FIBROBLAST DERIVED INDUCED PLURIPOTENT STEM CELLS
MANUFACTURED UNDER GOOD MANUFACTURING PRACTICE (GMP)
CONDITIONS FOR THE TREATMENT OF AUTOSOMAL RECESSIVE
DYSTROPHIC EPIDERMOLYSIS BULLOSA

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in

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(Stem Cell)

by

Aaron S. H. Schwertschkow

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Department of Biological Sciences
Abstract

of

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Dystrophic epidermolysis bullosa (EB) is a serious genetic skin blistering condition. There are several manifestations of this disorder, each corresponding to a difference in severity of the same symptoms. The least prevalent form of epidermolysis bullosa is termed autosomal recessive dystrophic epidermolysis bullosa, but is symptomatically the most severe. The condition is the result of a mutant collagen gene, COL7A1, which renders a person incapable of producing proteins that collectively form type VII collagen. Collagen is produced and secreted from keratinocytes in the skin and plays a quintessential role in anchoring the basal lamina to the dermis and epidermis. Without proper expression of the COL7A1 gene, the skin is incapable of anchoring to the underlying tissue resulting in particularly fragile skin that can be easily damaged by minor friction or even routine tasks such as eating.
Currently, there is no cure for autosomal recessive dystrophic epidermolysis bullosa, however, advancements in induced pluripotent stem cell (iPSC) technology offers hope for future treatments. There are many advantages in using iPSCs in EB patient treatment, two of the advantages being the evasion of graft rejection as well as avoiding ethical issues surrounding the use of human embryonic stem cells. By applying patient iPSCs, a patient’s own tissue can be engineered, and human embryos to generate pluripotent stem cells are not required.

The process of generating iPSCs begins with the reprogramming of mature skin cells by the application of an integrating, but also excisable lentiviral vector. The vector delivers four genes, Oct4, Sox2, c-Myc, and Klf4 into the skin cell’s nucleus. The addition of these four genes causes the adult cell to express genes normally only expressed during embryonic cell development. At this stage, the reprogrammed cell has properties similar to that of an embryonic stem cell and has the potential to differentiate into any tissue type. The integrated reprogramming vector, however, must be removed in order to produce a clinical grade product. To accomplish this, a LoxP / Cre recombinase approach is used. Cre recombinase is added to established iPSC colonies to excise the vector. Following vector excision a DNA plasmid targeting vector with the fully functional COL7A1 gene is introduced into the iPSCs. As the cells divide, the chance arises for homologous recombination to occur, the functional gene then replaces the mutant gene in the target locus. While the efficiency of homologous recombination is only between 2-10% we can select for successfully corrected iPSCs and expand these
cells to provide a relevant number of gene corrected pluripotent cells for clinical use. The corrected cells are then differentiated into keratinocytes and finally manufactured into dermal grafts which can be used for transplantation onto the patient.

While many of the complicated steps involved in the generation of a clinical product are demonstrated in this project, the primary objective revolves around the differentiation assay. This is one of the final steps in the entire process in the generation of clinical grade keratinocytes, and currently the least developed process. Our partners at Stanford University have routinely generated EB fibroblast derived iPSCs, excised the lentiviral vector, and corrected the cells via homologous recombination; however, they have not performed the differentiation and purification process required for the final keratinocyte population starting with iPSCs. A theoretical protocol has been generated by the Stanford Oro group for the derivation of keratinocytes from H9 hESCs. The task for this research project has been primarily to elucidate the efficacy of translating the Oro protocol from H9 hESC differentiation to iPSC differentiation. Furthermore, this project has been challenged with the task of adapting the original protocol in any way necessary resulting in a functional protocol for the reproducible differentiation of iPSCs into functional keratinocytes.
This project took place during the second year of four years of total CIRM funding for the EB project. During this time, remarkable progress has been made and the vast majority of the objectives have been met. At the time of completion of this work, the entire EB project is nearing the investigational new drug (IND) application phase which will represent an important milestone for the cause of novel EB therapy research as well as the field of regenerative medicine.

_______________________________, Committee Chair
Thomas Landerholm, Ph.D.
DEDICATION

This work is dedicated to my Father, my Mother, my Little Sister, and to all the people with Epidermolysis Bullosa.
ACKNOWLEDGEMENTS

I would like to thank all the people that have contributed to the success of this project. Dr. Thomas Peavy for his early support and encouragement which helped put me on this path and for urging me to apply for the Masters program. Dr. Thomas Landerholm and Dr. Christine Kirvan for their review of my work and help throughout the last two years. I would also like to thank Dr. Gerhard Bauer for guiding me to discovering my own confidence and his day to day dedication to teaching me to be an independent successful person. I would also like to give thanks to Jon Walker and William Gruenloh for their daily support and for always reminding me that I can achieve anything I put my mind and heart into. I would like to give a very special thanks to Brian “The Lab Wizard“ Fury, always willing to interpret hieroglyphics and turn a difficult situation into a success. Finally but certainly not least I would like to thank Astra Chang, her constant positive attitude transforms the most difficult days into good days, her many reviews of this paper were also most insightful and greatly contributed to its success.

Thank you to the people of California for having the bravery to vote for proposition 71, creating the wonderful and extremely productive California Institute for Regenerative Medicine. Thank you to Dr. Jan Nolta for accepting so many of the CIRM masters students into her laboratory and helping make this program not only a dream but a reality.
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LIST OF ABBREVIATIONS
(In Alphabetical Order)

bFGF basic fibroblast growth factor
CFR code of federal regulations
cGMP current Good Manufacturing Practice
COL7A1 collagen type VII gene
D10 DMEM with 10% fetal bovine serum and 2mM L-glutamine
DEB dystrophic epidermolysis bullosa
DMEM Dulbecco’s Modified Eagle’s Medium
DNA deoxyribonucleic acid
EB epidermolysis bullosa
FDA United States Food and Drug Administration
hESC human embryonic stem cell
HIV human immunodeficiency virus
HSC hematopoietic stem cell
ICC Immunocytochemistry
IND investigational new drug
iPSC induced pluripotent stem cell
MEF mouse embryonic fibroblasts
MSC mesenchymal stem cell
PBS phosphate buffered saline
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<td>PBS containing calcium and magnesium</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>QC</td>
<td>quality control</td>
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<td>RDEB</td>
<td>recessive dystrophic epidermolysis bullosa</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid (mRNA = messenger RNA)</td>
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<tr>
<td>UCD</td>
<td>University of California Davis (also UC Davis)</td>
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1. INTRODUCTION

Epidermolysis bullosa (EB) is a family of heritable, genetic, skin disorders that is foremost characterized by severe fragility of the skin. In the United States alone there are over 6.5 million people afflicted by one of the various forms of EB, with an annual prevalence of nineteen new cases for every 1 million births (Fine, 2010). The EB family of diseases consists of four major categories (Alper et al., 1978) as well as thirty-two phenotypically distinct subtypes (Feijoo et al., 2011; Uitto et al., 2005), based in part ultrastructurally on the amount of epidermal separation from the underlying basement layer. This epidermal separation of the skin and mucous membranes from the basal lamina making up the basement membrane results from the formation of blisters, often in response to minor mechanical stress. The effects of this skin erosion vary from minor blistering of the skin to lethality if other organs are involved (Feijoo et al., 2011; Shinkuma et al., 2012). There are four major categories of EB consisting of epidermolysis bullosa simplex (EBS), junctional epidermolysis bullosa (JEB), Kindler syndrome, and dystrophic epidermolysis bullosa (DEB), which is the least prevalent yet most gruesome of the manifestations of EB. Within the dystrophic category are both dominant and recessive forms. Recessive dystrophic epidermolysis bullosa (RDEB) is considered to be among the most devastating of all the heritable conditions known to afflict humans and is associated with the highest morbidity, with no effective therapy available (Fine, 2010). It is estimated that RDEB afflicts less than one case per 1 million newborns within the United States, and children born with this disease face a dismal life.
Rare and devastating diseases such as RDEB are referred to as “Orphan Diseases”. The United States Food and Drug Administration (FDA) through the “Orphan Drug Act” enacted by Congress in 1983, allows a special “Orphan Drug” designation for the development of therapies of these diseases. This is accompanied by accelerated approval and preferential funding for the development of such therapies. Through this project, a promising novel therapeutic is investigated to treat RDEB, a devastating disease that currently lacks an effective therapy. Herein RDEB is described in detail, followed by a discussion of the shortcomings of common approaches applied in attempt to ameliorate this disease and how this current project, overviewed in Figure 1 and described below in section 1.2.4, overcomes these shortcomings possibly leading to an effective therapy for RDEB.
Figure 1. Project Overview. Fibroblast-derived induced pluripotent stem cells manufactured under Good Manufacturing Practice (GMP) conditions are utilized for the treatment of autosomal recessive dystrophic epidermolysis bullosa.
1.1 Disease characterization of recessive dystrophic epidermolysis bullosa (RDEB)

1.1.1 Subgroups of epidermolysis bullosa and their clinical description

In the medical literature, epidermolysis bullosa was a term coined as early as 1886 (Koebner, 1886; Coulombe and Lee, 2012, Fine 2010). The etiology of EB or other mechanobullous diseases, however, was not elucidated until relatively recently. Application of transmission electron microscopy to the study of EB diseases in 1962 led to the first characterization, partitioning EB broadly into three major manifestations by ultrastructural findings (Pearson, 1962): EB Simplex (characterized abnormalities localized in the basal layer), Junctional EB (characterized by abnormalities localized in the lamina lucida) and Dystrophic EB (characterized by abnormalities occurring in the sub-lamina densa, also commonly referred to as the basal lamina). The characterization and classification of EB subtypes has been a lengthy progression that has become more accurate as laboratory techniques became more advanced and precise and is strongly based on the degree of tissue separation, clinically determined by assaying a biopsy of a recently formed lesion using transmission electron microscopy and immunofluorescence (Shinkuma et al., 2012).

