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### 14. ABSTRACT
This application addresses the FY11 PRMRP Topic Area, Epidermolysis Bullosa, and proposes to develop stem-cell based therapies for junctional epidermolysis bullosa (JEB), which is one of the most severe forms of epidermolysis bullosa (EB), a group of rare inherited skin blistering diseases. To accomplish this goal, we are proposing to develop stem-cell based therapies for EB using autologous induced pluripotent stem cells (iPSCs) derived from skin cells harvested from the same EB patient. During the second year of funding, we developed a novel integration-free protocol for the reprogramming of human primary fibroblasts and keratinocytes into clinically relevant iPSCs. The efficiency of our method surpasses all previously published reports and results in the generation of stable iPSC lines. The protocol was employed for the reprogramming of human JEB fibroblasts into iPSCs, which now allows us to address the possibility of gene correction via ZFNs in these human JEB iPSCs. Our developed iPSC generation protocol is applicable not only to JEB patients but also to patients with other inherited skin diseases, as well as veterans with chronic wounds. In addition to reprogramming, we have further optimized our method for the differentiation of iPSCs into keratinocytes, thus fulfilling major prerequisites for the successful accomplishment of the proposed study.

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

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Introduction

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

Keywords
- Epidermolysis Bullosa (EB)
- Junctional EB (JEB)
- Induced pluripotent stem cells (iPSC)
- Gene correction

Body

Aim 1: To determine the histocompatibility of iPSC-derived keratinocytes and mesenchymal cells.
Aim 1 has been reported in Dr. Dennis Roop’s report.

Aim 2: To determine the genetic stability of human iPSC generated from keratinocytes obtained from JEB patient biopsies.
In 2011, it was reported that the process of reprogramming into iPSC may introduce somatic mutations into the genome\(^1\). Our close examination of the paper showed that most of the iPSC were reprogrammed using viral vectors, and from cells that may have already contained somatic mutations.

We have completed Tasks 2.1-2.6 in the steps to ensure heterogeneity in the starting population and to rigorously re-evaluate the genetic stability of human iPSC. Tasks 2.1-2.5 have been reported in Dr. Dennis Roop’s report.

**Task 2.6.** Generate 5 independent mesenchymal cell lines from one of the sequenced iPSC lines from each patient and perform total exome sequencing on these lines.
Derivation of mesenchymal stromal cells from iPSC has been a challenge for whole field. Our laboratory (Tolar) has tested multiple approaches and finally has been able to develop a robust and reliable protocol to derive mesenchymal cells from pluripotent cells.

The experimental schema capturing the various cell fate induction pathways is as follows:

**SCHEMA**

As the mesenchymal cells adhere to plastic and display characteristic size (approximately 18-23 μm) and shape (spindle-like) we assessed these readouts first:
Next, we wished to confirm the phenotypical identity of these cells by assessment of expression of surface markers that traditionally define mesenchymal cells. This is shown here:

**P2 FLOW**

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Subcell Name</th>
<th>Count</th>
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</thead>
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</tr>
</tbody>
</table>

Collectively with Dr. Roop’s laboratory, we show that mesenchymal cells can be successfully derived from iPSC, as is evidenced by both morphology and antigen mapping by FACS (shown above).

**Aim 3. To develop methods to increase the homing of iPSC-derived Lin-/PDGFRα+ cells into injured epithelia.**

A report suggested that it might be possible to mobilize BM-derived cells into the circulation by systemically administering recombinant HMGB1, which results in increased homing of Lin-/PDGFRα+ BM cells into injured epithelia².

To confirm these observations, we proposed the following:

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

To this end we first isolated these cells from non-adherent bone marrow cell fraction of C57/Bl6 GFP transgenic mice. We next infused them (dose: 100,000 cells/mouse) intravenously into EB mice on day 1 of life. After 10 weeks we electively sacrificed the mice and harvested tissues. Upon histological examination we observed numerous donor (GFP positive) cells in recipient skin (Legend: Skin section with GFP+ donor cells...
[Of note, the GFP+ donor cells engrafted long term (at least 10 weeks) and in both major layers of skin, epidermis and dermis.]

