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AN INVESTIGATION OF EPIDERMOLYSIS BULLOSA AND AN ANALYSIS OF RESEARCH AND TREATMENT

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Abstract of the Thesis

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The dysfunction of genes encoding integral proteins of the extracellular matrix and the basement membrane zone create foundations for the disease epidermolysis bullosa. Various proteins reside in the basement membrane zone and are responsible for the strength of the epidermal tissue. The cure for this disease is elusive which poses a problem for modern medicine. In this analysis, the mechanisms of epidermolysis bullosa subtypes were investigated and the viability of future treatment protocols was assessed. The promising treatment options include stem cell, gene, fibroblast, and protein therapy. Strategies need to be investigated further in order to develop therapeutic cures which will improve patient survival rates.

Dedication Page

This thesis is proudly dedicated to my loving friends and family. I have been blessed with great people in my life who have always encouraged me to pursue my dreams. My parents have given me great support throughout my academic career and without them I wouldn't be where I am today. Thank you all for your advice, sacrifice, prayers, and love!

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List of Abbreviations

3D	3-dimensonal
AP	Alkaline Phosphatase
BMZ	Basement membrane zone
COLVII	Type VII collagen
COLXVII	Type XVII collagen
cDNA	Complementary deoxyribonucleic acid
c-myc	c-myc proto-oncogene
COL7A1	Gene encoding type VII collagen
COL17A1	Gene encoding type XVII collagen
DEB	Dystrophic epidermolysis bullosa
DNA	Deoxyribonucleic acid
DST	Gene encoding dystonin
EB	Epidermolysis bullosa
EBS	Epidermolysis bullosa simplex
ECM	Extracellular matrix
FERMT1	Gene encoding Kindlin-1
HB-EGF	Heparin-binding EGF-like growth factor
HCT	Hematopoietic cell transplantation
HPC	Hematopoietic progenitor cell
iPSC	Induced pluripotent stem cell
JEB	Junctional epidermolysis bullosa
JEB-H	Herlitz junctional epidermolysis bullosa
JEB-nH	Non- herlitz junctional epidermolysis bullosa
K5	Keratin 5 protein
K14	Keratin 14 protein
Klf4	Kruppel-like factor 4
KRT5	Gene encoding keratin 5 protein
KRT14	Gene encoding keratin 14 protein
KS	Kindler syndrome
LAMA3	Gene encoding α3 subunit of laminin-332

LAMB3	Gene encoding β3 subunit of laminin-332
LAMC2	Gene encoding γ2 subunit of laminin-332
mRNA	Messenger RNA
MEF	Mouse epidermal fibroblasts
MMC	Mitomycin C
MSC	Mesenchymal stem cell
Oct4	Octamer-binding transcription factor 4
PCR	Polymerase chain reaction
PLEC	Gene encoding plectin
RDEB	Recessive dystrophic epidermolysis bullosa
RDEB-HS	Recessive dystrophic epidermolysis bullosa, Hallopeau-Siemens type
RNA	Ribonucleic acid
Sox2	(sex determining region Y)-box 2
WT	Wild type

1 General Introduction

Epidermolysis bullosa (EB) is a group of rare, inherited genodermatoses characterized by recurring blisters and erosions of the patient's epithelial tissue. These wounds are caused by mechanical stress, minor friction, and trauma (Soro, Bartus, & Purcell, 2015). In patients blisters present on the skin and mucous membranes, creating problems within the respiratory system, digestive system, and urinary tract (Laimer, Prodinger, & Bauer, 2015). Lesions form on the esophagus and cause pain when the patient eats, while wounds on the respiratory system make it difficult to breath. These patients suffer immense pain, life-shorting complications, and face premature death. In the United States, it was reported that the overall incidence of the disease is 19 per 1,000,000 live births, while the prevalence of the disease is 8 per 1,000,000 live births (Fine, 2010).

There is no cure for this disease and current treatment focuses on reducing trauma to the skin. This is done by giving the patients cold compresses, protective dressings, and corticosteroids (Reddy & Wong, 1972). A variety of complications arise in other epithelialized and mesenchymal organs, including secondary disease phenotypes, such as nail dystrophy, pseudosyndactyly, and pigmentary disturbances. In more severe subtypes the patients experience a combination of malnutrition, failure to thrive, anemia, infection, organ defects. Scar tissue can develop in epithelial tissue when blisters begin to heal. Mild phenotypes are accompanied by localized blisters on the hands and feet, and clinically manifest in late childhood or adolescence (Laimer, Prodinger, & Bauer, 2015). The same mutated gene can result in different phenotypes in patients, adding to disease complexity (Bruckner-Tuderman & Has, 2014). Microscopy has elucidated the pathophysiology of the disease in the past, although the mechanisms involving this disease are not completely understood (Reddy & Wong, 1972). Animal and *in vivo* models have

helped researchers understand the underlying molecular mechanisms of the disease (Chamcheu et al., 2012). EB research in the past twenty years has contributed to increased knowledge about the functional significance of proteins within the dermal-epidermal adhesion networks (Bruckner-Tuderman & Has, 2014).