Recessive dystrophic epidermolysis bullosa, like the rest of the mechanobullous or “blistering diseaseas” is most broadly characterized by the perpetual blistering of regions of the body that are covered with epithelium (Fine and Mellerio, 2009). RDEB however, unlike the other forms of EB is significantly more severe. Hallmark characteristics of RDEB include skin blistering which begins already during the birthing
process, nail dystrophy of all ten digits, dyspigmentation (Fine, 2010), loss of hair or the complete absence of it, growth retardation and failure to thrive (primarily a result from the body’s perpetual use of energy for the purpose of healing rather than normal growth and weight gain), disfiguring scarification including ankyloglossia (a condition in which the membrane under the tongue becomes short and thick, effectively anchoring the tongue to the bottom of the mouth) (Fine et al., 2008), microstomia (reduction in the size of the oral aperture), greatly increased risk of infection as a direct result of the loss of a key physical barrier to pathogens which an intact skin provides (Siprashvili et al., 2010), esophageal strictures which may be so severe that the affected person loses the ability to eat and maintain proper nutrition (Fine et al., 2008), pseudosyndactyly also referred to as mitten deformities (this occurs when the hands and feet have undergone repetitive cycles of scarification that eventually force the hands and feet to form a single unit where the digits are no longer capable of individual movement) (Fine et al., 2005), urethral strictures, colitis, vaginal strictures (Fine et al., 2008) (which restrict normal sexual function), renal failure (Fine et al., 2004), and a significantly increased risk of squamous cell carcinoma (Fine et al., 2009). With all of the various forms of EB, symptoms may not be immediately apparent, often not presenting until months or even years after a child’s birth (Coulombe and Lee, 2012). Furthermore, while many of the same hallmark features of RDEB are shared by the various forms within the epidermolysis bullosa family, none have such an extreme phenotypic expression as in the recessive form of the disease (Fine, 2010).
1.1.2 Molecular characterizations of EB

Perhaps some of the most important advances in the characterization of the various forms of EB have been those on the molecular level which have allowed scientists to identify genetic mutations. In 2008, in excess of 1000 mutations spanning 10 genes responsible for the manifestations of all the known forms of EB had already been discovered (Dang and Murrell, 2008). The discovery of these gene mutations has made it possible to develop targeted gene therapies (discussed below in section 1.2.3) aimed at correcting the mutations and hoping to relieve the deleterious effects of these diseases. The current project presents one such therapy which holds great promise and focuses on the COL7A1 gene.

The dystrophic variant of epidermolysis bullosa is the result of individual heterogeneous mutations leading to the same phenotypic disease manifestation. Each of these mutations responsible for dystrophic epidermolysis bullosa is found within the gene responsible for coding type VII collagen (Varki et al., 2007), abbreviated COL7A1. The mutation in the collagen VII protein coding gene is also what makes recessive dystrophic epidermolysis bullosa (RDEB) distinct from the other forms of EB. Variations of EB can result from mutations in a number of other genes responsible for coding proteins other than collagen VII. For instance, mutations in genes coding for Keratin V, Laminin-332, and Kindlin-1 have all been found to be responsible for the phenotypic expression of epidermolysis bullosa simplex, junctional epidermolysis bullosa, and Kindler syndrome, respectively (Fine et al., 2008) . All of the listed proteins are also essential for the
endogenous function of the skin at various ultrastructural locations. When mutations occur in the genes responsible for the production of certain ultrastructural proteins, a loss of function will be initiated for this ultrastructure. Depending on which ultrastructure is affected, the EB category can be classified. While both the dominant and recessive forms of dystrophic epidermolysis bullosa are solely the result of a mutation within the COL7A1 gene, it is interesting to note that for some of the other major manifestations of EB such as EB simplex as well as junctional EB there are several different protein coding genes that may be affected, resulting in the disease phenotype.

1.1.3 Mutations in the gene COL7A1 leading to RDEB

The COL7A1 gene itself is 32 kilobases (Wertheim-Tysarowska et al., 2011) and is constructed of 118 exons (Pfendener et al., 2005). The COL7A1 gene is located on chromosome number 3, encompassing base pairs 48,601,505 to 48,632,592 (Entrez Gene). To date, over 530 mutations in the COL7A1 gene have been reported and recognized as responsible for RDEB (Wertheim-Tysarowska et al., 2011). The majority of these mutations are missense mutations (Wertheim-Tysarowska et al., 2011), in which a single nucleotide mutation results in a pathological amino acid alteration or a premature termination codon. Both types of mutation lead to the formation of truncated collagen VII protein, a severe reduction in the quantity of collagen VII, or the complete absence of it (Christiano et al., 1994). As mentioned previously, the mutations responsible for RDEB are heterogeneous and tend to be family specific. Due to the heterogeneity of the
mutations it is difficult to perform genetic screening for RDEB. The precise location of
the mutation will also have an impact on the severity of EB which more than justifies the
difficult task of studying the correlation between genotype and phenotype.

Collagen VII is a protein that in its normal state is quintessential for the stable
anchoring of the dermis to the basal lamina. Collagen VII begins as three protein
products that together wind around one another in a rope like fashion to form pro-
collagen. The pro-collagen is secreted by keratinocytes, although it is now believed that
fibroblasts may also secret pro-collagen (Woodley et al., 2003), which is then
enzymatically cleaved and arranged into long strands of mature collagen (Bruckner-
Tuderman et al., 1999). These mature collagen bundles are incorporated into anchoring
fibrils and then anchored into the basal lamina. They also extend into the dermis,
effectively anchoring the epidermis to the dermis being responsible for firm cohesion.
The anchoring of these two anatomical structures together affords humans, as well as
other animals an elastic and durable skin layer that is resistant to mechanical friction and
also provides a strong barrier against large molecules and germs (Briggaman and
Wheeler, 1975). The absence or paucity of collagen VII results in the characteristics of
RDEB, essentially culminating in a skin layer without any integrity. Without normal
collagen VII production even minor mechanical friction, such as rubbing or a scratch, can
effectively separate the epidermis from the dermis resulting in the formation of a new
blister.
1.2 Approaches for Therapies of Epidermolysis Bullosa

Currently, the outlook for RDEB patients is bleak. Measures to treat this disease are greatly restricted to the same treatments that were offered already thirty years ago, and are primarily palliative. Standard treatment regimens include the careful and thorough application of gauze to the affected areas of the skin where blistering has resulted in loss of the skin’s integrity. The careful application of gauze to the digits can also aid in the prevention of mitten hand and foot deformities which will afflict 100% of the patient population by the age of 20 (Fine et al., 2005). The application of antibiotics and Vaseline also help prevent infection and reduce the mechanical friction to the skin. In addition to these basic preventive measures, surgeries may be necessary as more serious life-threatening conditions manifest; these may be debilitating esophageal strictures, which develop in 90% of patients by the age of 35 (Fine et al., 2008), requiring dilation of the esophagus or even placing a feeding tube into the patient permanently. In addition to the daily struggles RDEB patients encounter, they are also at high risk of developing squamous cell carcinoma, with an average of 87% of patients having this aggressive life threatening form of cancer by age 45 (Fine et al., 2009).

Despite the fact that RDEB is an orphan condition that affects few people in comparison to other conditions, RDEB has seen a recent surge in research. A PubMed search for “Recessive Dystrophic Epidermolysis Bullosa” returns over 1000 published articles. In addition, there are now well known research institutions such as Stanford University, the University of California Davis and the University of Minnesota dedicating
substantial resources to finding a potential cure for this condition. This effort is not only ongoing in the United States but is also shared globally. Although novel approaches for treating EB are impressive, none have thus far proven to be the final solution. Nevertheless, while there is still not an optimal solution, there are many ideas that are showing great promise. There have been positive results from studies that have used protein-based injection (Woodley et al., 2004), bone marrow therapy, and many therapies that aim at gene correcting various cells such as fibroblasts (Woodley et al., 2003), keratinocytes (Chen et al., 2002), and their precursor cells. So far, however, only one therapy has been approved by the FDA to go forward in clinical trials as an investigational new drug (IND) for the treatment of EB. This effort is led by Stanford University, to which the current study is linked.

1.2.1  Protein-level therapeutic approach targeted towards COL7A1

Regarding the potential therapies of RDEB it is interesting to note that none are as simplistic and direct as the protein based approach. As has been described thus far, the underlying issue in RDEB patients is a mutation within the type 7 collagen gene which results in the absence or paucity of the important adhesion protein collagen VII. Rather than attempt a true curative approach to RDEB which would require genetic correction of the COL7A1 gene mutation, a task that is extremely complex using current methodologies, some investigators have taken the approach to restore function of the skin’s integrity at the protein level itself. Such an approach involves, for example, fibroblasts that have been biopsied from patients and transduced with a lentiviral vector
to express wild type collagen VII (Kern et al., 2009; Siprashvili et al., 2010). The collagen VII protein from *in vitro* cultured cells could then be isolated and injected into EB skin that had been grafted onto immuno-deficient mice. It has consequently been shown that the collagen VII protein migrates to the basement membrane zone of the skin graft, and for approximately two months is able to restore nearly normal levels of collagen VII (Siprashvili *et al*., 2010). The protein injection approach has some advantages over other methods; it is easy to apply, does not require any gene manipulations, and carries few if any ethical concerns. Similar injection procedures are currently routinely conducted with collagen for the purely cosmetic purpose of wrinkle reduction (Brown *et al*., 2011). While the approach may seem particularly appealing due to its simplicity, there are some fundamental shortcomings. First and foremost, the protein approach is not a cure, but rather a short duration treatment. Lifelong regularly occurring injections over the whole body will be needed. Also the intradermal injection of corrected collagen VII does not address the fact that many of the other epithelial regions, such as the interior of the esophagus, are not as easily accessible for such application. Finally, the problem of treating an orphan disease with a “protein drug” becomes another issue since the pharmaceutical industry does not easily manufacture drugs for orphan diseases (Pollastri, 2011). The development of a clinically applicable drug is very expensive as it has to go through FDA-prescribed drug manufacturing under Good Manufacturing Practice (GMP) conditions (described below), preclinical toxicology and efficacy testing in *in vitro* and *in vivo* models, then through Phase I, II and III clinical trials as an investigational new drug (IND), and finally through a post-
marketing study. A price tag of several hundred million up to over a billion dollars for such a drug is not unusual (Adams and Brantner, 2010). Drugs that can be used to treat common diseases easily recoup such an investment and turn a large profit over their patent duration. However, drugs that are used to treat only few people rarely come close to recouping the developmental expense. Large pharmaceutical companies therefore refrain from manufacturing orphan-disease drugs.