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

In a similar fashion as shown above in Task 3.1, we have isolated human mesenchymal stem/stromal cells (Lin-, PDGFRα+) and transplanted them into immune-deficient NOD/IL-2Rγc/Rag-/- (NOG) mice. After 1 week we observed donor cells in non-injured skin. Representative section is shown below:
Aim 4. To develop an efficient and safe method for the genetic correction of the defective gene in JEB-specific iPSC.

Based on two reports showed that zinc finger nucleases (ZFNs) can dramatically increase the efficiency of homologous recombination in iPSCs\(^3\),\(^4\), we outlined tasks to confirm these observations and eliminate concerns about off target events.

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

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

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

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

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

To be completed by Dr. Tolar once the genetically corrected mouse JEB cells are received from the Dr. Roop’s laboratory.
Key Research Accomplishments

- Developed a robust and reliable protocol to derive mesenchymal cells from iPSC.
- Established that mouse GFP+ donor cells engrafted long term (at least 10 weeks) and in both major layers of skin, epidermis and dermis, in C57/Bl6 GFP transgenic mice.
- Isolated human mesenchymal stem/stromal cells (Lin-, PDGFRa+) and transplanted them into immune-deficient NOD/IL-2Ryc/Rag/- (NOG) mice. After 1 week donor cells were observed in non-injured skin.

Reportable Outcomes


Conclusion

Severe generalized epidermolysis bullosa (EB) is an extremely painful, quality-of-life destroying incurable inherited skin blistering disorder. Although systemic therapy in the form of allogeneic bone marrow transplant has shown beneficial effects with recessive dystrophic epidermolysis bullosa (RDEB), this therapy has not proven as effective for patients with one of the most severe forms of EB, Junctional EB (JEB). To accomplish the main goal of the study, we proposed to develop a genome editing strategy for JEB patient-specific iPSCs using ZFN-induced homologous recombination, which is then followed by the differentiation of genetically corrected iPSC into keratinocytes and mesenchymal cells suitable for autologous transplantation. We proposed to employ both the mouse model for JEB to address the immunogenicity of iPSCs-based therapy, as well as actual human samples to move the study closer to the clinical trial.

The key accomplishments are induction of mesenchymal stromal/stem cells from of induced pluripotent stem cell, and skin engraftment of mesenchymal stromal/stem cells derived from mouse and human.

The steps accomplished during this report period are critical to address the next steps of our proposal and eventually develop a genome editing therapeutic strategy for JEB patients.
References


Advances in understanding and treating dystrophic epidermolysis bullosa
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Abstract
Epidermolysis bullosa is a group of inherited disorders that can be both systemic and life-threatening. Standard treatments for the most severe forms of this disorder, typically limited to palliative care, are ineffective in reducing the morbidity and mortality due to complications of the disease. Emerging therapies—such as the use of allogeneic cellular therapy, gene therapy, and protein therapy—have all shown promise, but it is likely that several approaches will need to be combined to realize a cure. For recessive dystrophic epidermolysis bullosa, each particular therapeutic approach has added to our understanding of type VII collagen (C7) function and the basic biology surrounding the disease. The efficacy of these therapies and the mechanisms by which they function also give us insight into developing future strategies for treating this and other extracellular matrix disorders.

“The outcome of any serious research can only be to make two questions grow where only one grew before.”
Thorstein Veblen, The Evolution of the Scientific Point of View, 1908.

Born to blister
Recessive dystrophic epidermolysis bullosa (RDEB) is a severe inherited skin disorder characterized by chronic skin blistering, diminished wound healing, joint contractures, esophageal strictures, pseudosyndactyly, corneal abrasions, and a shortened life span [1-3]. Affected individuals suffer intense pain throughout their lives, with few or no effective treatments available to reduce the severity of their symptoms. Along with the life-threatening infectious complications associated with this disorder, many individuals will develop an aggressive form of squamous cell carcinoma [4,5].

