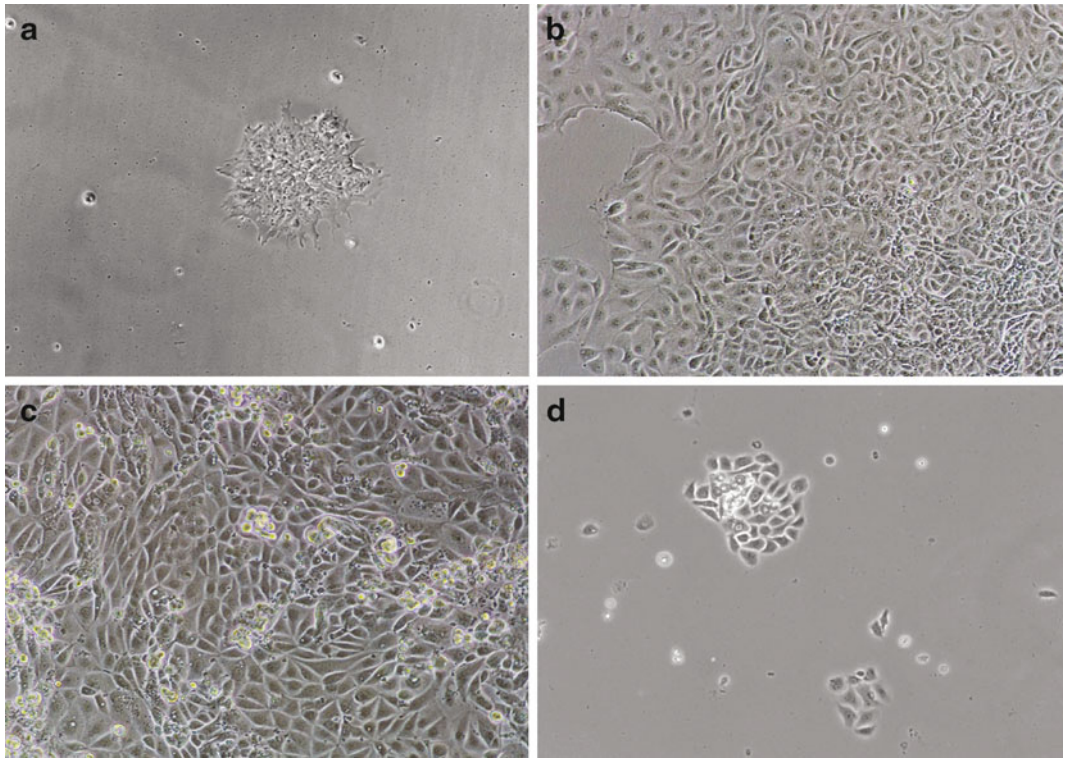

Fig. 2 The stages of iPSC differentiation during keratinocyte derivation. Human iPSCs generated with a modified mRNA-based approach from human neonatal fibroblasts were differentiated into keratinocytes using RA and BMP4. (**a**) Human iPSCs seeded at low density for differentiation on day 1 of differentiation. (**b**) An iPSC colony outgrown on a Geltrex/ColI-coated plate after the combined treatment with RA and BMP4 on day 14 of differentiation. (**c**) An iPSC colony outgrown on a Geltrex/ColI-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× objectives

8. Aspirate off DKSFM 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 ColI/ColIV-coated plates. We recommend using up to four 100 mm ColI/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 ddH_2O.
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 O_2 conditions (5 %). While iPSCs are maintained under low-oxygen conditions, the differentiation toward a keratinocyte lineage is performed under atmospheric O_2 (~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 × *g* for 10 min, and aspirate the supernatant. Gently resuspend the iPSC pellet in plain DMEM/F12, spin the cells down at 75 × *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

We are grateful for funding support from the National Institutes of Health (R01AR059947 and P30 AR057212), the US Department of Defense (PR110793), the Foundation for Ichthyosis and Related Skin Types (F.I.R.S.T.), and the Dystrophic Epidermolysis Bullosa Research Association (DEBRA) International.

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Induced Pluripotent Stem Cells in Dermatology: Potentials, Advances, and Limitations

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Cold Spring Harbor Perspectives in Medicine
www.perspectivesinmedicine.org

The discovery of methods for reprogramming adult somatic cells into induced pluripotent stem cells (iPSCs) has raised the possibility of producing truly personalized treatment options for numerous diseases. Similar to embryonic stem cells (ESCs), iPSCs can give rise to any cell type in the body and are amenable to genetic correction by homologous recombination. These ESC properties of iPSCs allow for the development of permanent corrective therapies for many currently incurable disorders, including inherited skin diseases, without using embryonic tissues or oocytes. Here, we review recent progress and limitations of iPSC research with a focus on clinical applications of iPSCs and using iPSCs to model human diseases for drug discovery in the field of dermatology.

Direct reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) through the ectopic expression of reprogramming factors (Takahashi and Yamanaka 2006; Okita et al. 2007; Takahashi et al. 2007; Wernig et al. 2007; Yu et al. 2007) has opened up a new era in research and therapy. Similar to embryonic stem cells (ESCs), iPSCs can be expanded indefinitely and are capable of differentiating into all three germ layers (Takahashi and Yamanaka 2006; Okita et al. 2007; Takahashi et al. 2007; Wernig et al. 2007; Yu et al. 2007). Traditional techniques for the isolation of human ESCs rely on the use of surplus in vitro fertilization embryos (Mitalipova and Palmarini 2006). Therefore, unlike iPSC technology, ESC-based techniques do not allow for the generation of genetically diverse patient-specific cells. Furthermore, the use of ESC-derived cells for therapeutic applications may result in immune rejection, which is not anticipated to be a concern if patient-specific iPSC-derived cells are returned to the same patient. Thus, 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 (Yamanaka 2007, 2009; Nishikawa et al. 2008; Takahashi 2012).