1.2.2. Systemic / Bone Marrow Transplantation approach

Therefore, capable academic centers view it as an important task and also as a moral obligation to develop other therapeutics, such as gene and cellular therapeutics that offer potential cures or long lasting treatments for orphan diseases. One of the most interesting and perhaps most promising potential therapies geared towards a partial cure is the use of bone marrow transplantation for treating recessive dystrophic EB. At the University of Minnesota, Minneapolis, a clinical trial (NCT00478244) was approved to use bone marrow transplantation for the amelioration of recessive dystrophic epidermolysis bullosa. The trial was approved after preclinical data could demonstrate that allogeneic bone marrow transplantation from healthy mouse donors into mice with dystrophic EB could actually prolong their lives and increase the quantity of type VII collagen produced at the dermis epidermis junction (DEJ) (Tolar et al., 2009; Wagner et al., 2010). In order to perform a complete reconstitution of a human EB patient’s bone marrow with healthy donor marrow, recipient bone marrow ablation prior to allogeneic
bone marrow transplantation is required. This is perhaps the greatest weakness of this approach of EB treatment as myeloablative therapy is highly toxic and did result in the death of one of the seven patients in the clinical trial prior to receiving the allogeneic bone marrow (Wagner et al., 2010). It is therefore clear that when a conditioning regimen is so extremely toxic that one in seven patients dies before the therapy can even be applied, that therapy may then be deemed highly risky and not easily applicable to all EB patients. In the United States, a therapy that results in the death of one patient out of seven does not meet the safety standards required in a Phase 1 clinical trial for non-malignant disease, per the FDA Code of Federal Regulations Title 21 (21 CFR). It is likely for this reason that trials of this kind did not proceed any further.

Therapeutic results on EB from this study were nonetheless very impressive and thought provoking. The principle investigator for this study, Dr. Wagner, found that in the patients that tolerated the ablation and subsequent reconstitution of the hematopoietic system, did indeed show a subtle, yet important increase in collagen VII at the dermis epidermis junction. Other studies have shown that a complete normalization of collagen VII at the dermis epidermis junction is not required in order to restore a functional dermis; in fact, an increase in wild type collagen as minute as 10% is sufficient for a statistically relevant increase in the stability of the skin, and therefore decrease in morbidity to the patient (Fritsch et al., 2008). The patients in the Wagner study increased their collagen VII levels above the 10% threshold, however with some mixed therapeutic results. Although they all had increased levels of collagen VII, they did not necessarily have the ultrastructures associated with mature and functional anchoring fibrils; but it
was noticed that the presence of collagen VII in its wild type form, even when not properly enzymatically rearranged into anchoring fibrils, was enough to introduce substantial stability of the skin (Wagner et al., 2010). All patients had increased clinical improvement, although this improvement was variable. Apart from the toxic myeloablative therapy required to condition the patients this method appears to address some of the most difficult problems in EB therapy. The use of allogeneic bone marrow transplantation is truly a systemic approach for EB therapy; not only would the skin cells be producing collagen VII, but also those that are in difficult to reach areas such as the esophagus.

This study also begs an important question: What is the mechanism by which an allogeneic bone marrow transplant is capable of ameliorating the effects of dystrophic EB? The reason is as of yet unknown. The Wagner publication is very careful to not directly address this issue. The current dogma in developmental biology is that differentiation is a one way path. For example, if a cell is destined for the mesoderm lineage, it cannot become a cell in the ectoderm. This dogma persists despite evidence to the contrary (e.g. Barzilay et al., 2009; Phinney and Prockop, 2007; Shinmura et al., 2011; Li et al., 2011) and as such we may soon see a paradigm shift. In the study by Wagner et al. the term bone marrow transplantation is used as a clinical term for what scientists specializing in developmental stem cell biology would call a mesenchymal stem cell (MSC) and hematopoietic stem cell (HSC) transplant. The most thought provoking portion of Wagner’s findings were in the assessments of chimerism using skin biopsies. Analyzing these biopsies, a median donor cell content of 20% was found, with
some cases having as high as 93% donor cell content (Wagner et al., 2010). If MSCs and HSCs are incapable of transdifferentiation under any circumstances, what indeed resulted in such high levels of chimerism? Although further discussion on the transdifferentiation of MSC is beyond the scope of the current report, follow up studies must surely be conducted. Some speculate that MSCs could have fused with the recipients’ keratinocytes, while others speculate that there may be more to the story. It would undoubtedly be informative to pursue the possibility of MSCs being able to transdifferentiate, particularly because MSCs are immuno-privileged (Rastegar et al., 2010), meaning that they do not cause a rejection reaction in the recipient. They are therefore more easily transplanted into a recipient than other cell types exhibiting high levels of human leukocyte antigens (HLA) that could lead to tissue rejection. Should MSCs be shown to be capable of transdifferentiation, they might be an extremely promising avenue for dystrophic EB therapy.

1.2.3. Gene therapy approach to treat EB

Perhaps some of the most complex yet most promising ventures in EB therapies are those incorporating gene therapy. Gene therapy is a general term describing the process in which a gene is introduced into a cell for the express purpose of correcting a genetic abnormality or altering the course of a disease. Gene therapy may be approached and accomplished in several different ways. In general, the strategies employed are to either insert a gene into the host’s genome (Biasco et al., 2012), or a mutant, non-functional gene in the host can be exchanged for a functional copy of the correct gene
The greatest advantage of gene therapy that sets it above other current possible approaches in EB therapies is that gene therapy may be curative. Gene therapy offers a permanent solution to the disease state by correcting a faulty gene, the cause of the disease itself whereas the other approaches in EB therapy only provide short term palliative value, or suboptimal disease correction.

The hurdle that must be passed before gene therapy can become successful, however, is not easily overcome. This hurdle is the safe and effective delivery of target genetic material into a host’s genome. There are currently several different ways of accomplishing such gene delivery in which most commonly a viral vector is used. Even among the viral vectors there are many choices, each having its own set of pros and cons. Some of the most common viral vectors used today for gene therapy applications are based on retroviruses, adenoviruses, and adeno-associated viruses (Ghazizadeh and Taichman, 2000). Retroviruses are capable of integrating their genetic information into the host’s genome. Therefore, if used as gene transfer vector, retroviruses provide a permanent gene integration solution. Retroviruses are RNA viruses, a subgroup of which is made up of lentiviruses. Retroviruses carry their genetic information in the form of two single strands of RNA and act by reverse transcription of their RNA into DNA after they have entered the target cells, through the activity a viral specific polymerase called “reverse transcriptase”. Finally, they use another viral specific enzyme called “integrase” to integrate the newly synthesized DNA into the host’s DNA. In order to make retroviruses suitable for therapeutic application they are commonly generated to be replication-defective by deleting genes required for viral replication (Ghazizadeh and
Taichman, 2000). In this manner the retrovirus can act as an effective means for gene delivery and integration of genes of interest without viral replication.

As mentioned previously, lentiviral vectors, which are HIV based, are a subclass of the retroviral family and are a current popular choice. In gene therapy it is common practice to use the term retroviral vectors for any gene therapy vectors derived from mouse oncoretroviruses such as the Moloney leukemia virus, and the term lentiviral vectors for any gene therapy vectors derived from HIV-1. One drawback of both retroviral and lentiviral vectors is that they integrate their newly synthesized DNA “quasi randomly” into the host cell genome (Biasco et al., 2012). This has caused some concern since such random integration of a vector into the host genome could possibly activate an oncogene if the integration site is close to such an oncogene, and the promoter present in the vector could trans-activate the gene. In a gene therapy clinical trial conducted in 2003 in France this possibility actually occurred. Several children developed a leukemia that correlated to the insertion of the retroviral vector (European Society of Gene Therapy, 2003). It was found, however, that “insertional oncogenesis” was due not just to retroviral gene transfer but that several other factors such as a strong selective pressure on the transduced cell population and the transfer, in this clinical trial, of a growth factor receptor gene whose expression was constitutively upregulated increased the chance of developing cancer many-fold. In hundreds of other gene therapy clinical trials using retro- or lentiviral vectors, no insertional oncogenesis was found. Recently, a safety study conducted on over 600 immunodeficient mice receiving human hematopoietic or mesenchymal stem cells transduced with retro-or lentiviral vectors was published (Bauer
et al., 2008) and showed no evidence that insertional oncogenesis would arise if the gene transduction was performed properly. Numerous other studies utilizing lentiviral vectors, to date have also failed to prove that lentiviral vectors promote oncogenesis on their own. It is even now considered that lentiviral vectors have a better safety profile compared to retroviral vectors since their integration pattern does not favor transcriptional start sites of genes in the host cell DNA. Furthermore, problems associated with seemingly random vector integration will eventually become clarified as bioinformatics sciences advance. In the meantime in the laboratory, scientists are also taking precautionary measures such as the use of linear amplification-mediated polymerase chain reaction (LAM-PCR) to detect where integration has or is likely to occur (Bartholomae et al., 2012; Schmidt et al. 2003).

Adenovirus and Adeno Associated Virus (AAV) based vectors are interesting alternatives in gene therapy for Epidermolysis bullosa. These are DNA viruses, and unlike retroviruses and lentiviruses they do not integrate the transferred gene into the host’s genome, rather the gene persists episomally. Therefore, insertional oncogenesis is not an issue with this group of viral vectors. Adenovirus and Adeno Associated Virus vectors also have the advantage of being capable of infecting both actively dividing and non-dividing cells, which broadens the cell targets for these vectors. In the laboratory, all described vector types have been used in experimental approaches of gene therapy for various forms of Epidermolysis bullosa. Some attempts have been successful while others have encountered various problems.
1.2.4. The Stanford U/UCD Approach Utilizing Gene Corrected Induced Pluripotent Stem Cells (iPSCs)

The Stanford/UC Davis vision for this project is to ameliorate the EB condition such that the quality of life is vastly improved for those who are afflicted. The course of action that has been deemed most suitable to address autosomal recessive dystrophic EB (RDEB) is a complicated one that incorporates several techniques and principles that have been previously described. In order to cure RDEB the patient’s keratinocytes must be genetically altered to express functional COL7A1 rather than the mutant variant. As there are many keratinocytes requiring this correction, a method had to be developed that allowed for massive expansion of these cells. With the necessity for expansion as well as gene correction, a plan was derived that would fulfill both objectives, which is carried out in the current project. Due to the remarkable advancements in stem cell biology many feats that were once considered impossible are now routinely performed in laboratories. One of the techniques that has emerged, and will increasingly play an important role in medicine, is the capability of advanced laboratories to generate induced pluripotent stem cells (iPSC) from a patient’s fibroblasts (Takahashi K and Yamanaka S, 2006). For RDEB patients it is our hope to translate this into the development of a novel therapy.