RDEB is caused by mutations in COL7A1, the gene that encodes for C7 [6,7]. One of the most severe types of epidermolysis bullosa, RDEB is typically inherited in an autosomal-recessive fashion. It results from transfer of the mutated COL7A1 copies from both parents, who carry the mutation, to the affected offspring [8]. C7 is the main component of anchoring fibrils, structures that attach the dermis to the epidermis at the dermal-epidermal junction [9-11]. The inability of these anchoring fibrils to form and function properly causes the epidermis to not adhere to the underlying dermis [12]. This loss of structural integrity causes the skin to become susceptible to even slight trauma and also hinders the skin from healing productively [13,14]. It is likely that the constant cellular stress from the skin trying to heal itself, along with the resulting chronic inflammation, is the main reason for the increased risk of squamous cell carcinoma in individuals with RDEB [5,15-17].

Owing to its nature and severity, RDEB presents unique challenges for developing successful therapies that simultaneously alleviate the plethora of complications while having a significant impact on survival and quality of life. Recent approaches such as allogeneic cellular therapy, gene therapy, and protein therapy [18-23] show
promise. Beyond the potential translational benefit of these studies, they have also significantly advanced our understanding of the biological properties of skin. Armed with this information and the recent technical advances, we believe the collective ability of multiple teams around the globe to both understand and treat RDEB is approaching a pivotal point in achieving effective, sustainable treatment options.

**Allogeneic cellular therapies: from bench to bedside**

Initial studies using allogeneic cells for the in situ treatment of epidermolysis bullosa included allogeneic fibroblasts [24-27] and mesenchymal stromal cells [28] and gene-corrected autologous epidermal stem cells [29]. These early studies using donor cells for local skin repair were crucial in demonstrating the capacity of allogeneic cells to correct this extracellular matrix disorder, but the benefits were limited to the site of application. Although the pathology of severe generalized RDEB is most apparent in the skin, its effects are numerous and systemic, and any therapy to treat the systemic manifestations requires broad delivery of C7 throughout the body. The prototype of cell therapy for genetic disorders is hematopoietic cell transplantation (HCT), which allows systemic and long-term distribution of donor cells in the recipient [30,31].

There is a growing amount of evidence describing the participation of cells with hematopoietic origin that are responsible for orchestrating and contributing to productive wound healing [32-35]. The process of wound healing in injured skin is complex, and a wide variety of cells from the bone marrow are recruited and participate in regulating inflammation, re-epithelialization, and extracellular matrix production [35].

Initial studies investigating the potential for bone marrow cells to treat extracellular matrix disorders confirmed this potential [36,37]. In a mouse transplantation model of RDEB, purified populations from the wildtype bone marrow were shown to home to injured skin and secrete C7 [37]. In turn, this improved the blistering phenotype and increased survival rates in treated mice. This approach was also shown to be effective in treating other forms of epidermolysis bullosa [38]. These studies provided the proof of principle needed for the first clinical trial using HCT to treat RDEB. The results from the initial patients enrolled in the clinical trial demonstrated the efficacy of HCT’s and also revealed new information about how the bone marrow contributes to wound healing [18]. The patients treated with HCT not only displayed an increase in C7 deposition (Figure 1) but also showed a substantial level of donor chimerism in the skin following transplant. Exactly which cell types are responsible for homing to the skin, producing C7, and contributing to high levels of donor chimerism is still being determined, but several studies of this phenomenon have uncovered potentially relevant mechanisms. For example, a recent study described a particular subset of bone marrow cells expressing the surface marker platelet-derived growth factor receptor alpha that respond to a homing signal in injured skin, high-mobility group box (HMGB1) [39]. This subset was shown to produce C7 in the transplanted mouse model of RDEB. Other studies have demonstrated that certain subsets of bone marrow or cord blood cells were capable of producing C7 and that production increased in the context of wound healing [35,40]. Although it remains to be seen whether this subset of cells can be enriched prior to transplant or whether particular homing signals can be manipulated in order to improve transplant efficacy, these findings improve our understanding of how HCT can treat extracellular matrix disorders [41-43].