Although iPSCs have tremendous potential for cell-based drug discoveries, cell therapy, and disease modeling, extensive analyses are still required to show the safety and reliability of the reprogramming technology. Until recently,

Editors: Anthony E. Oro and Fiona M. Watt
Additional Perspectives on The Skin and Its Diseases available at www.perspectivesinmedicine.org

 doi: 10.1101/cshperspect.a015164
Cite this article as *Cold Spring Harb Perspect Med* 2014;4:a015164

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progress in this area has been significantly impeded by the lack of efficient protocols for the differentiation of iPSCs into relevant adult lineages/tissues. This was especially apparent in the field of dermatology, which is unfortunate, because the skin may be an ideal tissue to initially apply an iPSC-based therapy. Skin is readily accessible, easy to monitor, and if an adverse event should occur, the affected area could be excised.

Nevertheless, significant advances have recently been achieved in the differentiation of both mouse and human iPSCs into keratinocytes (Bilousova et al. 2011a; Itoh et al. 2011; Bilousova and Roop 2013), melanocytes (Ohta et al. 2011), and fibroblasts (Hewitt et al. 2011); thus, opening the possibility of expanding iPSC technology into the field of dermatology. This article discusses the prospect of using iPSC technology as a tool to study the skin and its pathology and cure genetic skin diseases.

IN SEARCH OF PLURIPOTENCY

The remarkable phenotypic stability and low proliferative capacity of differentiated adult cells limit their applications in personalized regenerative medicine and have triggered an extensive search for sources of pluripotent stem cells suitable for the clinic.

One of the potential sources of pluripotent stem cells is ESCs. In mammals, embryonic development is characterized by a gradual decrease in differentiation potential and an increase in the specialization of cells as they commit to the formation of adult lineages and tissues that constitute the embryo. The developmentally versatile pluripotent ESCs residing in the inner cell mass of the blastocyst (Thomson et al. 1998) exist for a brief period of time during development and eventually differentiate into more specialized multipotent stem cells (Fig. 1). Whereas human pluripotent ESCs still hold great promise in regenerative medicine and drug discoveries, ethical concerns and the possibility of immune rejection of tissues derived from allogeneic ESCs have hindered the therapeutic application of these cells.

Attempts to derive pluripotent stem cells from adult somatic cells were influenced by early nuclear transfer experiments performed in the 1950s using frogs, *Rana pipiens* (Briggs and King 1952) and *Xenopus laevis* (Gurdon et al. 1958), as a model system. These early studies documented the feasibility of reprogramming adult frog somatic cell nuclei by the cytoplasm of enucleated unfertilized frog oocytes and generation of cloned frogs. Similar reports of successful nuclear reprogramming, either by transferring somatic cell nuclei into oocytes (Kimura and Yanagimachi 1995; Wakayama et al. 1998) or by fusing somatic cells with pluripotent stem cells (Ambrosi and Rasmussen 2005), were published. However, it was the cloning of Dolly the sheep (Wilmut et al. 1997) that showed the possibility of complete reprogramming of somatic cells from mammals back into the pluripotent state. Following somatic cell nuclear transfer (SCNT), the adult cell's nucleus is exposed to the cytoplasm of an unfertilized egg and returned to a pluripotent state from which it can develop an entire animal. Dolly's birth (Wilmut et al. 1997) and the first report of the derivation of human ESCs shortly thereafter (Thomson et al. 1998) led to wide speculations

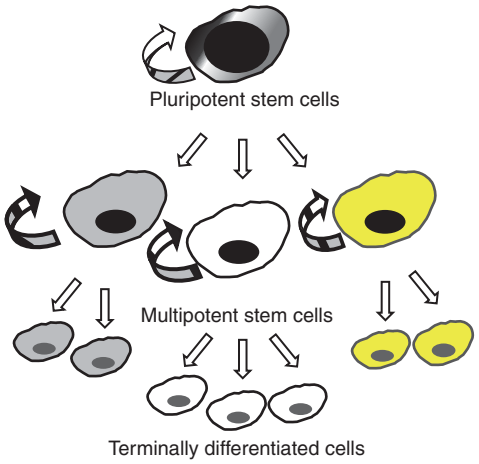

Figure 1. Stem cell hierarchy. Pluripotent stem cells have the capacity for self-renewal and only exist in an early stage of embryogenesis. They give rise to all types of more specialized multipotent stem cells of the adult organism. Multipotent stem cells also show a self-renewal capacity; however, they are committed to produce only a restricted range of adult somatic cells and terminally differentiated progeny.

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about the possibility of therapeutic cloning. However, the difficulty of obtaining donated oocytes, which are required to perform SCNT, as well as ethical concerns, limits the applicability of human therapeutic cloning in the clinic.

The success in using a cell from an adult animal to generate another healthy, fertile adult has shown that unfertilized eggs and ESCs contain a set of factors that can confer pluripotency to somatic cells. Encouraged by these findings, Takahashi and Yamanaka (2006) analyzed 24 genes that were specifically expressed in ESCs as candidates for factors that induce pluripotency in murine somatic cells. They identified a combination of four factors that when ectopically expressed was sufficient to revert differentiated mouse somatic cells into embryonic-like cells that were similar to ESCs. The factors were Oct-4 (also known as POU5F1), sex determining region Y (SRY)-box2 (Sox-2), Krüppel-like factor 4 (Klf-4), and c-Myc (Takahashi and Yamanaka 2006). The iPSCs resulting from this manipulation functioned in a manner undistinguished from mouse ESCs: they were capable of forming multiple cell types in vitro and in vivo, they expressed markers associated with pluripotent cells, and perhaps most importantly, they could be used to make fertile mice (Takahashi and Yamanaka 2006; Okita et al. 2007). A similar combination of factors (Takahashi et al. 2007; Wernig et al. 2007) as well as an alternative combination of Oct-4, Sox-2, Nanog, and Lin-28 (Yu et al. 2007) were later shown to be sufficient to reprogram human somatic cells into the pluripotent state (Fig. 2), thus, launching a new era in regenerative medicine and tissue engineering.