Therapy approaches and protocols under development may provide hope of curative treatments in the future (Soro, Bartus, & Purcell, 2015). Stem cells are being explored in a variety of therapies for EB with research involved in mesenchymal stem cells, induced pluripotent stem cells, and hematopoietic progenitor cells. The intent for stem cell therapy is to restore the missing protein from epithelial tissue. Stem cells are able to self-renew, generate differentiated cells, and provide proper tissue maintenance. Induced pluripotent stem cells (iPSC) are generated by introducing a specific set of genes to create reprogrammed adult cells. Mesenchymal stem cells (MSCs) are multipotent stromal cells which can differentiate into several cell types, such as epidermal cells. Researchers have used MSCs in an attempt to stimulate a regenerative response in patients with EB. Hematopoietic progenitor cells (HPCs) are collected from bone marrow, peripheral blood, and umbilical cord blood. HPCs are used in hematopoietic cell transplantation and undergo hematopoiesis; a process which creates new blood cells (Lane, Williams, & Watt, 2014).

Protein, gene, and fibroblast cell therapies have also been researched in EB subtypes. Protein therapy involves techniques which replace deficient protein in the epidermal tissue of patients (Remington et al., 2009). In gene therapy, a patient's epidermal cells are collected and injected with viral vectors. These vectors integrate into the genome and replace the nonfunctional gene. With this technique, the skin cells will contain a functional gene which can grafted back onto the patient (Mavillo et al., 2006). Fibroblast cells synthesize extracellular matrix and

collagen. These cells are an important for proper wound healing and maintenance of epidermal tissue integrity. Fibroblasts have been injected near blisters in an attempt to restore the missing epidermal proteins (Wong et al., 2008).

This thesis provides an analysis of the disease EB and developing treatments. Establishing an effective therapy will be possible once the molecular mechanisms and pathogenesis of the disease are better understood.

1.1 Disease subtypes

There are four major subtypes of EB; epidermolysis bullosa simplex (EBS), junctional epidermolysis bullosa (JEB), dystrophic epidermolysis bullosa (DEB), and Kindler Syndrome (KS) (Table 1). These subtypes differ by both gene mutated, and by phenotype (Fine et al., 2008).

Blistering in EBS patients occurs within the epidermis (Soro, Bartus, & Purcell, 2015). In severe EBS, also known as Dowling-Meara, blisters form on the skin and mucous membrane but improve with age. Localized EBS, also known as Weber-Cockayne, is a mild version of EBS with blistering manifesting in late childhood through adulthood. The clinical signs of localized EBS are localized blisters and hardening of the hands and feet. In generalized EBS patients present with mild blisters at birth. EBS is responsible for about 75% of disease cases and is commonly associated with dominantly inherited mutations in the genes keratin 5 (*KRT5*) or keratin 14 (*KRT14)*, encoding the proteins keratin 5 (K5) and keratin 14 (K14), respectively (Laimer, Prodinger, & Bauer, 2015). These fibrous proteins are needed for the cytoskeletal formation of basal keratinocytes, which are necessary for healthy skin function. Keratinocytes

are cells which reside in the epidermal tissue and produce keratin proteins. Without the presence of K5 or K14, strong keratin networks will not form (Gostyńska, et al., 2015).

Mutations in the plectin gene (*PLEC*) and dystonin gene (*DST*) have also been found to cause EBS. These genes encode the proteins plectin and dystonin, respectively (Gostyńska, K. et al., 2015). These proteins both bind to keratin in keratinocytes. Dystonin is a cytoskeletal linker, and anchors intermediate filaments to hemidesmosomes in epithelial cells (Bruckner-Tuderman & Has, 2013). Plectin is a main component of hemidesmosomes and functions to provide proper skin adhesion, tissue strength, and mechanical stress resistance. Mutations in *PLEC* result in EBS with muscular dystrophy, pyloric atresia, or cardiomyopathy. However, the complete plectin involvement in EBS is not known (Gostyńska et al., 2015).