**Induced pluripotent stem cells: evidence-based approaches**

Along with HCT, another option for future therapies in RDEB would be the use of cells derived from personalized induced pluripotent stem (iPS) cells [44-46]. In principle, iPS cells offer an inexhaustible supply of cells capable of differentiating into almost all cell types of the body.
They have already been used in ex vivo modelling of many genetic diseases [47-49]. Skin cells isolated from both patients with RDEB and patients who suffer from the closely related disorder, junctional epidermolysis bullosa (JEB), can be reprogrammed into iPS cells that can be used to investigate the mechanisms of mucocutaneous destruction and wound healing in disorders with deficiencies in the protein complexes that support structural integrity of the epidermis and extracellular matrix of the dermis [50,51]. Furthermore, keratinocytes isolated from a healthy patch of skin from a patient with RDEB were reprogrammed into iPS cells [52]. The healthy patch of skin was determined to be a result of somatic mosaicism [53-55], and iPS cells derived from this healthy patch produced functional, biologically relevant levels of C7. These cells, and similar cells derived from mosaic patches in JEB individuals [56-58], represent a serendipitous opportunity for therapeutic use and a spearhead for the future of autologous cellular therapy [59]. RDEB iPS cells can also be differentiated into keratinocytes and fibroblasts, the two cell types that produce C7 in the skin, and can be used to construct full-thickness three-dimensional skin equivalents [60-62]. Fibroblasts, keratinocytes, and skin equivalents produced from iPS cells could be used therapeutically to treat localized, topical wounds. In addition to differentiation into skin cells and reconstruction of epidermis and dermis, recent studies showed that RDEB iPS cells can generate cells with surface markers similar to those expressed by human hematopoietic cells [63]. Intense efforts are under way to derive transplantable human iPS cell-derived hematopoietic stem cells that can be used for HCT [64-70]. These advances, along with the allogeneic HCT being used today, are the first steps needed in developing a more comprehensive therapy for RDEB. Other simultaneous advances in genome engineering should eventually allow a patient’s cells to be gene-corrected and then reprogrammed into iPS cells for use in autologous therapy.

**Gene therapy: both inside and outside of the COL7A1 locus**

Although allogeneic HCT is the most effective and widespread cellular therapy of genetic disorders to date, it requires a human leucocyte antigen-matched donor, and the HCT process itself can be life-threatening [71-75]. Autologous transplant would be a preferred option. Multiple approaches have been used for correcting COL7A1, including retroviral vectors, self-inactivating retroviral or lentiviral vectors, and retroviral vectors encoding a 3’ pre-trans-splicing molecule [19,76-78]. These approaches demonstrated that transduced cells were capable of producing functional and biologically significant levels of C7. Although viral-mediated transgenesis is an efficient way of correcting a genetic defect in patients’ cells [79-83], correcting the endogenous mutation in situ in the genome could offer benefits over the use of viral vectors. Endogenous correction ensures physiological transcriptional control of COL7A1 and expression at biologically appropriate levels and—because the transgene is designed to not integrate in the host genome—reduces off-target, potentially oncogenic events caused by random insertion mutagenesis. Recently, genome-editing strategies using zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have demonstrated the ability to target specific sites in the human genome and correct endogenous mutations [84-87]. TALENs have been used successfully in combination with homology-directed repair to correct the COL7A1 mutation in human fibroblasts from patients with RDEB (Figure 2) [87]. These corrected fibroblasts were capable of producing wildtype C7 with minimal off-target genomic effects. Moreover, these cells could be reprogrammed into...
ips cells and, when xenotransplanted into immunodeficient mice, generated human skin-like structures with apparently normal C7 deposition. These data support the possibility that in situ correction of the COL7A1 locus leads to physiological C7 production and could offer therapeutic benefit to individuals with RDEB. Although TALEN correction appears to be a superior option to previous gene therapy methods, TALEN construction must be tailored to the particular COL7A1 loci that harbor the specific RDEB mutations, which can be both costly and labor-intensive [88]. As there are hundreds of causative mutations for RDEB characterized to date, using this approach on a larger scale may be challenging [89]. With the advent of clustered regulatory interspaced short palindromic repeats and associated proteins (CRISPR/Cas), the ability to correct multiple genetic mutations in human cells might have become considerably easier [90-92], although their off-target profile needs to be carefully analyzed [93].