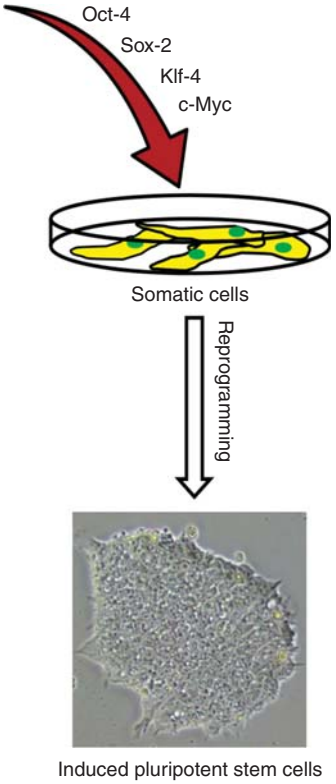

Figure 2. Reprogramming human somatic cells with four transcription factors. Both mouse and human somatic cells can be reprogrammed into embryonic stem cell–like iPSCs as a result of exogenous expression of four transcription factors: Oct-4, Sox-2, Klf-4, and c-Myc. An alternative combination of Oct-4, Sox-2, Nanog, and Lin-28 can also be used to achieve reprogramming of human somatic cells. The resulting iPSCs resemble ESCs at the phenotypic, functional, and molecular levels.

THE GENERATION OF iPSCS

Since the development of the first protocols for the generation of mouse iPSCs, followed by the generation of human iPSCs, a variety of reprogramming protocols have been published and various combinations of reprogramming factors in the form of transcription factors or microRNAs (miRNAs) have been used. The initial protocols for the generation of both mouse and human iPSCs relied on the use of integrating retro- and lentiviral vectors to deliver the exogenous reprogramming factors into somatic cells (Takahashi and Yamanaka 2006; Takahashi et al. 2007; Yu et al. 2007; Wernig et al. 2008; Sommer et al. 2009, 2012). However, the observation that the expression of reprogramming factors was only essential in the initial steps of reprogramming and silencing of exogenous reprogramming factors must occur for the successful directed differentiation of iPSCs (Takahashi and Yamanaka 2006; Okita et al. 2007) has opened the possibility for developing genome integration-free approaches without the risk for per-

manent genetic modifications caused by insertional mutagenesis. A number of genome integration-free techniques for the generation of iPSCs have subsequently been published with a focus on accelerating the transition of iPSC-based technology into the clinic.

Somatic cells have been successfully reprogrammed with expression plasmids encoding the reprogramming factors (Okita et al. 2008) and adenoviruses (Stadtfeld et al. 2008; Zhou and Freed 2009). However, these approaches are not considered clinically safe because of a potential risk of genomic integrations and mutagenesis. Similarly, the use of PiggyBac transposons (Yusa et al. 2009) may be associated with point mutations and chromosomal rearrangements. Recently, iPSCs were generated with a set of miRNAs alone (Anokye-Danso et al. 2011). However, the method for the delivery of reprogramming miRNAs also relies on the use of integrating lentiviral vectors (Anokye-Danso et al. 2011), and the only report of reprogramming with direct transfections of mature miRNAs showed an extremely low efficiency (Miyoshi et al. 2011). More clinically relevant approaches require a DNA-free delivery of reprogramming factors to avoid the potential integration of the exogenous DNA into the genome. Examples of DNA-free reprogramming approaches are shown in Figure 3.

One potentially safe approach to generate iPSCs is to use small chemical compounds to substitute for the expression of reprogramming factors. Many small molecules that can func-

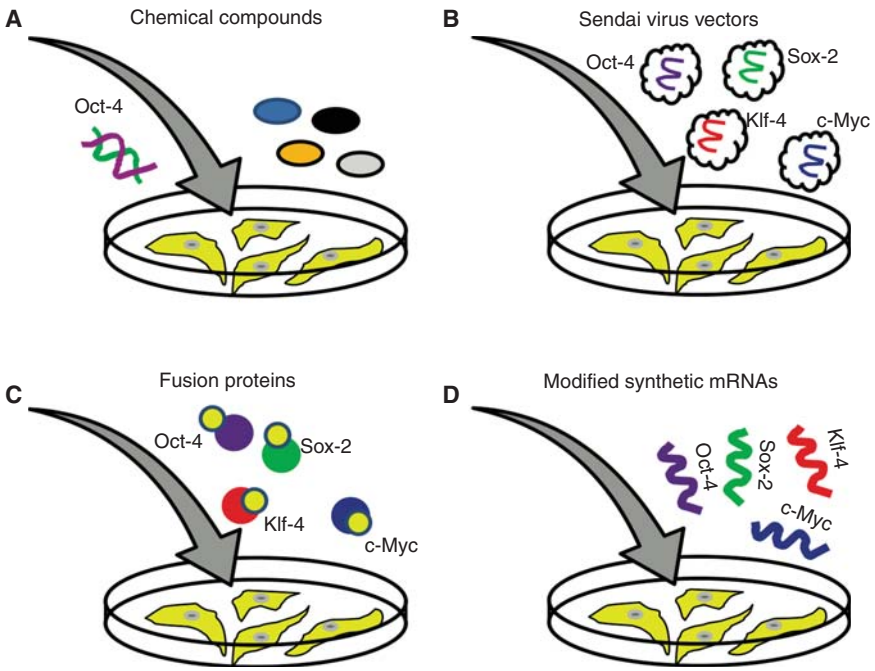

Figure 3. Clinically relevant DNA-free approaches to reprogram human somatic cells into iPSCs. (*A*) Chemical compounds that substitute for the expression of ectopic reprogramming factors have been identified and may be used for the reprogramming of human somatic cells. However, exogenous expression of Oct-4 is still required for this method to produce iPSCs. (*B*) RNA-containing Sendai viruses can be used to deliver reprogramming factors into somatic cells. The Sendai viral genome is not able to integrate into the host genomic DNA, and viral vectors are slowly diluted during the robust cell division of iPSCs. (*C*) Reprogramming can be achieved with purified recombinant reprogramming proteins fused to either HIV-TAT or polyarginine peptides. Both peptides are cell-penetrating peptides and can deliver large cargo proteins into cells. (*D*) The direct transfection of modified synthetic messenger RNAs (mRNAs) encoding the reprogramming factors can also be used to reprogram somatic cells.