Junctional epidermolysis bullosa is an autosomal recessive disease. This subtype is caused by mutations in the *LAMA3*, *LAMB3, LAMC2*, and *COL17A1* genes. A defining feature of JEB is blister formation in the lamina lucida; a component of the basement membrane zone (Soro, Bartus, & Purcell, 2015). JEB is further divided in to main groups; Herlitz (JEB-H), and non-Herlitz (JEB-nH). The symptoms of JEB-H are blistering at birth with blisters present in the skin, mouth, and digestive tract. The presence of blisters in both the mouth and digestive tract cause pain while eating. Patients with this form of JEB tend to be malnourished, experience slow growth, have nail deformities, and deformed enamel. Granule formation occurs in the respiratory system and causes pain with each breath. Further complications with this subtype include hair loss and fusion of the fingers and toes. Recurring blisters form in the eye and result in visual impairment. JEB-nH is a milder version of JEB, with blister formation limited to the mechanical joints of hands, feet, knees and, elbows. In JEB-nH no scarring or granulation occurs in patients (Fine & Mellerio, 2009).

Dystrophic epidermolysis bullosa is caused by mutations in the type VII collagen gene (*COL7A1*). The protein encoded by the *COL7A1* gene, type VII collagen (COLVII), is a component of anchoring fibrils found at the epidermal-dermal junction. These fibrils anchor the dermis to the epidermis, and are transcribed in both keratinocytes and fibroblasts (Soro, Bartus, & Purcell, 2015). In DEB there is either an insufficient amount, or absence of, COLVII at the epidermal-dermal junction. This absence or truncation causes the skin layers to separate (Nevala-Plagemann, Lee & Tolar, 2015). There are two main forms of recessive dystrophic epidermolysis bullosa (RDEB); severe and intermediate.

In generalized severe recessive DEB there are two premature stop codons within the gene. Truncated COLVII results in the absence of anchoring fibrils (Soro, Bartus, & Purcell, 2015). Clinical signs of this subtype include epithelial blistering, scarring, nail dystrophy, malnutrition, and fusion of the toes and fingers. Vision loss has been reported on patients due to eye inflammation, blistering, and scarring (Fine & Mellerio, 2009). Epidermal tissue scarring leads to an increased risk of developing squamous cell carcinoma. Generalized intermediate recessive DEB is a milder form of RDEB (Laimer, Prodinger, & Bauer, 2015). In this subtype mutations in *COL7A1* create abnormal, but partially functional protein (Soro, Bartus, & Purcell, 2015). There is scarring in the limited area where blisters form. Nail and teeth deformities can occur along with an increased risk of squamous cell carcinoma (Laimer, Prodinger, & Bauer, 2015).

In some cases, DEB has an autosomal dominant mode of inheritence. The clinical signs for generalized dominant DEB include mild blisters, scarring, fingernail loss, and deformed fingers and toes (Nevala-Plagemann, Lee & Tolar, 2015). RDEB patients have a higher risk of

cancer with most patients developing squamous cell carcinoma by the time they turn 40 years old (Fine & Mellerio, 2009).

The fourth major subtype of EB is Kindler Syndrome (KS). This subtype is caused by mutations in the gene *FERMT1* which encodes the protein kindlin-1. Clinical signs of KS include skin blistering, pigment anomalies, skin atrophy, scarring, and photosensitivity (Kiritsi et al., 2012). Other manifestations include dental abnormalities such as gingivitis and periodontitis. Patients have an increased risk of nonmelanocytic skin tumors (Laimer, Prodinger, & Bauer, 2015). Kindlin-1 is involved in integrin activation and cell adhesion (Kiritsi et al., 2012). This protein influences keratinocytes to proliferate and to adhere to both fibronectin and laminin. Keratinocytes are also responsible for normal migration to epithelial wounds in the skin (Laimer, Prodinger, & Bauer, 2015).

1.2 Disease pathogenesis

The human skin consists of two main layers; the epidermis and dermis. Extracellular matrix (ECM) and basement membrane (BMZ) proteins link these layers together, resulting in healthy, elastic, and functional skin. In EB the genes which encode necessary proteins to hold skin layers together are mutated. Complete protein loss results in severe phenotypes, while truncated proteins are known to cause milder forms of the disease (Nevala-Plagemann, Lee, & Tolar 2015).

Mutations have been described in 18 genes encoding for keratin filament components, disturbing the anchoring fibrils of the skin and mucous membranes (Laimer, Prodinger, & Bauer, 2015). The BMZ regulates the stratified squamous epithelia in epithelial tissue.

Hemidesmosomes attach cells to the ECM and are located on the inner basal surface of keratinocytes. This ECM attachment occurs through intracellular and extracellular proteins.