**Protein therapy: translation of basic scientific insights**

Protein therapy has been used for other inherited disorders of enzyme production due to the inherent capacity of affected cells to take up the missing enzyme [94,95]. Using protein therapy to treat inherited defects of structural protein production has been limited in comparison, but recent studies have demonstrated exciting results, specifically in using C7 protein therapy in pre-clinical models of RDEB [96,97]. Intradermal injections of C7 resulted in the stable incorporation of recombinant C7 into the basement membrane zone and corrected the phenotype in a murine model of RDEB. Intravenous injection of recombinant C7 into RDEB mice resulted in systemic biodistribution and deposition of C7 in wounded skin, but not in unaffected skin sites and internal organs [20]. It is likely that the soluble nature of C7 (unlike other collagens that aggregate and collect in the bloodstream) underlies both the safety and efficacy of systemic C7 infusion [98]. In addition, topical C7 application not only improves the phenotype in an RDEB murine model but may accelerate wound healing in skin that produces functional C7 as well [21]. Thus, the ability of C7 to promote healing in normal skin highlights its importance in coordinating cell migration and extracellular matrix organization in skin repair [13]. The necessary dosing levels and repeated applications of using recombinant C7 for RDEB patients has yet to be determined, although the initial pre-clinical studies have shown promising results with levels that should be attainable for clinical settings.

**Future: finding a cure for the incurable**

The future of medicine, including the quest to decrease suffering in individuals with RDEB, will involve a nuanced understanding of mechanisms underlying patient-specific therapies and combinatorial approaches to achieve the best possible outcomes. Although cellular therapies have been effective in ameliorating the severe generalized phenotype of RDEB, additional modifications, including local application of recombinant homing signals (such as HMGB1) or topical C7 therapy to remaining wounds, will likely complement the use of systemic cellular therapy. Also, inclusion of multiple cell types, such as hematopoietic stem/progenitor cells, mesenchymal stromal cells, fibroblasts, or keratinocytes, alone or after HCT, may speed and enhance wound healing. Local administration will be required in sites where systemic cell therapy offers little benefit, such as in the eyes, where limbal cell transplantation has been shown to be effective in treating other types of corneal disorders or trauma [3,99-101].

Certain aspects about why particular therapies are effective at treating RDEB are unknown, but findings from one therapy can give clues to questions that remain about another. For instance, the finding that intravenous injection of recombinant C7 results in C7 deposition at the dermal-epidermal junction of injured skin may have implications regarding the mechanisms of HCT for treating RDEB. It is conceivable that cells are not required to be in close proximity to the dermal-epidermal junction in order to produce the C7 that is deposited there. Rather, owing to the soluble nature of C7, cells from the graft could produce C7 in another site (such as the bone marrow), which is then taken up by the bloodstream and distributed systemically to injured skin. Thus, the beneficial effects of donor cells that are present near the dermal-epidermal junction following HCT can be amplified by these distant C7-producing cells. It has also been hypothesized that donor cells, such as those used in allotopic fibroblast therapies, may not only be producing their own functional C7 but inducing recipient keratinocytes and fibroblasts to produce increased levels of mutant C7 as well, through induction via heparin-binding epidermal growth factor-like growth factor signaling [102]. Investigating such possibilities may help discover or define new roles for cell types or signals that were not previously known to be important for wound healing or extracellular matrix production in RDEB, other genodermatoses, and acquired skin disorders and injuries.