tionally replace exogenous reprogramming factors and enhance the efficiency of reprogramming have been identified (Zhang et al. 2012). However, the requirement for the exogenous expression of Oct-4 in the human reprogramming protocol that uses a cocktail of defined small molecules limits the applicability of this approach in the clinic (Zhu et al. 2010; Zhang et al. 2012). Nonetheless, a recent study by Hou et al. (2013) found a set of small-molecule compounds that can reprogram mouse cells into iPSCs without the expression of any exogenous reprogramming factors, suggesting that a complete combination of chemical compounds suitable for the reprogramming of human somatic cells may be identified in the future.

Another possibility for exogenous DNA-free reprogramming uses a nonintegrating RNA-containing Sendai virus (Fusaki et al. 2009; Ban et al. 2011). Sendai virus is a negative-strand RNA virus that belongs to the *Paramyxoviridae* family (Nakanishi and Otsu 2012). It replicates in the cytoplasm of infected cells, does not go through a DNA phase that can integrate into the host genome, and has been successfully used to introduce foreign genes in a wide spectrum of host cells (Nakanishi and Otsu 2012). Sendai viral vectors are currently being applied in gene therapy studies for cystic fibrosis and vaccines (Nakanishi and Otsu 2012), and have been shown to induce reprogramming with high efficiency (Fusaki et al. 2009; Ban et al. 2011). Although Sendai viral vectors are slowly diluted during the robust cell division of iPSCs, more studies are required to confirm the clinical safety of reprogramming with this approach.

Several reports indicate that iPSCs can also be generated without the use of any nucleic acids by fusing reprogramming factors with cell-penetrating peptides (Kim et al. 2009; Zhou et al. 2009). The identification of a group of proteins with an enhanced ability to cross the plasma membrane in a receptor-independent manner has led to the discovery of a class of protein domains with cell-penetrating properties. The fusion of these domains with heterologous proteins is sufficient to allow their rapid transduction into different cells in a concentration-dependent manner. The most commonly used cell-penetrating peptides are the HIV-1 transcriptional activator TAT protein and polyarginine (Frankel and Pabo 1988; Green and Loewenstein 1988). The fusion of reprogramming factors with TAT or polyarginine protein transduction domains has been shown to be sufficient for reprogramming somatic cells (Kim et al. 2009; Zhou et al. 2009). Although being potentially the safest approach, the low efficiency of iPSC generation with cell-penetrating proteins (0.001%) and technical difficulties associated with protein purification prevent an easy transition of this methodology into the clinic. A more promising approach for the transgene-free generation of iPSCs may be the use of synthetic modified mRNAs encoding the reprogramming factors. This approach has been shown to reprogram a variety of cell lines with the efficiency that surpasses that of the other integration-free protocols (Warren et al. 2010, 2012). The only disadvantage of mRNA-based reprogramming, however, is that mRNA molecules must be delivered into the cells every day until the reprogramming is complete.

DONOR CELL TYPES FOR REPROGRAMMING

Unlike ESCs, which can be derived only from the developing embryo, iPSCs have been generated from multiple easily accessible cell types, such as dermal fibroblasts, keratinocytes, melanocytes, peripheral blood $CD34^+$ cells, etc.

Fibroblasts are the most commonly used cell type for reprogramming because of their accessibility and easy culture conditions. As a result, the reprogramming process has been extensively studied in these cells and shown to follow an organized sequence of events, which begins with the down-regulation of somatic gene expression (Polo et al. 2012). The first step of reprogramming in fibroblasts requires a mesenchymal-to-epithelial transition (MET) (Esteban et al. 2012), which is followed by the activation of the early pluripotency markers *SSEA-1* and alkaline phosphatase before bona fide pluripotency genes *NANOG* and *OCT-4* become involved and provide an independence from exogenous factor expression (Polo et al. 2012). The

requirement for MET as a first step in reprogramming of fibroblasts may partly explain why cells of epithelial origin, such as keratinocytes, are easier to reprogram (Aasen et al. 2008; Gadue and Cotsarelis 2008). Although the exact mechanism of reprogramming remains unknown, the removal of epigenetic modifications, which restrict expression of pluripotency genes in somatic cells, seems to play a predominant role in the process (Polo et al. 2012; Watanabe et al. 2013).

Somatic cells show different reprogramming capacity depending on their origin, rate of proliferation, and gene expression profiles, and selecting the appropriate donor line for reprogramming may be critical not only to achieve the highest efficiency and quality of reprogramming, but also to attain maximum differentiation capacities of the resulting iPSCs. For instance, epigenetic profiling of iPSCs revealed that the reprogrammed cells retain epigenetic marks of the cell type of origin (Kim et al. 2010, 2011), although these marks disappear on continued passaging (Polo et al. 2010). This short-term genetic memory may provide an advantage in achieving the higher differentiation efficiency of early-passage iPSCs into a target adult lineage/tissue, which might be particularly relevant to iPSCs derived from keratinocytes or melanocytes. Not only can keratinocytes and melanocytes be reprogrammed with a higher efficiency than fibroblasts (Aasen et al. 2008; Utikal et al. 2009), the residual epigenetic memory in keratinocyte- and melanocyte-derived iPSCs may allow for easier differentiation back into the corresponding original cell type for cell replacement. One drawback in using skin cells such as keratinocytes and melanocytes for reprogramming is the potential accumulation of DNA mutations caused by continuous exposure of the skin to UV light. However, this problem can be avoided if biopsies are taken from non-sun-exposed sites such as armpits.