Dystonin and plectin are the intercellular proteins which interact with keratin intermediate filaments. In addition, plectin serves as a link between the actin microfilaments, microtubules, and intermediate filaments of the cytoskeleton. Connections made by plectin provide mechanical maintenance and tissue elasticity. The two transmembrane proteins of hemidesmosomes are COLXVII and Very Late Antigen-4 (α6β4-integrin) (Bruckner-Tuderman & Has, 2014). The α6β4-integrin binds to laminin-322 extracellularly. The β4-subunit of α6β4-integrin binds to plectin and assist in the formation of the epithelial cytoskeleton (Seltmann et al., 2015). COLXVII interacts with three proteins; plectin, dystonin, and the β4-subunit. Anchoring fibrils are important for hemidesmosome stability (Bruckner-Tuderman & Has, 2014). As stated previously, COLXVII mutations result in JEB, while plectin mutations are associated with EBS.

The genes *LAMA3*, *LAMB3*, and *LAMC2* encode the α3, β3, and γ2 subunits of laminin-332, respectively (Floeth & Bruckner-Tuderman, 1999). Laminin-332 mediates an extracellular matrix (ECM)-epithelial interaction by assisting with the attachment of the epidermis to the underlying layers of the skin. The skin is fragile without laminin-332 (Tsuruta et al., 2008). The gene *COL17A1* encodes the assembly of type XVII collagen (COLXVII). This protein provides structural support to connective tissue and assists with epidermal attachment. Without COLXVII, the skin is not protected against mild trauma (Laimer, Prodinger, & Bauer, 2015). Mutations involving JEB are being actively studied (Väisänen et al., 2005).

Anchoring fibrils pass through the lamina lucida, a component of the BMZ, and consist of the proteins laminin-322 and COLXVII. These anchoring fibrils emerge from the plasma

membrane and end in the lamina densa; another component of the BMZ which lies above the dermis. Anchoring fibrils from the lamina densa extend into the dermal ECM and are composed COLVII. These fibrils further attach to the basement membrane, connecting the dermis to the basement membrane. Laminin-322 and type IV collagen secure the anchoring fibrils, composed of COLVII to the basement membrane. Type I collagen then attaches this complex to the dermal fibrils (Bruckner-Tuderman & Has, 2014).

Kindlin-1, encoded by the *FERMT1* gene, is a fundamental protein participating in adhesion contacts in basal keratinocytes, periodontal tissue, and the colon. Loss of function mutations in the *FERMT1* gene impairs anchorage of the actin cytoskeleton with the ECM (Laimer, Prodinger, & Bauer, 2015). Understanding the complete molecular mechanism of epithelial attachment will enable the development of valid therapies to fight against fragility, scarring, and therefore cancer (Fritsch et al., 2008).

1.3 Mortality rates

With no data for calculating the risk for specific causes of death, Fine et al., 2008 published information about the cause-specific risk of death for the first 15 years of life in EB patients. Inherited EB patients die prematurely with death usually occurring during childhood. Patients with JEB subtypes have a higher risk of childhood death. It was estimated that 10-44.7% of children with this subtype die within their first year, usually due to failure to thrive. By age 15 in two different forms of JEB, the risk of death increased to 61.6% in JEB-H, and 48.2% in JEB-nH. In both JEB subtypes, it was found that 14% of patients passed away from respiratory failure. In RDEB-HS patients, squamous cell carcinoma is the major cause of death in patients,

along with a 12.3 % risk patients with RDEB-HS pass away from renal failure by the age of 35 (Fine et al., 2008).

Fine et al., 2008 published that there is a lack of understanding about this disease in non-dermatologist physicians. It is necessary to have accurate EB data and subtype descriptions available for doctors to correctly diagnosis and treat the disease. Proper wound care will prevent infections and delay premature death, while advancements in synthetic wound dressings and daily care will improve the quality of life of EB patients. This progress has decreased the mortality rate in non JEB patients (Fine et al., 2008).

Loh et al., 2014 published the EB Disease Activity and Scarring Index because there was insufficient data about the disease activity of EB. This index has enabled physicians to assess disease activity and determine whether the symptoms are improving in patients. An accurate scoring system allows clinicians and researchers to evaluate the disease and provide a correct diagnosis to patients (Loh et al., 2014).

2 Experimental approaches

Technological advances have enabled scientists and clinicians to utilize electron microscopy and immunofluorescence mapping to identify the level of blistering, and diagnose EB in patients (Chamcheu et al., 2012). EB research has shifted from solely short-term wound care to developing curative therapies.