Along with new discoveries, critical information will become available following the treatment of these patients. Whether the new approaches are deemed successful will not only be evaluated by the long-term improvement of their daily lives but also by the reduction of the associated risks of RDEB, including squamous cell carcinoma and systemic infections.
Integrating treatments for these complications will be necessary moving forward, as will expanding the use of novel therapies to more complicated cases. It also remains to be seen whether patients treated with cellular, genetic, or protein therapies develop an acquired immune response to antigens derived from the newly synthesized C7 that was not present before therapy, similar to the related autoimmune disorder epidermolysis bullosa acquisita [103-105]. No anti-C7 antibodies were detected initially in patients who received bone marrow transplant, but the long-term results remain to be determined [18]. In the case of HCT, reduced intensity conditioning and using alternative sources of hematopoietic cells may help improve survival rates and lessen the associated risks, such as graft-versus-host disease and infections. Further improvements and adjustments to these novel approaches will hopefully be made.

**Abbreviations**

C7, type VII collagen; HCT, hematopoietic cell transplantation; iPSC, induced pluripotent stem (cell); JEB, junctional epidermolysis bullosa; RDEB, recessive dystrophic epidermolysis bullosa; TALEN, transcription activator-like effector nuclease.

**Disclosures**

The authors declare that they have no disclosures.

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**References**


Preconditioning of mesenchymal stem cells for improved transplantation efficacy in recessive dystrophic epidermolysis bullosa

Christopher Perdoni1, John A McGrath2 and Jakub Tolar1*

Abstract

Introduction: The use of hematopoietic cell transplantation (HCT) has previously been shown to ameliorate cutaneous blistering in pediatric patients with recessive dystrophic epidermolysis bullosa (RDEB), an inherited skin disorder that results from loss-of-function mutations in COL7A1 and manifests as deficient or absent type VII collagen protein (C7) within the epidermal basement membrane. Mesenchymal stem cells (MSCs) found within the HCT graft are believed to be partially responsible for this amelioration, in part due to their intrinsic immunomodulatory and trophic properties and also because they have been shown to restore C7 protein following intradermal injections in models of RDEB. However, MSCs have not yet been demonstrated to improve disease severity as a stand-alone systemic infusion therapy. Improving the efficacy and functional utility of MSCs via a pre-transplant conditioning regimen may bring systemic MSC infusions closer to clinical practice.

Methods: MSCs were isolated from 2- to 4-week-old mice and treated with varying concentrations of transforming growth factor-β (TGFβ; 5-20 ng/mL), tumor necrosis factor-α (TNFα; 10-40 ng/mL), and stromal cell-derived factor 1-α (SDF-1α; 30 ng/mL) for 24-72 hours.

Results: We demonstrate that treating murine MSCs with exogenous TGFβ (15 ng/mL) and TNFα (30 ng/mL) for 48 hours induces an 8-fold increase in Col7a1 expression and a significant increase in secretion of C7 protein, and that the effects of these cytokines are both time and concentration dependent. This cytokine treatment also promotes a 4-fold increase in Tsg-6 expression, a gene whose product is associated with improved wound-healing and immunosuppressive features. Finally, the addition of exogenous SDF-1α to this regimen induces a simultaneous upregulation of Col7a1, Tsg-6, and Cxcr4 expression.

Conclusions: These data suggest that preconditioning represents a feasible method for improving the functional utility of MSCs in the context of RDEB stem cell transplantation, and also highlight the applicability of preconditioning principles toward other cell-based therapies aimed at treating RDEB patients.