APPLICATION OF iPSCS IN DERMATOLOGY

Essentially all of the most devastating forms of inherited skin diseases such as the epidermolysis bullosa (EB) subtypes and congenital ichthyoses (Khavari 1997; DiGiovanna and Robinson-Bostom 2003; Smith 2003) are caused by known monogenic defects, which, in theory, should enable their correction at the genetic level. The correction achieved in iPSCs derived from a patient with a skin disease may be followed by the differentiation of these corrected iPSCs into autologous skin cells for transplantation. In addition to genetic skin disorders, iPSC technology may potentially be applicable to wound healing, depigmentation disorders, and cosmetic dermatology.

The successful development of iPSC-based therapies for inherited skin disorders depends on four important steps (Fig. 4). First, cells need to be isolated from a patient's skin biopsy. Second, these cells have to be reprogrammed into iPSCs. Third, the genetic defects in generated iPSCs need to be corrected by safe approaches, preferentially through homologous recombination (HR). Fourth, these genetically corrected patient-specific pluripotent cells have to be differentiated into the cell type relevant to their disease, followed by transplantation onto the same patient as an autograft. Uncorrected iPSCs may also be used as a source of disease-relevant patient-specific cells for in vitro disease modeling and in vivo xenograft modeling, which would offer platforms for yielding new insights into disease mechanisms and drug discovery.

Reprogramming Cells from Patients with Inherited Skin Diseases

The generation of iPSC lines from patients with genetic disorders provides an unlimited supply of cells for studies and opportunity to repair gene defects in vitro, as long as iPSCs can be differentiated into relevant cell types. To date, human iPSCs have been generated for several genetic skin disorders including type VII collagen (Col7)-deficient recessive dystrophic epidermolysis bullosa (RDEB) (Itoh et al. 2011; Tolar et al. 2011), *LAMB3* gene-deficient junctional epidermolysis bullosa (JEB) (Tolar et al. 2013), p63 mutant ectrodactyly, ectodermal dysplasia, and cleft lip/palate (EEC) syndrome (Shalom-Feuerstein et al. 2013), epidermolytic hyperkeratosis with a dominant N188S keratin 1

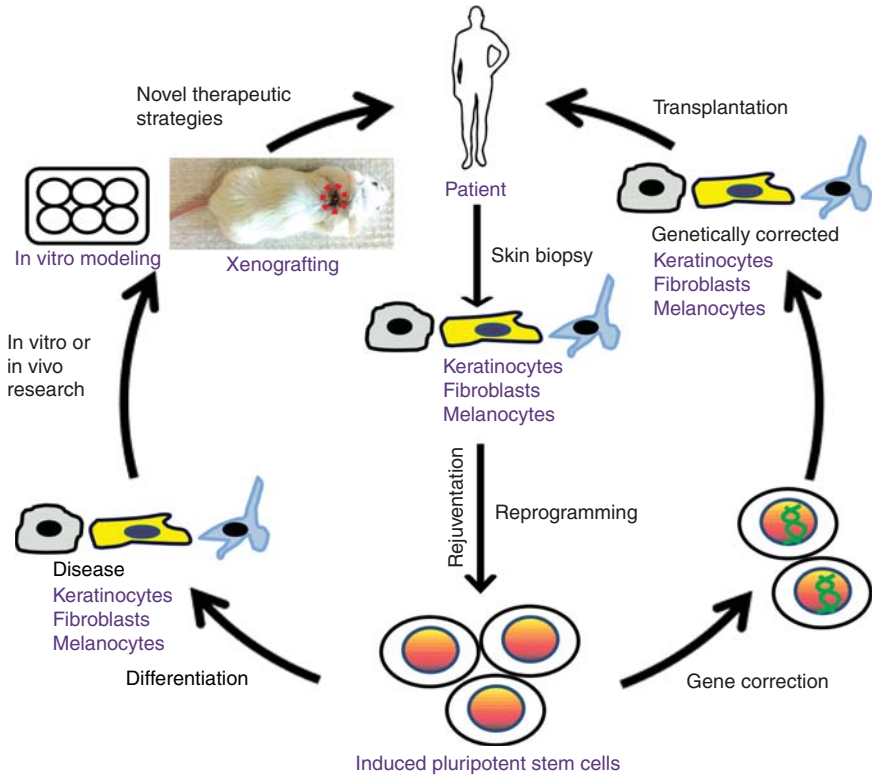

Figure 4. Dermatological applications of iPSCs. The generation of iPSCs allows for the development of stem cell replacement strategies to treat genetic skin disorders and modeling these diseases with the goal to producing novel therapeutic options for patients. Initially, cells need to be isolated from a skin biopsy of a patient with a genetic skin disease. Suitable cell types for reprogramming are keratinocytes, melanocytes, and fibroblasts. During reprogramming, cells acquire ESC properties and undergo "rejuvenation," as can be determined by the elongation of telomeres and restoration of the mitochondrial function. The resulting iPSCs can then be genetically corrected by HR. The corrected iPSC clones can be differentiated into relevant cell types for transplantation and returned back to the patient as an autograft. Alternatively, uncorrected iPSCs can be differentiated into cell types relevant to the disease of interest for modeling the disease in organotypic cultures or recapitulating the disease phenotype in vivo by transplanting onto immunodeficient mice as a xenograft.