The following describes the steps outlined in Figure 1 (above), many of which were performed by the author of the current report. Fibroblasts are easily harvested by a physician from an individual suffering from RDEB through a simple skin punch biopsy. The fibroblasts can then be sent to a laboratory, such as the UC Davis Good Manufacturing Practice Facility, where a patient fibroblast master cell bank can be
established. Following adequate expansion of the patient’s fibroblasts the technology pioneered by Yamanka and adapted by Stanford University scientists is employed to generate iPSCs. Using a lentiviral vector, four genes namely Oct4, Sox2, c-Myc, and Klf4, are delivered and integrated into target fibroblasts. These genes are “early acting genes” (Nichols et al., 1998; Niwa et al., 2000; Avilion et al., 2003; Cartwright et al., 2005; Li et al., 2005), and are naturally present in the human genome but are upregulated only in early embryonic development. In the past, introducing four genes was accomplished by transducing a cell with four separate lentiviral vectors; however, the design of the Stanford vector allows all four genes to be delivered to the patient’s fibroblasts using a single lentiviral vector. All 4 genes fit into the carrying capacity of a lentiviral vector, and can be driven by the use of just one promoter (EF1 alpha) using 2A sites and Internal Ribosome Entry Sites (IRES) within the vector. Such sites allow for the continued translation of all m-RNAs transcribed from the integrated vector DNA. In addition, delivery of all four genes in a single vector further reduces the chance of oncogene activation through quasi random integration by decreasing the number of vector insertions within the host genome. The addition of these four early acting genes to the patient’s cultured fibroblasts causes the cells to express gene products that are normally expressed only during embryonic development. These gene products stimulate intracellular signaling cascades inducing the cell to acts as if it were an embryonic stem cell. This results in the fibroblast being “re-programmed” into a pluripotent stem cell, which is now called an “induced” pluripotent stem cell (iPSC). It has repeatedly been demonstrated that iPSCs are fully capable of differentiation into all three germ layers,
mesoderm, endoderm and ectoderm (Takahashi and Yamanaka, 2006). The capacity for the iPSC to be differentiated into a cell of the ectodermal lineage is very appealing for the development of a treatment for RDEB, as it means the potential for iPSCs to differentiate into keratinocytes, the cells primarily responsible for secreting type VII collagen. Taking into account epigenetic preprogramming, it makes most sense to use starter cells for iPSC generation that are similar in function to the types of cells that are to be derived in the final product. Although fibroblasts are not of the ectoderm lineage they are similar in function to cells of ectodermal origin in part in that they have also been shown to secrete type VII collagen, however, less than keratinocytes.

The generation of iPSCs and subsequent differentiation of these pluripotent cells into keratinocytes has distinct advantages over other methods currently sought after by other research groups. First, the generation of keratinocytes derived from patient iPSCs results in the avoidance of graft rejection. The final product, functional keratinocytes, will present the same human leukocyte antigen (HLA) markers presented by all of the patient’s other cells. In essence, the final product truly belongs to the patient - it is a product derived from their own body and will not illicit an immune response. This is therefore a superior method over other methods seeking transplantation of foreign derived cells, such as allogeneic bone marrow transplantation which requires immunomyleoablative therapy as a conditioning regimen.

An issue that is of concern to modern scientists in the field of stem cell biology is the ethical consideration. While there is yet great public controversy of the ethics behind
the use of human embryonic stem cells (hESC), which are shown in the current work, to be fully capable of differentiation into keratinocytes, the use of iPSCs avoids the ethical issues surrounding hESCs entirely. The process of generating an iPSC does not require the destruction of an embryo at any stage. iPSCs, if generated and cultured properly from normal skin fibroblasts are as functional as any hESCs and present the same pluripotency markers.

Subsequent to the generation of iPSCs from patient fibroblasts gene therapy for COL7A1 is required. This adds a second layer of complexity to the project. Additionally, safety concerns are raised by the use of both iPSCs and gene therapy, which we address throughout the project. While a fully differentiated cell, such as a keratinocyte, does not pose a threat to a person after transplantation, a pluripotent cell such as an iPSC could cause problems if transplanted. Pluripotent cells can develop teratomas, tumors comprised of derivatives of all 3 germ layers. In fact, the actual functional in vivo test for a pluripotent stem cell is the teratoma formation test in an immuno-deficient mouse. However, teratomas must not arise in patient treated with iPSC derived keratinocytes. Therefore the complete removal of any pluripotent cell is required for this therapy. In addition, if the reprogramming vector remains active the cells would not easily differentiate into keratinocytes, since the pluripotency factors would continue to be expressed, maintaining the cells in the pluripotent state. For these reasons, the approach of the current project is to ensure that the iPSCs are no longer forced into their pluripotent state by the lentiviral insert containing the early acting genes. In order to shut down the driving force behind the pluripotency, the four early acting genes must be silenced.
Taking this further, to improve safety, these genes are not merely silenced but are fully removed from the cells. To achieve this complete removal, the vector gene cassette is flanked by LoxP sites. LoxP sites are specifically recognized by the enzyme Cre recombinase which splices the LoxP sites, thereby excising the lentiviral vector. While the short LoxP sites remain in the genome, they do not pose a threat since the promoter and messages from the early acting genes are absent. After the excision process is enacted, individual colonies are tested and verified via PCR for vector removal. The removal of the lentiviral vector meets requests by the FDA for safety of iPSC derived therapeutic products.

The next step will be the gene correction of the successfully produced iPSCs after vector removal, achieved by homologous recombination. This is a major improvement over conventional gene insertion strategies, since the defective COL7A1 gene is actually repaired, in exactly the same place and under the same promoter that is used in a normal healthy human genome. For this step a DNA plasmid targeting vector with the fully function COL7A1 gene is introduced to the iPSCs. As cell division occurs the chance arises for the plasmid to replace the mutant gene. When homologous recombination is successful the functional gene is introduced into the cells genome and the mutant variant of COL7A1 is removed. The success rate of homologous recombination is low with an approximate efficiency of the recombination process being between 2 and 10% (Wolf-Dietrich H, 2007), however, using large cell populations allows for selection for those few colonies that have undergone the desired homologous recombination. The colonies that are selected for passage are tested for successful homologous recombination to
ensure that the targeted DNA has indeed been repaired. We work with very large cell populations which provide us with ample opportunities to successfully generate at least the single colony that is perfectly repaired. This colony that has undergone gene therapy for COL7A1 can then be dramatically expanded to provide a clinically relevant number of gene corrected cells.

The number of cells required for the final differentiation is minimal. We have found at the UC Davis Good Manufacturing Practice facility that even a single small iPSC colony can be differentiated into functional keratinocytes. These keratinocytes may then also undergo immense expansion into the final product of COL7A1 gene corrected fully differentiated keratinocytes. The keratinocytes may then be applied to the patient at the Stanford University hospital clinic in a manner that our collaborating physician feels most comfortable, which may be the expansion of the keratinocytes into a manufactured dermal graft, or a keratinocyte/collagen combination that can be sprayed onto the patient with a cell spray gun.

1.3 The Good Manufacturing Practices (GMP) Facility and Current Good Manufacturing Practices (cGMP) at the University of California Davis

In order for the joint effort between Stanford University and the University of California, Davis to be successful, the EB project must pass through several check points before the cellular therapeutic may be suitable for a phase I clinical trial. In order for a therapy to proceed from the laboratory bench to the patient’s bedside the Food and Drug
Administration (FDA) has stipulated a series of defined regulations that must be adhered to in order to assure that the highest standards of safety are met to protect the persons receiving a novel therapeutic. These regulations are outlined in great detail within the Code of Federal Regulations (CFR), specifically in CFR 21 Part 58 as well as CFR 21 Parts 210 and 211, which together dictate the standards called Good Laboratory Practice (GLP) and current Good Manufacturing Practice (cGMP) respectively.

Good Manufacturing Practices have played an integral role in protecting the lives of Americans already since the early 20th century. After an incidence with tetanus infected antiserum against diphtheria which caused the death of several children in St. Louis, MO, Congress enacted the Biologics Control Act of 1903, for the first time mentioning the term “Good Manufacturing Practice” (Food and Drug Administration, 2009). Since the Biologics Control Act of 1903, Good Manufacturing Practice facilities had to be built for the manufacturing of Biologics (therapeutics derived from living sources), and have been required to remain current in their practices ensuring that the laboratories continue to evolve their safety standards and maintain strict quality control, and quality assurance measures. Before a therapeutic can enter a clinical trial, extensive tests in the laboratory have to be performed to demonstrate safety and efficacy in both in vitro and in vivo experiments. Good Laboratory Practice guidelines decree that all laboratory experiments are well documented and such documents are available, at all times, for an FDA audit. All documents are maintained in a laboratory notebook which does not leave the laboratory, remains in a locked compartment after laboratory hours, and contains even the source documents (anything that an original note was written upon,
including a piece of scrap paper or even a Kim wipe, should it have been used) for anything that may pertain to the testing of a product. Good Laboratory Practice guidelines also dictate that all the materials must be able to be traced back to their origin for the purpose of complete characterization of a product to assure its safety when it gets translated into a therapeutic. This includes certificates of analysis from all the reagents used for the purpose of manufacturing a product.

Principal to a cGMP facility is adherence to Standard Operating Procedures (SOP). A Standard Operating Procedure is essentially a script that describes in minute detail all the steps that must be completed while performing the manufacturing of a product. There are Standard Operating Procedures for all actions performed while in the Good Manufacturing Practice facility, from the moment a technician (or even the professor!) enters the gowning room to the facility to the moment they leave. Standard Operating Procedures are quintessential to maintaining a true Good Manufacturing Practice facility as they ensure that the facility is capable of reproducing all work accomplished and manufacture in the most controlled environment possible. At the UC Davis Good Manufacturing Practice Facility, Standard Operating Procedures are tightly regulated by the facility director, the medical director, and the scientific director. All three directors must concur on a Standard Operating Procedure before it may be signed into standard practice.