Understanding the molecular mechanisms of disease pathogenesis is important for proper development of targeted therapies. Protein and fibroblast curative techniques have been studied in EB research. Mesenchymal stem cells (MSCs), induced pluripotent stem cells (iPSC), and hematopoietic progenitor cells (HPCs) have also been studied in various cell therapy and gene

therapy techniques. These therapies, which involve the use of either allogenic or autologous stem cells, have led to more information about disease subtypes, and potential treatments (Soro, Bartus, & Purcell, 2015). It is important to note that most EB studies focus on the RDEB and JEB subtypes because they can present severely in patients.

2.1 Protein therapy

In a study conducted by Remington et al., 2009, the intradermal injection of recombinant COLVII into RDEB murine models has been found to lead to the formation of anchoring fibrils, and promote adequate wound healing. Murine models were established by targeted inactivation of the *COL7A1* gene. The *COL7A1* null mice lacked COLVII at the BMZ and displayed the severity and clinical phenotype of human RDEB patients. Purified COLVII was injected into the *COL7A1* null mice on the day after their birth. The recombinant COLVII was detected six hours after injection at the epidermal-dermal junction of the BMZ. After, these mice were subjected to weekly injections of recombinant COLVII in order to keep a functional population of COLVII within the BMZ. Immunogold labeling of the mice epidermis displayed both COLVII at the BMZ and anchor fibril formation. However, COLVII was only observed in the skin and not in internal mucous membranes (Remington et al., 2009). Other studies have shown that the intravenous injection of COLVII in mice has led to improved RDEB phenotype. It was observed that COLVII was recruited to wounded skin and repairing the damage, but the mechanisms of this homing is not fully understood (Soro, Bartus, & Purcell, 2015).

2.2 Gene therapy

In RDEB patients, viral vectors have allowed a functional *COL7A1* gene to integrate into the patient's cells. The corrected cells then grow into thin sheets, and grafted onto the patient

(Soro, Bartus, & Purcell, 2015). Mavillo et al., 2006 published work involving successful transplantation of genetically modified stem cells in a 36 year old patient suffering from nonlethal JEB. The patient was deficient in the β3 subunit of laminin-332 protein, which is encoded by the *LAMB3* gene. The JEB patient's epidermal stem cells were transduced with a retroviral vector which expressed *LAMB3* complementary DNA (cDNA). This *LAMB3* cDNA encoded for the β3 subunit of laminin-332. The patient went through nine skin grafts which resulted in the regeneration of genetically corrected epidermis. An immunofluorescence analysis was conducted at 4 months post-procedure and indicated the expression of functional laminin-332. After one year, there was a lack of blistering and immune response, indicating a possible method for EB therapy (Mavillo et al., 2006).

2.3 Bone Marrow stem cell therapy

Mesenchymal stem cells have been reported to encourage wound healing in murine models (Sasaki et al., 2008). Preclinical trials in murine models have been shown to improve symptoms of the disease phenotype in RDEB patients (Kiuru et al., 2010). As a result, MSCs have been noted to influence rapid wound repair in RDEB patients. The use of autologous MSCs as a therapeutic cure in RDEB murine models was studied by Alexeev et al., 2013. MSCs were collected from the bone marrow of newborn mice. The skin was analyzed by immunofluorescence and was observed to contain 15% WT COLVII in epithelial tissue. A mechanical stress test was applied and there was no major separation in the epidermal-dermal junction, leading researchers to believe that the use of MSC from bone marrow contributed to the partial restoration of COLVII in epithelial tissue (Alexeev et al., 2013; Nevala-Plagemann, Lee, & Tolar, 2015).

Cognet et al., 2010 studied the injection of mesenchymal stem cells near chronic ulcerations. The wounds began to heal when COLVII was present in the epidermal-dermal junction. However, after four months blistering and ulceration started up again (Conget et al., 2010; Nevala-Plagemann, Lee, & Tolar, 2015). The safety procedures of bone marrow-MSC therapy need to be improved and further research needs to be conducted to develop optimal therapies (Soro, Bartus, & Purcell, 2015).

Hematopoietic cell transplantation (HCT) is the transplantation of multipotent stem cells. These stem cells can come from three sources; bone marrow, peripheral blood, or umbilical cord blood. HCT has many complications and is preferably used when the patient is up against a life-threatening disease, such as EB. HCT therapies will improve along with progress to placenta based therapies. Umbilical cord blood has advantages over bone marrow, as there is less of a risk of infection from the donor to the recipient (Nevala-Plagemann, Lee, & Tolar 2015).