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mutation (Bilousova et al. 2011b), and epidermolysis bullosa simplex (EBS) with a dominant R125C keratin 14 mutation (Bilousova et al. 2012). In addition, iPSCs have been obtained from patients with dyskeratosis congenita (DC), a disease of telomere maintenance that manifests itself in multiple organ failures, cancer, and age-associated skin phenotypes such as dyskeratotic nails, delayed wound healing, and hair loss (Agarwal et al. 2010; Agarwal and Daley 2011; Batista et al. 2011).

Attempts have also been made to correct the defects in iPSCs generated from skin disorders. In the study by Tolar et al. (2013), a *LAMB3*-encoding lentivirus was used to correct keratinocytes derived from JEB-iPSCs (Tolar et al. 2013). In another study by Tolar et al. (2011), transient gene correction of *COL7A1* gene-deficient RDEB-iPSCs was achieved via the transfection with an expression plasmid encoding the wild-type human *COL7* gene (Tolar et al. 2011). The "corrected" RDEB-iPSCs were differentiated into structures resembling skin and cells of a hematopoietic lineage through teratoma formation (Tolar et al. 2011). Wild-type, congenic bone marrow–residing mesenchymal cells have

been previously shown to engraft into the skin, regenerate injured epithelia, and restore Col7 production in blistered areas of RDEB patients (Tolar et al. 2009) and mouse models (Fujita et al. 2010; Tamai et al. 2011). Thus, corrected iPSCs may provide an unlimited source of autologous cells of both epidermal and mesenchymal lineages for the treatment of RDEB and potentially other skin blistering diseases.

Differentiation of iPSCs into Skin Cells

The ability to differentiate iPSCs into relevant cell types is an important prerequisite for the successful development of iPSC-based treatment strategies. Keratinocytes, melanocytes, and fibroblasts have been recently obtained from iPSCs. Mouse and human iPSCs have been differentiated into keratinocytes by sequential applications of retinoic acid and bone morphogenetic protein-4 (BMP4) (Bilousova et al. 2011a; Itoh et al. 2011; Bilousova and Roop 2013). Mouse iPSC-derived keratinocytes were shown to form an epidermis and skin appendages when grafted with mouse fibroblasts onto athymic nude mice (Bilousova et al. 2011a), whereas human iPSC-derived keratinocytes were able to establish a functional organotypic skin in culture (Itoh et al. 2011). Keratinocytes were also generated from RDEB-iPSCs (Itoh et al. 2011), JEB-iPSCs (Tolar et al. 2013), EEC-iPSCs (Shalom-Feuerstein et al. 2013), and EBS-iPSCs (Bilousova et al. 2012) and were shown to recapitulate the phenotypes of corresponding diseases when grown in vitro. Melanocytes can be derived from iPSCs by supplementing the culture with Wnt3a, stem cell factor, and endothelin-3 (Ohta et al. 2011), and the treatment of iPSC cultures with EGF and BMP4 can produce fibroblasts (Hewitt et al. 2011). Although protocols for the differentiation of iPSCs into the main skin cell types now exist, rigorous studies have not been performed to determine whether these iPSC-derived cells are identical to their normal skin equivalents.

Customized Gene Correction Therapy

Current technologies for gene transfer into somatic skin cells suffer from many limitations. One of the biggest obstacles is the availability of safe vectors that are able to produce long-lasting therapeutic effects with low risk of insertional mutagenesis. Plasmid- and adenoviral vector-based approaches for gene delivery are inefficient in inducing a long-term corrective effect in keratinocytes and associated with biosafety concerns (Khavari 2000; Ortiz-Urda et al. 2002). Thus, a genetic correction therapy via HR may be the only permanent cure for inherited skin diseases, especially for those involving recessive loss-of-function mutations, as occurs in JEB and RDEB. Because the epidermis is continuously renewed by adult stem cells residing in the proliferative basal layer, any permanent genetic correction must target this stem cell population. However, to date, no one has reported the successful use of HR technology to correct a defective gene in human keratinocyte stem cells. The only successful clinical trial for permanent gene correction of a blistering disease, in this case, JEB, was performed by Mavilio et al. (2006) and used a retroviral vector to restore expression of laminin 322. That trial was placed on hold because of safety concerns with the use of retroviral vectors (De Luca et al. 2009).

There are several approaches to induce HR that can be coupled with the generation of iPSCs to achieve genetic repair of inherited skin diseases. Enhanced HR in iPSCs could be achieved using chimeric molecules composed of a nuclease domain and separate, customized DNA-recognition domains. These chimeric molecules can introduce a double-strand break (DSBs) in a specific DNA sequence. The presence of exogenous donor DNA carrying the correct gene sequence and homology with the sequence flanking the DSB triggers HR and replacement of the defective gene with the corrected one (Carlson et al. 2012). Zinc-finger nucleases (ZFNs) (Kim et al. 1996; Bibikova et al. 2003; Porteus and Baltimore 2003) and transcription activator-like effector nucleases (TALENs) (Boch et al. 2009; Moscou and Bogdanove 2009; Christian et al. 2010) are the main representatives of chimeric endonucleases capable of introducing specific DSBs into genomic DNA and widely used as tools for research and gene therapy. In addition to ZFNs and TALENs, a novel

nuclease-based technique has recently been adapted to achieve genomic editing in human iPSCs. This technique uses the type II prokaryotic CRISPR (clustered regularly interspaced short palindromic repeats)/CRISPR-associated (Cas) systems, which when directed by short RNAs can induce precise cleavage at endogenous genomic loci with high efficiency (Jinek et al. 2013; Mali et al. 2013). ZFN-, TALEN-, and CRISPR/Cas-based gene modification strategies have been successfully used on human and mouse ESCs and iPSCs (Hockemeyer et al. 2009, 2011; Mali et al. 2013). In addition, a recent study by Osborn et al. (2013) shows the potential for using chimeric endonucleases in personalized genome-editing strategies for the treatment of inherited skin disorders. Using TALENs, the investigators achieved a specific heterozygous correction of *COL7A1* gene mutation in primary fibroblasts isolated from a RDEB patient. These corrected fibroblasts were then used to generate iPSCs suitable for the differentiation into corrected autologous keratinocytes (Osborn et al. 2013). The proof-of-principal research by Osborn et al. (2013) provides an important set of data that warrants further studies on the applicability of nuclease-based genome-editing approaches for the correction of inherited skin diseases.