A Standard Operating Procedure will contain the following information and adheres to a standard format:
Quality Control (QC) is yet another vital aspect that holds high importance within a GMP facility. QC ensures that each and every product that is manufactured has been held to the highest standards of production. In a regular laboratory it is common practice that individuals work by themselves. However, mistakes can occur even when the individual is paying attention. In a Good Manufacturing Practice facility, manufacturing is always carried out by at least two individuals, one that is manufacturing and the other one taking on the function of quality control. A mistake is far less likely to occur when a QC member is present to help catch routine mistakes. In such a way, for instance simple miscalculations are very rare within the facility. QC however is not restricted to just controlling the quality of product manufacturing, but also controls the quality of the facility itself as well as the product, to final completion. The facility undergoes daily quality control including the monitoring of the pressures of each room in relation to one another (this is particularly important when vectors are being manufactured in a room and there is a possibility for the vector to become aerosolized, negative pressure in a vector production room can ensure that even in such a case a vector cannot escape the
room), temperature of each room (to ensure that ambient manufacturing temperatures remain constant within 2 degrees Celsius at all times of the year), temperature of each incubator (manually performed to ensure that the incubators’ digital meters do not report false values), temperature of each refrigerator (to ensure that if a temperature alarm did not sound the products are not lost costing the facility capital and time), temperature of each liquid nitrogen freezer (to ensure that long term storage has not been compromised).

On a weekly level fyrine testing is performed on each incubator (to ensure the proper range of carbon dioxide is maintained), and the cleanliness of the facility is also tested through two vigorous methods. One of the methods uses touch plates whereas the other uses settling plates; both are agar plates that support the growth of microorganisms, such as bacteria and fungi. Settling plates are exposed to room air for 3 hours, and touch plates are used to touch surfaces and pick up microorganisms from them. After exposure, these plates are incubated for two weeks and colony forming units (CFU) are assessed. These assays are administered as decreed by the FDA regulation “Selected USP General Notices and Chapters, VI/59-64”. Should a colony forming unit assay result in a value of 4 colonies, “Low risk action” (facility cleaning) must be taken where as if a value of 6 colonies is derived “High risk action” (shut down and complete scrub down) must be taken. To date, after two years in operation, the UCD Good Manufacturing Practice facility has not encountered a colony forming unit value of 4, this attests to the remarkable cleanliness of the facility.

The final product generated under Good Manufacturing Practice conditions is never released without quality control (QC) and quality assurance (QA). In order to pass
quality control and quality assurance, each manufactured product, such as the keratinocytes, must undergo the following tests:

1. Gram stain (in order to visualize gross bacterial contamination)
2. Sterility (14 day sterility test to determine growth of microorganisms)
3. Mycoplasma (PCR for mycoplasma sequences and culture for mycoplasma)
4. Endotoxin (Limulus Amebocyte Lysate Test)
5. Viability (A viability test is prescribed by the FDA for a cellular product such as the keratinocytes generated within the Good Manufacturing Practice)
6. Potency (this is not required for phase I clinical trials which are required only to determine the safety of a product however the potency of the keratinocytes could feasibly be determined by staining for type VII collagen in order to determine if they are secreting adequate quantities of the protein product)
7. A certificate of analysis must also be generated for the final product that will leave the facility
2. METHODS

2.1 Culture of Epidermolysis Bullosa (EB) Fibroblasts and Generation of EB Derived Induced Pluripotent Stem Cells (iPSCs)

2.1.1 Passage

EB fibroblasts are harvested from an EB patient at Stanford Medical Center via punch biopsy, cultured and sent to us at UC Davis by Stanford medical scientists at passage number 3. The fibroblasts are then expanded into a master cell bank containing over 100 cryovials by passage 5. All EB fibroblasts used experimentally in the current research project are at passage 5.

2.1.2 EB fibroblast media

EB fibroblasts are cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% Fetal Bovine Serum and 2mM L-glutamine. This media formulation is commonly referred to as D10 media.

2.1.3 Transduction of EB fibroblasts (Generation of EB Derived Induced Pluripotent Stem Cells)

EB fibroblasts are plated in a 6 well plate 24 hours prior to lentiviral vector transduction. The volume of vector to be added is dependent on the desired multiplicity of infection (MOI), the number of EB fibroblasts that are to be transduced, as well as the titer of the vector. The formula used to determine the volume of vector is:
For this project the numbers of EB fibroblasts seeded prior to transduction were 40,000, 50,000 and 100,000. The desired multiplicity of infection was 50 and 100. The vector was titered at $10^8$.

A volume of 500μL of transduction solution is prepared for each well of a 6-well plate in which the EB fibroblasts are seeded. Protamine sulfate is added at a volume of 1μL for each 500μL of transduction solution to be used. Following the application of the transduction solution, the EB fibroblasts are incubated for 2 hours at 37°C and 5.0% CO$_2$ after which twice the volume of media as transduction solution used, is added to each well. The cells are then returned to and incubated at 37°C and 5.0% CO$_2$ for 24 hours. Following the full day incubation period the transduced EB fibroblasts were lifted from the 6-well plates using 500μL TrypZean (Sigma) per well and passaged onto mouse embryonic fibroblasts (MEF), described further below. MEFs were plated the day before at a density between 200,000 and 250,000 cells per well in a 6-well plate.

iPSC colonies were consistently visible after 14 days in culture.

2.2 Stanford Induced Pluripotent Stem Cell (iPSC) Culture Media and Maintenance

Stanford iPSC media is specifically formulated to culture iPSCs generated using Stanford’s 4-Factor Lentiviral Vector developed by Marius Wergnig. This media is used
following transduction of the EB fibroblasts to support the development of iPSC colonies and is replaced daily with 2 ml warmed fresh media.

To prepare Stanford iPSC media Knock Out Dulbecco’s Modified Eagle Medium/Nutrient Mixture F-12 (KO DMEM/F12, Gibco/Invitrogen) is supplemented with a proprietary blend containing human Fibroblast Growth Factor basic (bFGF), beta-Mercaptoethanol, Minimal Essential Medium-Eagle with Non-Essential Amino Acids, L-glutamine, Knock Out Serum Replacer (KSR).

2.3 Assessment of EB Fibroblast Transduction and iPSC Generation

Two methods were used to assess the successful transduction of EB fibroblasts and subsequent generation of iPSC colonies. The first method is morphological analysis of the iPSC using phase light microscopy. iPSC that are forming colonies should have the same morphological colony characteristics as an ESC colony, tight colony boarders, even color throughout the colony, flat, and round or oval in shape, as shown in Results.

The second method for the evaluation of EB fibroblast derived iPSCs is through the use of immunocytochemistry. There are numerous cell markers that definitively label a cell as pluripotent. The same pluripotent markers are found on ESCs and on iPSCs. ESCs can therefore be used as controls for comparison when assessing pluripotency gene upregulation in iPSCs.
Antibodies used for the analysis of EB fibroblast transduction are anti-Sox2 and anti-Oct3/4. These antibodies were specifically chosen for the assessment of EB fibroblast transduction due to the fact that they are essential early acting genes for the maintenance of pluripotency as well as both being genes that are inserted into the EB fibroblasts genome through the 4 factor lentiviral vector.

An additional antibody, SSEA4 and Tra-1-60 were also applied to provide additional substantial evidence that the four-factor vector had initiated pluripotency in EB fibroblasts.

The same immuno-stains are applied to human embryonic stem cells for comparison and control.

2.4 Immunocytochemistry

Each well of the 6 well plate containing cells were gently washed two times with 500μl PBS containing calcium and magnesium (PBS++). The cells were fixed in 500μl of 2% paraformaldehyde per well. Plates were incubated in a 4°C refrigerator for 10 minutes to allow fixation to take place. Following 10 minutes of refrigeration the cells were washed three times with 500μl PBS++ keeping the last PBS++ wash in the well. The plates could then be stored at 4°C for up to 12 months or until ready to perform the immunocytochemistry.
Permeabolizing and blocking solutions were prepared as follows. The blocking solution used consisted of 1% goat serum in PBS++. The permeabolizing solution was made up of 2% Triton X-100 in PBS++ with 1% goat serum. For the permeabolizing-blocking solution, 500μl of the permeabolizing and 500μL of the blocking solution were added to each well. Primary antibody solutions were to diluted as suggested by the manufacturer and applied to the wells for staining. The 6-well plates were then wrapped in parafilm and incubated in a 4°C refrigerator overnight. Each well was then washed three times with 500μl PBS++. In a dark room the appropriate secondary antibodies were added to each well. Following an hour of incubation with the secondary antibody the cells were washed three times with PBS++ and three drops DAPI mounting media added to each well. Staining was subsequently imaged by fluorescence microscopy.

2.5 Culture of Mouse Embryonic Fibroblasts (MEF)

MEFs form the support system for ESC colonies. They are plated before ESC colonies are passaged. Increasing the density of MEFs in a plate as well as increasing the concentration of bFGF in the hESC media will provide the hESC with healthier morphology and will help the hESCs remain in an undifferentiated state (Thomson et al., 2005).
2.5.1 Media for MEFs

Fresh media for the MEFs is changed every day with D10. The media formulation is as described in EB fibroblast media.

2.5.2 Plating of Mouse Embryonic Fibroblasts

MEFs frozen in cryovials were quickly thawed until only a small ice crystal remained then carefully suspended in D10 media. The suspended cells were centrifuged at approximately 300-400g for five minutes at room temperature. The supernatant was aspirated to ensure that any remaining dimethyl sulfoxide (DMSO) from the freezing media in which the cells were stored was removed from the MEFs. This is important as DMSO is toxic to the cells. The washed cells are resuspended in fresh D10 media and plated at a density between 200,000 and 250,000 cells for each well in a 6-well plate.

2.6 Culture of human Embryonic Stem Cells (hESC)

2.6.1 Media for human Embryonic Stem Cells

Human embryonic stem cells require a specialized media to support their growth called ESC media. ESC media consists of KO DMEM/F12 supplemented with beta-mercaptoethanol, Knock Out Serum Replacer (KSR), Glutamax (Gibco/Invitrogen), MEM-NEAA, and bFGF.
2.6.2 Human embryonic stem cell culture

Human embryonic stem cells are thawed from a cryovial and suspended in ESC media (described above). The ESCs are centrifuged at approximately 150-200g for three minutes. The media and DMSO are then removed and the ESC pellet is suspended in ESC media. The pellet is then broken up gently to disassociate the ESC colonies from one another and plated on prepared MEF plates. The ESCs are fed every day with ESC media between five to seven days and are then ready for passage. ESCs are passaged by first marking morphologically correct colonies with a microscope marking objective and then scratching the colonies out with a pipette tip. The media, containing broken up ESC colonies, is passaged in a 1:3 ratio onto MEF plates.

2.6.3 Human embryonic stem cell passage

The hESCs used within these experiments for the purpose of providing positive controls for iPSCs characterization and negative controls for keratinocyte differentiation were obtained at passage 32 from the ESC Corp at the Institute for Regenerative Cures, University of California Davis Medical Center in Sacramento, CA..