Wagner et al., 2010 first initiated the clinical trials of allogenic HCT therapy in seven RDEB patients (Nevala-Plagemann, Lee, & Tolar 2015). Patient ages ranged from 15 months to 14 years old. To begin the process, patients were treated with immunomyeloation chemotherapy. Then, the children underwent stem cell transplantation. Wagner et al., 2010 hypothesized that new COLVII would assemble after transplantation, along with WT-COLVII from the allogeneic cells, improving integrity.

The localization of COLVII was determined and specimen samples were collected at 1 mm away from site of erosions. The expression of COLVII was assessed with immunofluorescence staining by staining with anti-COLVII antibodies. Transmission electron microscopy was used to observe anchoring fibers beneath the lamina densa, although they were

thin and lacked cross-banding. Allogeneic bone marrow was seen to partially correct the COLVII deficiency seen in EB, as skin and mucous membrane improved in patients (Wagner et al., 2010).

However, two patients died in this study. One patient died from hemorrhagic cardiomyopathy from the chemotherapy. Due to this death, six patients completed chemotherapy and transplantations. The second death occurred post-transplantation as a result of graft rejection and infections (Wagner et al., 2010).

This study shows that bone marrow has the potential to improve RDEB phenotypes. Wagner et al., 2010 observed an increase of COLVII expression at the basement membrane of RDEB patients followed by allogenic stem cell therapy. However, it is unclear whether WT-COLVII from the allogenic donor, or newly formed COLVII are responsible for the improvement. A better understanding of the physiology of the clinical responses after the transplantation will help identify the stem cells responsible for the improvement. (Wagner et al., 2010).

A protocol for bone marrow transplantation with reduced toxicity was established in a set of experiments conducted by Geyer et al., 2015. In this study, hematopoietic progenitor cells (HPCs) were examined in RDEB patients. Authors hypothesized that RDEB associated wounds will encourage HPCs to differentiate into COLVII-producing keratinocytes. These keratinocytes will restore the function and influence production of anchoring fibrils. In these trials, it was examined whether HPCs can be programmed into the human skin of two RDEB patients. Chemotherapy treatments had reduced toxicity compared to protocol conducted by Wagner et al., 2010 discussed previously. Patient 1 was eighteen months old and had a homozygous mutation

in *COL7A1*. The BMZ of the patient was not expressing COLVII. On day 21 of post-allogenic HPC transplantation there was reduced blister formation. After two months there was increased epithelialization and after three months blister healing was observed. Patient 2 carried a heterozygous mutation in the *COL7A1* gene. Six months after the allogenic HPC transplantation, there were fewer separations between the epidermis and dermis. After eighteen months, there was a decreased amount of visible blisters and lesion depth (Geyer et al., 2015).

Clinical improvements need to be addressed as *COL7A1* mRNA levels did not stabilize. HPC-derived differentiated cells were present for a short amount of time as progenitor cells and were not able to be sustained in host patients. There have been cases, such as described by Wager et al., 2010, which *COL7A1* expression is present in the tissue, but not at the BMZ. Due to associated risks with allogeneic therapies, Geyer et al., 2015 stated that there is an increased research focus on patient-derived iPSCs because they provide an unlimited resource of the patients' own cells (Geyer et al., 2015).

2.4 Fibroblast cell therapy

The use of fibroblasts in cell therapy has led to partial restoration of COLVII in RDEB mouse studies. This exact mechanism of COLVII production is unclear (Soro, Bartus, & Purcell, 2015). In studies conducted by Wong et al., 2008, authors assessed the clinical benefits of intradermal injections of unaffected donor fibroblasts in five RDEB patients, as these WT-fibroblasts produce COLVII. Skin samples were taken from injected sites and biopsies were conducted at two weeks and three months after injections. After three months it was found that patients possessed an increased amount of COLVII at the dermal junction zone. Future studies

will need to examine the longevity and viability of COLVII in RDEB patients under this protocol (Wong et al., 2008).

2.5 Induced pluripotent stem cells in JEB patients

In a set of experiments conducted by Umegaki-Arao et al., 2014, it was observed that iPSCs can be induced from the revertant keratinocytes of JEB patients. Revertant mosaicism (RM) occurs when a mutation is spontaneously corrected in a cell. This phenomenon bypasses the need to artificially correct the gene. RM has been seen to occur in diseases such as EB and has been studied for a possible use in treatment (Lai-Cheong, McGrath, & Uitto, 2011). Umegaki-Arao et al., 2014 hypothesized that keratinocytes from revertant iPSCs would express functional proteins and be similar to the original revertant keratinocytes.