The Potential of Cellular Reprogramming for Tissue Rejuvenation

The generation of iPSCs coupled with gene targeting may solve many obstacles that are associated with gene correction in somatic cells. Unlike somatic cells, iPSCs can be expanded indefinitely, allowing for the easier selection and expansion of corrected clones. In addition, the process of reprogramming leads to the "rejuvenation" of fully competent cells as shown by the down-regulation of senescent pathways, elongation of telomeres, and restoration of the mitochondrial function (Marion et al. 2009; Suhr et al. 2009, 2010; Prigione et al. 2010; Lapasset et al. 2011). Reports indicate that iPSCs can be derived from very old patients (Dimos et al. 2008; Lapasset et al. 2011), and these iPSCs can be differentiated into fibroblasts that show a "rejuvenated" phenotype with respect to telomere length and mitochondrial function (Lapasset et al. 2011). Reprogramming also restores telomere length in cells derived from patients with autosomal dominant DC, caused by heterozygous mutations in the catalytic protein component of telomerase *TERT* (Agarwal et al. 2010), promising novel therapeutic benefits for these patients. Thus, reprogramming may hold a key toward tissue rejuvenation and provide insights into mechanisms underlying aging and longevity. Although it is now clear that cells from older patients can be reprogrammed, more studies will be needed to better understand how age impacts iPSC quality and how the "rejuvenating" properties of reprogramming can be used for reversing skin aging and improving outcomes of stem cell–based therapies for skin disorders. As a particular example, the generation of iPSCs from a patient with a genetic skin disorder may potentially provide a source of "rejuvenated" adult skin stem cells that are most likely exhausted as a result of unsuccessful attempts to repair blistered tissues. The exhaustion of keratinocyte stem cells was especially apparent in the reported case of using genetically corrected keratinocyte stem cells for the treatment of a patient with JEB (Mavilio et al. 2006). Also, the "rejuvenating" properties of reprogramming may potentially be applicable for the treatment of chronic wounds in the elderly, providing autologous, young-like skin cells for transplantation.

Revertant Mosaicism as Natural Gene Therapy

Although ZFNs, TALENs, and CRISPR/Cas systems can dramatically increase the efficiency of genetic correction in iPSCs, extensive genetic analysis will be required to eliminate the concern of offtarget events that may occur if iPSCs are genetically corrected with these approaches. Naturally occurring somatic reversions may offer an alternative source of genetically corrected cells suitable for transplantation.

"Natural" somatic reversions of inherited mutations are observed in many human genetic diseases such as Fanconi anemia (Kalb et al.

2007), Bloom syndrome (Ellis et al. 2001), Wiskott–Aldrich syndrome (Stewart et al. 2007), as well as inherited skin disorders (reviewed in Lai-Cheong et al. 2011; Pasmooij et al. 2012). There are a number of reports of revertant mosaicism in patients with inherited skin disorders including RDEB (Jonkman et al. 1997), JEB (Pasmooij et al. 2007), EBS (Schuilenga-Hut et al. 2002), and ichthyosis with confetti (Choate et al. 2010). Revertant mosaicism manifests as patches of normal skin in patients with genetic skin disorders. These patches represent a clonal outgrowth of cells with acquired secondary mutations that negate the effects of the primary mutation and reverse the phenotype of the disease. Several mechanisms are responsible for the second "correcting" gene event in revertant cells and include gene conversion, second-site mutation, intragenic crossovers, etc. (Lai-Cheong et al. 2011; Pasmooij et al. 2012). Thus, the generation of iPSCs from spontaneously revertant skin cells in diseases such as RDEB or JEB may provide an unlimited supply of naturally corrected cells for a cell replacement therapy.

iPSCs for Disease Modeling

Although skin cells can be easily isolated from a patient's biopsy, expanded, and used for modeling inherited skin disorders in organotypic cultures, their short lifespan limits their applicability for in vitro studies. Immortalization of somatic cell lines relies on the constitutive expression of oncogenes, which may dramatically influence the phenotype of cell types of interest. The iPSC technology provides a renewable source of patient-specific cells, which, on subsequent differentiation into relevant cell types, can be used to generate patient-specific organotypic or in vivo xenograft models (Fig. 4). The skin cells derived from disease-specific iPSCs maintain their phenotype in 3D cultures, as shown by the differentiation of RDEB-iPSCs into keratinocytes (Itoh et al. 2011), and may be invaluable for research on many aspects of skin biology and inherited skin diseases. The ability to genetically manipulate iPSCs will also allow the modeling of skin diseases by targeted mutagenesis of the relevant genes without the use of ESCs. This may be of particular importance for modeling rare inherited skin disorders such as RDEB, EBS, and JEB. A low incidence of these diseases worldwide limits the accrual of data for research and impedes progress in developing therapeutic strategies for these skin conditions.

CHALLENGES IN iPSC RESEARCH

Although considerable progress has been made in deriving iPSCs from patients and differentiating them into tissues of interest, the use of iPSCs as a platform for studying diseases and development of therapies is just emerging. There are several challenges that must be addressed before the transition of iPSCs into the clinic. They include the safety of methodologies for the generation, genetic correction, and differentiation of iPSCs, and the high cost associated with the repair of genetic defects in patient-derived iPSCs and subsequent transplantation of corrected iPSC-derived cells back into the patient.

The first major challenge is in the reprogramming method itself. Although genome integration-free approaches are widely used for the generation of iPSCs, the safety and reliability of these methods are currently under investigation.