2.7 Assessment of human embryonic stem cells

Human embryonic stem cells were characterized by immuno-staining using the same procedure described above for the assessment of induced pluripotent stem cells.
Antibodies used for the characterization of hESC are anti-Sox2, anti-Tra-1-60, anti-c-MYC, and anti-Oct3/4. Each of these antibodies binds to early acting genes that are indicative of pluripotency and the maintenance of undifferentiation.

2.8 Differentiation of iPSC and ESC into Keratinocytes-Oro Protocol

Following the generation of gene corrected iPSC colonies from EB patient fibroblasts, functional keratinocytes must be produced. In order to derive keratinocytes from a pluripotent stem cell a differentiation protocol must be utilized to drive the differentiation process. The differentiation protocol uses several types of media (listed below) at different specific time points in the process which help instruct the lineage differentiation and specialization of the cells. The same protocol may be used on hESCs and iPSCs. hESCs were used as a control for the differentiation process in order to test the Oro-Protocol before attempting the complicated procedure on iPSCs.

2.8.1 Differentiation Medias

Components of each of type of media used in the differentiation protocol described below, is listed here. Please note that concentrations not reported are due to proprietary restrictions (Stanford University and University of California Davis).
W8 Media:

The W8 media consists of DMEM/F 12 Knockout medium supplemented with Knock Out Serum Replacer (KSR), L-glutamine, MEM-NEAA, β-Mercaptoethanol, and bFGF.

CF1 Conditioned W8 Media:

$3 \times 10^6$ CF1 MEFs are plated in a 10cm dish overnight washed three times with PBS, and W8 media added for 24 hours. After 24 hours the now conditioned media is collected from the dish. After all the media has been conditioned, collected, and filtered, 1/2X bFGF is added.

FAD Differentiation Media:

DMEM/F12 is prepared in a 3:1 ratio and supplemented with 10% FetalClone II, 5μg/ml insulin, 0.5μg/ml hydrocortisone 21-hemisuccinate sodium salt, $10^{-10}$ mol/L cholera toxin, 1.37ng/ml 3,3’,5-Triiodo-L-Thyronine sodium salt, 24μg/ml adenine hydrochloride, 10ng/ml human recombinant epidermal growth factor, 25ng/ml BMP4, and 1μM retinoic acid.

Defined Keratinocyte Serum-Free Medium (DKSFM):

DKSFM is purchased from Invitrogen already prepared. It consists of a 500ml media that is supplemented with a 1ml intellectual property protected supplement.
2.9 Differentiation – Oro protocol

**Day 1**

Pluripotent stem cells are cultured on CF1 feeders in 6-well plates. The morphologically correct colonies are selected and manually passaged onto Matrigel coated 6-well plates and fed with CF1 conditioned W8 media.

**Day 2, 3, & -Ectoderm Lineage Initiation**

1μM retinoic acid is added to CF1 conditioned W8 media. Cells are fed with this media for 3 day changed daily.

**Day 5- Differentiation**

Media feeding the cells are replaced with FAD medium for 4 days changing media every day.

**Day 9 – Selection**

Cell cultures are suspended as single cells using 0.05% trypsin/EDTA and passaged at a 1:3 ratio onto gelatin coated 6cm dishes in DKSFM. The media is changed every other day until expanded keratinocyte colonies are visible by phase light microscopy.
2.10 Expansion

Keratinocyte colonies are marked under a light microscope. Non-keratinocyte cells are carefully aspirated off the culture plate. The remaining colonies are lifted with 0.05% trypsin/EDTA. These colonies are plated onto CF1 feeders and passaged two more times onto Matrigel coated dishes.

2.11 Assessment of iPSC/hESC Differentiation

The iPSC/hESC derived keratinocyte are assessed and characterized using immunocytochemistry. The immunocytochemistry protocol is identical to that described in the assessment of EB-fibroblast transduction and iPSC generation. The antibodies used for this assessment are anti-P63, anti-K18 (keratin 18), anti-K14 (keratin 14), anti-Oct3/4 (pluripotency marker) diluted as recommended by the manufacturer (various). P63, K18, and K14 are definitive markers of keratinocytes while Oct3/4 is used as a control for pluripotency. In this experiment one additional antibody was used in addition to the Oct3/4 pluripotency control, the anti-Sox2 antibody.
3. RESULTS

3.1 Transduction of EB fibroblasts and Wild Type fibroblasts

A lentiviral vector was used to transduce recessive dystrophic epidermolysis bullosa patient fibroblasts to generate induced pluripotent stem cells. The lentiviral vector contains four early acting genes Sox2, Oct4, Klf4, and c-Myc, flanked by LoxP sites, which when integrated into the EB fibroblast is capable of inducing pluripotency. Transduced fibroblast were stained for Sox2 and Oct3/4 in order to illustrate the functionality of the lentiviral vector (Figure 2). To further elucidate the complete reprogramming of the EB fibroblasts to an embryonic state, the cells were also stained for SSEA4 (Figure 4). In parallel with the transduction of EB fibroblasts, the lentiviral vector was also applied to unafflicted fibroblasts for comparison. The same antibody stains, Sox2, Oct3/4, and SSEA4, were applied to the wild type fibroblasts (Figures 3 and 5 respectively).
Figure 2. Transduced EB Fibroblast Immunocytochemistry Sox2, Oct3/4. (A) EB fibroblasts transduced with the Stanford 4-factor lentiviral vector. Positive immunostaining for Sox2 as well as Oct3/4 indicate successful transduction of EB.
fibroblasts. (B) Non-transduced EB fibroblasts are negative for lentiviral vector pluripotency products substantiating the efficacy of the lentiviral vector.
Figure 3. Transduced Wild Type Fibroblasts Immunocytochemistry Sox2, Oct3/4. (A) Wild type fibroblasts transduced with Stanford 4-factor lentiviral vector. Positive immunostaining for Sox2 and Oct3/4 are indicative of successful re-programming of fibroblasts into an early developmental state. The DAPI/overlay shows precisely the cells (Yellow) that have co-expression of Sox2 and Oct3/4. (B) Wild type fibroblasts not
transduced with Stanford 4-factor lentiviral vector stained negatively for Sox2 and Oct3/4 expression.
Figure 4. Transduced EB Fibroblast Immunocytochemistry. SSEA4 (A) EB fibroblasts transduced with the Stanford 4-factor lentiviral vector. Positive immunostaining for SSEA4 provides evidence of pluripotency gene activation. (B) Non-transduced EB fibroblasts are negative for SSEA4 staining lending additional evidence that the lentiviral vector is indeed initiating pluripotency in EB fibroblasts.
Figure 5. Transduced Wild Type Fibroblasts Immunocytochemistry, SSEA4. (A) Wild type fibroblasts transduced with Stanford 4-factor lentiviral vector. Staining for SSEA4 was not as pronounced in the wild type transduced fibroblasts as in the EB fibroblasts transduction, never-the-less, SSEA4 is visible. (B) The non-transduced wild type fibroblasts are entirely negative for SSEA4.
3.2 Morphological Findings of iPSC

Following the application of the lentiviral vector to diseased fibroblasts, plates were observed for colony formation. Within 14 days of transduction colonies were consistently visible. The colony formation resulted in an initially morphologically heterogeneous population of colonies. Many of the colonies did not grow robustly and died following several days of feeding (Figure 6 C3). Others began to differentiate at the edges of the colony (Figure 6 C1-C3). Some of the colonies also did not have a homogeneous coloration within the colony, indicating that the colony was either differentiating from the center of the colony or undergoing apoptosis (Figure 6 C2). Despite the fact that many of the transduced fibroblasts resulted in morphologically unacceptable colonies, there were never-the-less a number of choice colonies with high quality morphological features (Figure 6 A1-A3). These colonies had tight defined edges without breaks or rough edges, a homogenous colored interior, and a flat growth pattern on the plate. The colonies with the best morphological features were marked with a marking objective and observed until the colony filled up approximately 70% of a marking objective. These colonies were then passaged and expanded until a relevant number of colonies and plates were prepared to enter the differentiation process (Figure 6 B1-B3).
Figure 6. iPSC Colony Morphology Comparison. (A1,A2,A3) Early stage EB fibroblast derived induced pluripotent stem cells with promising colony morphology. (B1,B2,B3) Choice induced pluripotent stem cell colonies after selection and expansion exhibiting ideal morphological features. (C1) iPSC colony with irregular shape, rough edges, poor overall colony morphology. (C2) Severely heterogeneous coloration within the colony and rough colony edges, indicative of premature differentiation. (C3) iPSC colony that is in the process of apoptosis, colony edges are compromised.
3.3 Morphological Findings of hESC

As an ideal control for iPSCs undergoing the differentiation process, hESC colonies were selected and differentiated. The hESC colonies selected for differentiation however, must also be morphologically immaculate and selected by the same criteria as the iPSC colonies. Therefore hESC were propagated and only the best colonies were selected for differentiation (Figure 7).
Figure 7. hESC Colony Morphology Comparison. (A1, A2, A3) Selected H9 human embryonic stem cell colonies portraying ideal morphological features. These colonies would be differentiated or further propagated. (B1) hESC colony with tight boarders but a very dark brown/black center, indicative of a necrotic colony. (B2) hESC colony with differentiating edges. (B3) hESC colony with dark brown coloration, and rough edges, indicative of premature differentiation.
3.4 Pluripotency Assessment of iPSC and hESC control