Keratinocytes were isolated from areas of healthy and affected skin of two JEB patients. Affected skin was deficient in COLXVII. Retroviruses were used to transduce cells, and after five days, cells were seeded on Mitomycin C (MMC)-treated mouse embryonic fibroblasts (MEF) in human embryonic stem cell medium. Colonies appeared, and were expanded on the MMC-treated MEF. DNA was extracted from the iPSCs and analyzed. Exons of the *COL17A1* gene were amplified by a polymerase chain reaction (PCR) technique. The PCR products were sequenced and observed on polyacrylamide gel electrophoresis. This enabled researchers to compare the genotypes between the original keratinocytes from the patient's revertant skin and the keratinocytes derived from the iPSCs (Umegaki-Arao et al., 2014).

Cells were then stained with alkaline phosphatase (AP) to allow visualization with confocal microscopy. These iPSC colonies were then subcutaneously injected in mice along with matrigel which resulted in tumors after two months. Tumor formation confirmed the presence of

all three epidermal germ layers formed from the iPSCs colonies, which displayed the pluripotency of the iPSCs. Differentiation occurred by placing iPSCs in retinoic acid and bone morphogenic protein 4. The use of global gene expression profiling enabled the authors to determine that revertant iPSC keratinocytes were similar to the original revertant keratinocytes taken from the patient's healthy skin (Umegaki-Arao et al., 2014).

3D-skin equivalents were used to identify that revertant iPSC keratinocytes expressed COLXVII in the BMZ, as opposed to mutant iPSC keratinocytes derived from affected skin. Keratin markers were confirmed by immunostaining. Revertant iPSCs were grafted on the back of immunocompromised mice and successfully regrew healthy human skin. Umegaki-Arao et al., 2014 were able to derive keratinocytes from the patient's own iPSCs. These researchers state that patient-derived stem cells can provide patients with an unlimited amount of cells for treatment. This therapy does not have an artificial correction phase and is a safer way to correct keratinocytes (Umegaki-Arao et al., 2014).

2.6 Induced pluripotent stem cells in RDEB patients

The use of genetically corrected iPSCs as a cell therapy for RDEB has also been examined under protocol. In experiments conducted by Wenzel et al., 2014, scientists used an iPSC-based cell therapy to genetically correct fibroblasts, which proved to be potentially successful in long term treatments for RDEB patients. Wetzel et al., 2014 developed iPSCs from *COL7A1* mutant mice, which contained 10% of normal COLVII levels. These mice displayed blistering on the skin which resembled the human disease RDEB. Both fibroblasts and keratinocytes secrete COLVII, and these cell types can be derived from iPSCs to restore levels in the RDEB mice (Wetzel et al., 2014).

The *COL7A1* mutant mice displayed RDEB clinical signs, such as skin fragility and nail dystrophy. Authors generated fibroblasts from dermal tail tips from WT mice and *COL7A1* mutant mice, using a loxP-flanked polycistronic lentiviral vector which expressed Oct4, Klf4, Sox2, and c-Myc. These fibroblasts from the *COL7A1* mutant mice were then genetically corrected by Flpe recombinase. An optical luciferase reporter was used to trace clones, and the iPSC lines were tested for integrity. AP staining results were compared between WT iPSCs, *COL7A1* corrected iPSCs, and embryonic stem cells; which were used as a control. The *COL7A1* corrected iPSCs were deemed viable to use in the series of experiments (Wetzel et al., 2014).

COL7A1 gene corrected iPSCs were differentiated into keratinocytes expressing specific markers, as well as terminal markers of stratified epithelial differentiation. The proliferation of the keratinocytes was limited, as they senesced after two to three passages during the *in vivo* experiments (Wetzel et al., 2014). A fibroblast differentiated protocol was then established; iPSCs were treated with retinoic acid for five days and spread on gelatin-coated dishes. Fibroblasts were found to have positive fibroblast markers, expressed COLVII RNA, and secreted soluble COLVII protein similar to WT-COLVII. These fibroblasts also exhibited down regulation of stem cell markers, and were able to be maintained for up to 30 passages (Wetzel et al., 2014).

The fibroblasts were injected into *COL7A1* mutant mice and weren't rejected by the host. They were detected after 16 weeks post-injection. The presence of the corrected fibroblasts did not increase heparin-binding EGF-like growth factor (HB-EGF) expression in the mice. Expression of HB-EGF indicates an immune response, which is usually seen from allogeneic donors. There were no incidences of tumor growths post-procedure, which indicated that the use of iPSCs can potentially provide viable, long lasting treatment for RDEB (Wetzel et al., 2014).