Second, iPSCs, like ESCs, are predisposed to teratoma formation when they are not completely differentiated into somatic cells. Although efficient differentiation protocols have been developed, currently available cell purification technologies may not completely separate out the differentiated cells from undifferentiated iPSCs. This may pose a risk of transplanting undifferentiated or partially differentiated iPSCs into the patient. In addition, several recent genomic and epigenetic analyses have suggested that genomic abnormalities such as the accumulation of mutations and aberrant DNA methylation of certain single bases occur in iPSCs, either by reprogramming itself or subsequent culture conditions (Mayshar et al. 2010; Gore et al. 2011). However, the observed genomic abnormalities and accumulation of mutations may be caused by the method used for the generation of iPSCs, rather than reprogram-

ming itself. Indeed, a recent study indicates that iPSCs generated by nonintegrating approaches show a significantly lower number of somatic mutations than the cells generated with integrating approaches (Cheng et al. 2012). Therefore, more extensive and thorough genomic and epigenetic studies must be performed before applying iPSCs in the clinic.

Although using a patient's own cells to generate iPSC-derived cells is thought to eliminate the concern with immune rejection, one report indicates that iPSCs may be immunogenic (Zhao et al. 2011). This study assessed the immunogenicity of iPSC-derived teratomas formed by subcutaneous injection of undifferentiated mouse iPSCs. Given that teratomas are a type of tumor, their rejection by the recipient is more likely an indication of tumor immunity and may not be relevant to immune responses triggered by somatic cell transplantation. Indeed, a recent publication indicates that terminally differentiated cells derived from mouse iPSCs do not trigger an immune response in syngeneic recipients (Araki et al. 2013), suggesting that iPSC-derived cells might be well tolerated by the immune system. Nevertheless, further studies will need to be performed to rule out any possibilities of an iPSC-mediated immune response in patients. Another aspect of immune rejection with iPSC-based therapy is related to gene correction. This may be of particular importance for the genetic correction of skin diseases with homozygous null mutations of relevant genes such as RDEB. Reintroduction of a protein unfamiliar to the host may trigger an immune response and eventual rejection of corrected iPSC-derived cells. Prescreening for patients with compound heterozygous mutations or the expression of truncated, nonfunctional forms of the protein of interest may partially solve the issue of immune rejection on transplantation of corrected cells.

CONCLUSION

Because of its easy accessibility, the skin is an attractive organ to test novel concepts of regenerative medicine, and iPSC-based therapeutic strategies for the treatment of inherited skin diseases are a great example. The potential of iPSCs for generating platforms to better understand disease mechanisms, drug screening, and, ultimately, iPSC-based therapeutic approaches is enormous and offers the possibility for truly personalized medicine. iPSCs would not only eliminate the need for generating ESCs from fertilized human embryos, but also avoid the complication of immune responses inevitable during the transplantation of allogeneic cells. Although still in its youth, the iPSC field is gaining momentum and it holds great promise for changing the way we treat patients with inherited skin diseases and reversing age-associated conditions that affect the skin. It is no surprise that the 2012 Nobel Prize in Physiology or Medicine was coawarded to Sir John Gurdon for his pioneering discovery that the cytoplasm of frog oocytes contained factors that could reprogram the nucleus of adult somatic cells to generate viable frogs, and Shinya Yamanaka for discovering the actual identity of these reprogramming factors, which opened the door for using iPSC-based technology for the treatment of a variety of human diseases including skin diseases.

ACKNOWLEDGMENTS

We are grateful for funding support from the National Institutes of Health (R01AR059947 and P30 AR057212), U.S. Department of Defense (PR110793), Foundation for Ichthyosis and Related Skin Types (FIRST), and Dystrophic Epidermolysis Bullosa Research Association (DEBRA) International. We apologize to investigators whose work was not cited because of space constraints.

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Induced Pluripotent Stem Cells in Dermatology: Potentials, Advances, and Limitations

Ganna Bilousova and Dennis R. Roop

Cold Spring Harb Perspect Med 2014; doi: 10.1101/cshperspect.a015164

Personnel Report

Name:	*Dennis Roop*
Project Role:	*PI*
Contract Identifier	W81XWH-12-1-0606
Nearest person month worked:	1
Contribution to Project:	Dennis R. Roop, Ph.D. primary responsibility was to supervise the research project which focused on developing stem cell-based therapies for Junctional Epidermolysis Bullosa (JEB).
Funding Support:	

Name:	*Ganna Bilousova*
Project Role:	Key Personnel Faculty
Contract Identifier	W81XWH-12-1-0606
Nearest person month worked:	2
Contribution to Project:	Dr. Bilousova performed the experiments outlined in the awarded research project including: generating mouse induced pluripotent stem cells (iPSCs) using an mRNA-based method of reprograming, performing skin graft assays to determine the histocompatibility of iPSC-derived keratinocytes, generating clonal keratinocyte cell lines from JEB skin biopsies, generating iPSC lines from clonal keratinocyte cell lines using an mRNA-based method of reprograming, and using zinc finger nucleases to genetically correct the defective genes in JEB-specific iPSC
Funding Support:	

Name:	Igor Kogut
Project Role:	Key Personnel Faculty
Contract Identifier	W81XWH-12-1-0606
Nearest person month worked:	4
Contribution to Project:	Dr. Kogut assisted Dr Bilousova in performing the experiments the awarded research project including: generating mouse induced pluripotent stem cells (iPSCs) using an mRNA-based method of reprograming, performing skin

	graft assays to determine the histocompatibility of iPSC-derived keratinocytes, generating clonal keratinocyte cell lines from JEB skin biopsies, generating iPSC lines from clonal keratinocyte cell lines using an mRNA-based method of reprograming, and using zinc finger nucleases to genetically correct the defective genes in JEB-specific iPSC
Funding Support:	