Prior to differentiation the pluripotency of established iPSC colonies had to be fully assessed. To accomplish this, iPSC colonies with healthy morphology were selected and stained for two pluripotency markers Sox2 and Tra-1-60. hESC colonies with similar morphology were also selected to act as a natural control for the pluripotency staining and comparison group for iPSC colonies. The hESC colonies were stained for Sox2, Tra-1-60, c-Myc and Oct3/4. iPSC colonies were not stained for c-Myc and Oct3/4 due to the fact that transduced fibroblasts had already been successfully stained for Oct3/4. Since the lentiviral vector contained a single gene cassette carrying Oct3/4, Klf4, Sox2 and c-Myc it was safely assumed that staining positively for Sox2 and previously for Oct3/4 provided enough evidence that all four early acting genes were upregulated. The iPSC colonies stained positive for the expression for both Sox2 as well as Tra-1-60 indicating that the early acting gene cassette was still actively expressing Sox2, Klf4, c-Myc, Oct3/4 as well as the additional pluripotency marker Tra-1-60 in colony formation (Figure 8). The hESC colonies stained positively for all the pluripotency markers as well, Sox2, Tra-1-60, c-Myc and Oct3/4 (Figure 9). Together these data indicate that the genes expressed in both the iPSC colonies as well as the hESC colonies are identical. This staining also qualified the iPSCs to be considered truly pluripotent.
Figure 8. iPSC Colony Immunocytochemistry for Pluripotency Markers. (A1) DAPI nuclear stain, indicates cell nucleus. Here one can clearly see how many cells comprise a typical colony (A2) Sox2, pluripotency marker and early acting gene contained in the Stanford 4-factor lentiviral vector (A3) Tra-1-60, pluripotency marker (B1) DAPI/Sox2 Overlay (B2) Sox2/Tra-1-60 Overlay (B3) DAPI/Sox2/Tra-1-60 Overlay
Figure 9. Pluripotency Control Immunocytochemistry hESC. (A1) DAPI, nuclear stain (A2) c-Myc, pluripotency marker (A3) Oct3/4, pluripotency marker (B1) DAPI/c-Myc Overlay (B2) DAPI/Oct3/4 Overlay (B3) DAPI/c-Myc/Oct3/4 Overlay (C1) DAPI (C2) Sox2, pluripotency marker (C3) Tra-1-60, pluripotency marker (D1) DAPI/Sox2 Overlay (D2) Sox2/Tra-1-60 Overlay (D3) DAPI/Sox2/Tra-1-60 Overlay.
3.5 Differentiation Control - hESCs

Following the propagation of ideal hESC colonies (Figure 10 A1), the Oro-Protocol was initiated. The ESC colonies were passaged onto matrigel and fed with preconditioned W8 media with added bFGF and retinoic acid to drive the pluripotent colonies into ectoderm lineage differentiation. The ESC colonies began differentiation within the first 24 hours of adhering to the matrigel coated plates. While differentiation was not driven at the same pace for each hESC colony, some colonies took longer to begin differentiation than (Figure 10 A2 A3 B1), all colonies had reached complete differentiation by the 9th day after Matrigel plating (Figure 10 B2). On the 9th day of differentiation, after 4 complete days of FAD media feeding, the colonies still maintained their hESC circular colony form, however, the individual cells were morphologically keratinocytes as opposed to hESC cells. These colonies that presented the fullest differentiation, which were the vast majority, and lacked any pluripotent looking areas were selected for passage and further expanded (Figure 10 B3). The entire plate was covered with 100% confluent cells, the majority of which look morphologically to be keratinocytes, following only two days of expansion. The cells were again lifted and transferred back onto CF1 MEFs (Figure 10 C1) resulting in again a heterogeneous cell population of MEFs and keratinocytes. The keratinocytes expanded rapidly, growing in dense cobble stone like colonies, and were again transitioned to Matrigel. When the selected colonies were initially lifted from the CF1 feeders some MEFs were inevitably lifted as well (Figure 10 C2). This caused some concern, however, under the selective pressure of the defined keratinocyte serum free medium (DKSFM) which suppresses all
other cell types other than keratinocytes, the keratinocytes quickly emerged as the dominant cell type. These particularly pure keratinocytes, lacking any other contaminating looking cells, were expanded with great success in only a matter of a few days (Figure 10 C3). The final keratinocytes were ideal in their morphological features.
Figure 10. hESC Differentiation (A1) Pre-keratinocyte differentiation selection of hESC colonies. All colonies selected for differentiation were of equable quality (A2) 24 hours following hESC colony transfer to Matrigel coated plates, some differentiation is already visible in the lower left colonies, colony in the upper right maintains pluripotent colony morphology. (A3) 48 hours post-ectoderm lineage induction, some colonies have visibly initiated differentiation to a further degree than others. (B1) 72 hours post-ectoderm initiation, all colonies have initiated ectoderm lineage differentiation to varying degrees. (B2) 4 days of FAD media have resulted in complete differentiation of colonies; colonies maintain their hESC colony shape while single cells have distinct keratinocyte morphology. (B3) Confluent keratinocytes on gelatin coated dishes. (C1) Heterogeneous cell population containing CF1 feeders and many keratinocytes; some areas show dense keratinocyte colony expansion. (C2) Keratinocytes colony formation with some collaterally transferred MEFs plated on Matrigel. (C3) Final pure keratinocyte colony, with excellent morphological features (cobble stone-like colony).
3.6 Differentiation of Epidermolysis Bullosa Fibroblast Derived Induced Pluripotent Stem Cells.

The EB fibroblast derived iPSCs underwent the same differentiation sequence protocol as the hESC controls with some minute differences in the protocol to increase the efficiency of the protocol. The iPSCs were first selected for morphology (Figure 11 A1) using the same criterion as the hESC from the iPSCs generated from EB fibroblasts. They were then plated onto Matrigel and ectoderm initiation was progressed through the use of conditioned W8 media supplemented with bFGF and retinoic acid. The initial stages of the differentiation protocol, the first four days, progressed similarly to the hESC control (Figure 11 A2 A3 B1). Following the 4th day of FAD media treatment however the colonies had not differentiated completely (Figure 8 B2). Therefore the FAD treatment lasted an additional day to further drive the differentiation process (Figure 11 B3), the extra 24 hours of FAD media treatment, however, did not result in a complete differentiation. The colonies were carefully lifted and expanded on gelatin with some success, never-the-less some pluripotent looking cells remained despite the fact that DKSFM media was used to feed the cells (Figure 11 C1). The colonies were transferred per Oro-protocol onto MEFs (not shown) and carefully passaged again onto Matrigel covered plates (Figure 11 C2) resulting in a heterogeneous looking population of pluripotent cells and morphologically correct keratinocytes. The colonies with the least pluripotent looking areas were passaged further onto Matrigel with continued use of DKSFM and resulted in a more keratinocyte pure population (Figure 11 C3).
Figure 11. iPSC Differentiation (A1) Pre-differentiation iPSC selection, morphologically correct iPSC colony. (A2) 24 hours post iPSC passage to Matrigel coated plates, colonies begin ectoderm lineage initiation. (A3) 48 hours differentiation, promising ectoderm lineage initiation with a pluripotent region of the colony remaining in the center. (B1) 72 hours following ectoderm lineage initiation, heterogeneous colony with some pluripotent regions remaining. (B2) FAD media treatment day 4, colony not entirely differentiated, some pluripotent cells remain. (B3) FAD media treatment day 5, pluripotent areas continue to persist in the colony, incomplete differentiation. (C1) Keratinocyte expansion with some heterogeneity on gelatin using DKSFM driving selection. (C2) Matrigel expansion of keratinocytes, portions of colony expansion remain pluripotent despite DKSFM selection. (C3) Further selective transfers of promising colonies leads to a more pure keratinocyte population with morphologically correct keratinocytes.
3.7 Assessment of Differentiation Assay for hESC control

The assessment of the differentiation assay for hESC was particularly important. As the vanguard of the differentiation process and basis upon which the rest of the iPSC differentiation assays were to be based upon it was vital to obtain definitive results indicating keratinocyte differentiation. Should the keratinocyte markers prescribed by Stanford, p63, K14, and K18, not have been expressed by the H9 hESCs, the fate of the iPSC differentiation would likely also be failure. The assessment of the control differentiation would also act as a milestone for the project as it would be the proof of concept, that pluripotent stem cells could be differentiated under Good Manufacturing Practice conditions to generate definitive keratinocytes. The stains were performed following standard procedures and the results indicated that all three definitive keratinocyte makers were expressed following the differentiation assay (Figure 12). With positive results obtained from all three hESC control differentiations, the experiment could progress to the iPSC differentiation phase.
Figure 12. hESC Derived Keratinocytes Immunocytochemistry. (A1) DAPI. (A2) p63, keratinocyte marker (A3) DAPI/p63 Overlay. (B1) DAPI (B2) K18, keratinocyte marker (B3) DAPI/K18 Overlay (C1) DAPI (C2) K14, keratinocyte marker (C3) DAPI/K14 Overlay
3.8 Assessment of Differentiation of EB fibroblast derived iPSC

After the fifth day of FAD media treatment of the EB fibroblast derived iPSC colonies initial stains were conducted to establish the identity of the cells that maintained the morphological features of the pluripotent colony as well as to determine if pluripotent cells had remained in the differentiated outskirts of the original colony. The wells were stained for two pluripotency factors Sox2 and Oct3/4. It was determined that the centers of the colonies were as suspected positive for both pluripotency markers (Figure 13 B2 D2). The outermost regions of the colonies, which had acquired early keratinocyte morphology, did not stain positive for either pluripotency markers (Figure 13 B1-2 D1-2).
Figure 13. iPSC Early Differentiation Pluripotency Immunocytochemistry (A1) Phase (A2) DAPI, portrays cells outside of the pluripotent region (B1) Oct3/4 pluripotency marker labeling an undifferentiated region of the colony (B2) DAPI/Oct3/4/Phase Overlay, edges of the undifferentiated colony are clearly visible with DAPI labeling cells that are not expressing the Oct3/4 pluripotency marker undergoing keratinocyte
differentiation (C1) Phase (C2) DAPI (D1) Sox2, pluripotency marker (D2) DAPI/Sox2/Phase Overlay, characterizes the colony similarly to the DAPI/Oct3/4/Phase Overlay, a pluripotent receding center with defined edges is flanked by DAP labeled differentiating cells lacking pluripotency markers.
iPSC derived keratinocytes were stained following the purification of the cell population. The immunostains were used to determine if the cells at the final stage were definitively keratinocyte in nature. The definitive keratinocyte markers were characterized as per Stanfords request, namely p63, K18, and K14. As is observed in Figure 14, all the keratinocyte markers were positively characterized lending substantial evidence that the differentiation of EB fibroblast derived iPSC could result in a keratinocyte population. This along with the morphological characterization of iPSC derived keratinocytes provides evidence of a successful differentiation process achieved not only in the hESC control group but also the EB patient cell group.
Figure 14. iPSC Derived Keratinocytes Immunocytochemistry (A1) DAPI, nuclear stain (A2) p63, keratinocyte marker (A3) DAPI/p63 Overlay (B1) DAPI (B2) K14, keratinocyte marker (B3) DAPI/K14 Overlay (C1) DAPI (C2) K18, keratinocyte marker (C3) DAPI/K18 Overlay
4. DISCUSSION AND CONCLUSIONS

It has been the objective of this work to substantiate the preliminary findings of Stanford University regarding the potential therapeutic application of epidermolysis bullosa fibroblast-derived induced pluripotent stem cells for the treatment of RDEB and to further the development of a clinical grade final product for the treatment of patients with RDEB. For this purpose, the described study has adjusted the initial research protocols written by Stanford University in such a way that they can be used to generate Standard Operating Procedures enabling Good Manufacturing Practice manufacturing of a clinical grade product for patient treatment. The Stanford / Oro lab-protocol for keratinocyte differentiation had initially been conceived in the context of H9 hESC differentiation however, in this study modifications were made improving upon the original protocols and achieving notable efficacy and specificity in differentiating iPSCs into keratinocytes. Such successful differentiation had not been achieved previously, and particularly not under Good Manufacturing Practice and Good Laboratory Practice conditions. Therefore the results of this study not only satisfy the obligations of this CIRM funded project but more importantly, will bring a pluripotent stem cell based therapeutic closer to the clinical application of treating a patient suffering from this terrible disease.