3 General Discussion

The proteins of the epidermal-dermal junction prevent shearing forces from separating the epithelial cells. Mutations in the genes encoding the basement membrane proteins result in EB skin disease subtypes. Protein, fibroblast, gene, and stem cell therapy research will provide more information about the molecular mechanisms which underlie this disease. The evolution and improvement of valid therapies is becoming a major focus of the field. Therapies are continuing onto preclinical trials, while few have gone into clinical trials in gene, protein, and stem cell therapy (Bruckner-Tuderman & Has, 2014).

3.1 Interpretation and Caveats

According to authors Nevala-Plagemann, Lee, & Tolar, 2015, the past 10 years have contributed to therapy advancements in the placenta-based therapy field, contributing to the increased success of RDEB treatment possibilities. Experimenting with the use of HPCs, iPSCs, fibroblasts, and MSCs have contributed to the progress of developing a cure. Umegaki-Arao et al., 2014 was able to utilized patient's revertant keratinocytes into iPSCs, and develop functional keratinocytes expressing COLXVII. This autologous cell therapy protocol will allow for an unlimited resource of cells for the patient.

There is no cure due to a variety of problems and unknowns scientists experience in their research pertaining to EB. Problems with gene therapy lie within the viral vectors. There is a risk of unintended incorporation of the viral vector in the patient's genome, increasing the complexity of this therapy (Bruckner-Tuderman & Has, 2014). There are limitations where the corrected skin graft can be placed. In patients with severe phenotypes the blisters and erosions within internal mucus membranes are difficult to get to and correct (Soro, Bartus, & Purcell, 2015).

In the Umegaki-Arao et al., 2014 experiments, the murine models did not have JEB, rather they exhibited the same collagen mutations seen in JEB patients. The authors still argue that this technique can open doors for the use of autologous iPSCs from RM epidermis. However, this technique falls short in patients who do not have RM epidermis (Umegaki-Arao et al., 2014).

Protein therapy and stem cell therapy have provided information about DEB mechanisms. The gene *COL7A1* provides a useful target to study, as this gene alone causes DEB. COLVII can also be produced by both keratinocytes and fibroblasts. Studies involving allogenic fibroblasts and mesenchymal cells (from bone-marrow) result in COLVII expression which last several months (Bruckner-Tuderman & Has, 2014). In the study conducted by Wagner et al., 2010, the patients were exposed to high risk stem cell therapy resulting in two deaths, although COLVII at the basement membrane zone was discovered post-procedure in surviving patients (Wagner et al., 2010). Factors which determine clinical responses are not fully understood. Future experiments need to be conducted to correctly assess the risk-benefit ratio (Bruckner-Tuderman & Has, 2014).

3.2 Future Direction

Viable animal models will prove useful to address the unknowns and associated risks related to EB, and potential treatment options. Murine models will continue to help scientists understand the mechanisms of disease, blistering, cancers, and abnormal wound healing related to EB. These models have increased our understanding of disease in the past, as well as secondary effects mediated by signaling pathways and other systems that modify disease phenotypes (Fritsch et al., 2008).

Possible directions of future studies include identifying an optimal population of HPCs for therapy purposes and improving HPC homing. A greater understanding of the nature of HPCs in the dermis will only lead to positive RDEB therapies involving HPCs (Geyer et al., 2015).

Without accurate knowledge of exact mechanisms of EB mutations, cures for this disease will remain elusive. Active research is being conducted and treatment options are being studied in both animal models and in the clinical setting. Research will increase the understanding of the disease and lead to the development of efficient patient therapies. Cures and treatment plans within the near future may be primarily based on the individual patient and create personalized therapies incorporating gene, stem cell, and protein based therapies, as the disease and each subtype are fairly complex (Bruckner-Tuderman & Has, 2014). As molecular and clinical understanding of the disease grows, EB classification systems will continue to be updated. The quality of life for these patients will improve with time (Fine et al., 2008).

Tables

Table 1. EB subtypes. This table displays each epidermolysis bullosa subtype (column 1), abbreviation of each subtype (column 2), and the gene mutation responsible for each subtype (column 3).

EB subtype	**Abbreviation**	**Gene Mutations**
Epidermolysis Bullosa Simplex	EBS	*KRT5, KRT14, PLEC, DST*
Junctional Epidermolysis Bullosa	JEB	*LAMA3, LAMB3,LAMC2*, *COL17A1*
Dystrophic Epidermolysis Bullosa	DEB	*COL7A1*
Kindler Syndrome	KS	*FERMT1*

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