In this study, the Stanford 4-factor lentiviral vector was proven to be capable of reprogramming both wild type healthy fibroblasts as well as the EB patient fibroblasts into induced pluripotent stem cells. It should be noted that the iPSCs generated were
manufactured, under Good Manufacturing Practice conditions, adhering to the same
guidelines and procedures as required by the FDA for clinical grade product
manufacturing. In addition, many techniques for iPSC expansion, passaging,
cryopreservation and differentiation under Good Manufacturing Practice conditions were
pioneered in this project by the author, as this is the first iPSC derived product being
developed with the intent to file an Investigational New Drug (IND) Application with the
FDA.

The project demonstrated that the first step in the Stanford University / UC Davis
plan, to generate clinical grade iPSCs from patients with RDEB can be achieved as such has
been accomplished in the course of this project. While the efficacy of transduction is as of yet relatively low, as can be seen in both the wild type fibroblast and EB fibroblast
transductions, it is nevertheless adequate and will be subject to improvement in future
optimization studies. Currently, at the UC Davis Institute for Regenerative Cures a new
attempt is already underway, jointly conducted by the Good Manufacturing Practice
Facility and the Stem Cell Program Vector Core to produce a new batch of clinical grade
high titer Stanford lentiviral reprogramming vector with the goal of increasing the
transduction and therefore reprogramming efficiency. This will lead to further attempts in
generating high quality, morphologically and cytogenetically correct iPSCs from EB
patient fibroblasts. As the yield of iPSC generation is anticipated to increase with the
refinement of the Good Manufacturing Practice protocols, the Good Manufacturing
Practice facility will be able to increase iPSC production, feasibly to a point where
clinically relevant cell numbers can be produced for each individual patient within a
reasonable timeframe. This again brings stem cell based, personalized medicine closer to
the person who needs it, and particularly to patients suffering from an orphan disease.

As our commitment at the UC Davis Institute for Regenerative Cures to finding
effective therapies for patients in need is truly very high, we are additionally constantly
attempting to develop new and improved techniques to achieve this goal. In addition to
conducting the experiments requested by Stanford University, my colleagues and I at the
UCD Good Manufacturing Practice facility have taken it upon ourselves to also reach
such goals through our own research. Most recently, we have acquired one of the latest
technologies available to generate integration free iPSCs, a micro RNA cell
reprogramming strategy, and we are in the process of attempting to generate integration
free iPSCs, which can be immediately applied to the RDEB project. The obvious
advantage would be that no reprogramming vector excision would be necessary and the
generated iPSCs would pass the scrutiny of regulatory agencies including the FDA more
easily. Through high motivation, persistence, and an affinity for innovation we and our
fellow scientists will move the field of iPSC derived therapeutics forward, as many other
diseases dependent on tissue regeneration will be able to benefit from the new methods
developed in our facility.

Aside from the generation of iPSCs, extensive improvement was made on the
keratinocyte differentiation protocol. This differentiation protocol has been known as one
of the most difficult steps in the entire scope of the project. Before the Stanford / Oro lab
protocol reached the UCD- Good Manufacturing Practice Facility it had been developed
for H9 hESC differentiation only. Through this project, our goal was in part to adapt the protocol to iPSC differentiation, which in theory should function equally, but had not yet been successfully demonstrated at Stanford University laboratories. In order to better understand the differentiation process prior to the iPSC differentiation attempts and to perform it in a reproducible, Good Manufacturing Practice compliant manner, several experimental runs were conducted using large quantities of H9 hESCs. Initially, the differentiation protocol was not easy to follow, written in a very limited manner for a specialized laboratory dedicated to the field of keratinocyte research and therefore lacked comprehensive description of many nuances that would make following the protocol more efficient. Examples of such included a lack of methodology which thus had to be developed by the author for the preparation of Matrigel for the specific purpose of pluripotent cell differentiation to the keratinocyte phenotype. Through experimental trial and error, as well as copious hours of literature researching, the protocol was substantially rewritten by the author of the present report to meet the standards of the Good Manufacturing Practice facility. It is now a comprehensive protocol and Standard Operating Procedure that can easily be followed and reproduced by any personnel in the Good Manufacturing Practice laboratory or elsewhere, which is of fundamental importance to prudent scientific research.

While the re-written Stanford / Oro lab differentiation protocol ran smoothly and efficiently according to plan using H9 hESCs, it did not immediately translate into a functional protocol for the differentiation of iPSCs into keratinocytes, an unfortunate phenomenon which had also been observed previously in the Oro lab. The iPSC
differentiation culture was much more technically challenging and required a good amount of troubleshooting and fine tuning. The first problem with the iPSC differentiation process emerged directly after the media change from CF1 conditioned W8 media supplemented with ½ bFGF and retinoic acid to FAD media. Already in the early stages of the shift from the ectoderm lineage initiation to the keratinocyte differentiation process, it had become abundantly clear that the iPSC colonies were, for reasons still unknown, not readily differentiating at the same pace as the H9 hESCs. This same issue presented itself consistently at the same differentiation point from plate-to-plate as well as when the entire differentiation assay was repeated conducted. Great care was taken to ensure that all the conditions were identical in the iPSC differentiation as in the H9 hESC differentiation process. For example, the same stock solutions of key reagents such as BMP4 and retinoic acid were used in both differentiation assays. The dilution of Matrigel was kept identical, which is important as the area of concern with Matrigel is a possible alteration of the differentiation potential of pluripotent cells if it is not applied in a highly consistent fashion. Without a better option immediately available, it was reasoned that the time iPSCs get exposed to the FAD media should be increased by 25%. The time increase in the keratinocyte differentiation phase did have the desired effect on the iPSC colonies, which further differentiated, however they did not differentiate to completion as the H9 hESC colonies did. As already mentioned, other key concerns were the BMP4 and retinoic acid concentrations, which are key reagents in driving the differentiation process. In future attempts the effects of increased BMP4 and retinoic acid concentrations will be tested. Another possibility for the difficulties in the
differentiation of the iPSC into keratinocytes may lay in that we were working with iPSC that did not have the 4-factor pluripotency integrated vector (lentiviral reprogramming vector) excised. It is probable that following vector excision using the Cre-Lox system, that the differentiation assays into keratinocytes will be more efficient, and that the differentiation will likely be on pace with the hESC controls with the vector-excised-iPSC fully differentiating to completion.

Despite initial incomplete differentiation of the iPSC colonies to definitive keratinocytes, it could be demonstrated that the differentiation process was dramatically improved with additional manual selection steps. At the end of the FAD media phase, only the most promising areas of the cultures with visible, almost complete differentiation were selected for manual passage rather than the entire plate, following the technique that had been applied to H9 hESCs. The areas that were selected were those that did not have the typical iPSC coloration and had characteristic keratinocyte morphology. This method of manual selection is physically straining as it takes at least an hour under the dissection microscope to select the perfect regions to passage from a single 6-well plate. As a proof of principle experiment, or to manufacture a clinical grade product for a single patient, this method is feasible. However, should this process be required for cell lines to be generated for every patient to be treated, such manual selection and passaging would no longer be a manageable task. It should also be noted that the skill level in recognizing the correct morphology of keratinocytes would require special training of every technician working on such manual dissections, and these technicians would have to become specialists in recognizing the various stages of
keratinocyte differentiation. For Good Manufacturing Practice manufacturing purposes, these technicians would have to undergo proficiency testing on this subject. This is therefore an issue that must be addressed in the future if the process of generating keratinocytes from iPSCs should be industrialized by a company wishing to treat EB patients.

Two fully funded years remain in this CIRM funded study and already many of the goals have been accomplished. Of the overall objectives, only two steps have yet to be fulfilled under Good Manufacturing Practice conditions: Lentiviral reprogramming vector excision and homologous recombination for the COL7A1 gene. Although the lentiviral vector excision has not yet been performed under Good Manufacturing Practice conditions, this step is not anticipated to pose a great challenge to the laboratory due to the fact that the LoxP/Cre recombinase excision system is well studied and has previously been tested and successfully accomplished at the collaborating Stanford University laboratory several times, using the identical vector. Established protocols can therefore be followed and adapted to Good Manufacturing Practice conditions. The second, more challenging objective, homologous recombination must still be performed under Good Manufacturing Practice conditions. In order to truly correct the EB phenotype, mutant COL7A1 (the gene coding for collagen type VII), must be removed and replaced with a functional version. The current methodology prescribed by Stanford in accomplishing this step in the EB gene therapy process is to use a targeting DNA plasmid that, during cell division will replace the mutant COL7A1 gene. This has also been accomplished, although with low efficiency by the Stanford group and is still a
subject of research at their campus for the purpose of increasing recombination efficacy. As soon as the Stanford team has definitively determined the method of gene correction to be used for the clinical application, this method will then be transferred to the Good Manufacturing Practice facility for adaptation and further testing.

During the short time that the Stanford/UCD Good Manufacturing Practice team have been working on a novel therapeautic for RDEB much progress has been made. The remaining goals for the clinical grade product are at this point only issues of optimization. Currently, talks are already being held how to best submit an investigational new drug (IND) application which would be a major step forward for the field of regenerative medicine, as this would be the first IND application filed for an iPSC derived clinical grade product. With dedication, compassion, and the willingness not to shy away from problems, but working to solve them, this project will no doubt become successful and will importantly increase the quality of life of individuals with RDEB that have currently no hope for any working treatment.
LITERATURE CITED


http://www.fda.gov/AboutFDA/WhatWeDo/History/ProductRegulation/100YearsofBiologicsRegulation/ucm070022.htm


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