Introduction

Epidermolysis bullosa represents a spectrum of blistering diseases that vary in genetic etiology, molecular phenotype, and clinical severity [1]. Of the major epidermolysis bullosa subtypes, one of the most profound in terms of clinical presentation and progression is recessive dystrophic epidermolysis bullosa (RDEB). RDEB is characterized by loss-of-function mutations within the collagen type VII gene (COL7A1), ultimately manifesting as the reduced presence of type VII collagen protein (C7) within the dermal–epidermal junction (DEJ) [2,3]. Normally, C7 is synthesized and secreted as procollagen homotrimers by keratinocytes and dermal fibroblasts, and further processed and assembled within the extracellular space into anti-parallel dimers, which polymerize into anchoring fibrils [3,4]. Anchoring fibrils provide a structural attachment between the epidermal basement membrane and papillary dermis, thus strengthening the DEJ [5]. In RDEB, however, the diminished presence of functional C7 precludes DEJ integrity and results in the blisters and erosions seen clinically.
Various strategies have emerged with regards to approaching RDEB therapy, including genetic correction of RDEB cells [6-9], intradermal injection of allogeneic fibroblasts [10,11], as well as intraderal [12,13] and systemic [14] injection of recombinant C7. While intraderal fibroblast injections have been shown to improve wound healing in selected areas of ulcerated human RDEB skin, the remaining techniques have yet to be tested in RDEB patients, and none have looked at systemic responses in these populations. In contrast, the use of hematopoietic cell transplantation (HCT) has been demonstrated to promote systemic wound healing and to ameliorate the disease phenotype in pediatric RDEB patients [15,16]. Specifically, healthy allogeneic donor cells contained within the hematopoietic graft are capable of homing to the site of mucocutaneous injury, engrafting, and promoting repair at wounded recipient tissue sites [17]. However, taking into consideration that RDEB patients are already hypersensitive to infections due to the loss of mucocutaneous barriers, the immuno-myeloablative conditioning regimens required for transplantation may exacerbate these predispositions while also introducing additional risks [16].

Reducing the degree of immunomyeloablative conditioning used for transplantation must be weighed against the patient’s likelihood of developing graft-versus-host disease, a major immune complication associated with HCT. A potential solution to this balancing act involves the use of nonhematopoietic mesenchymal stem cells (MSCs), which not only exhibit local immunosuppressive properties [18,19] but also serve as secretory sources for adhesive molecules, anti-apoptotic and anti-fibrotic growth factors at injured tissue, and other bioactive molecules that support local progenitor cells [20-22]. The co-administration of MSCs within HCT protocols has previously been shown to promote hematopoietic engrafment in the settings of hematologic malignancy [23] and recovery from breast cancer chemotherapy [24], while infusions of MSCs alone have shown additive benefit relative to HCT in the context of osteogenesis imperfecta [25]. Additionally, while the exact mechanisms involved in HCT-mediated amelioration of RDEB are not completely understood, it is thought that nonhematopoietic cells within the graft, including MSCs, may be largely responsible [17].

The potential role for MSCs in RDEB therapy was most recently supported by Alexeev and colleagues [26], who used a Col7a1−/− mouse model to demonstrate that intraderal injections of wild-type MSCs could partially restore the basement membrane by increasing local C7 expression to 15% that of wild-type mice. However, we previously found that the use of wild-type MSCs as a stand-alone systemic infusion therapy was insufficient to rescue Col7a1−/− mice from their typical early death, despite the ability of wild-type MSCs to express Col7a1 mRNA, albeit at relatively low levels [27]. While these shortcomings may in part be due to the current inefficiencies of systemic MSC infusions, they are also confounded by the very short lifespan (2 to 3 days) of RDEB pups. Additionally, within murine RDEB models, simply increasing the number of transplanted MSCs to enhance cumulative C7 expression potentiates the odds of infusional toxicity, where entrapment of donor cells in pulmonary capillaries and/or peripheral organs results in recipient dysfunction. Thus, although intraderal injection of MSCs throughout affected body surfaces of RDEB patients would be an arduous process, the previous results from Alexeev and colleagues [26] provide evidence that if systemic infusions of MSCs are able to reach cutaneous areas in sufficient quantities, restoration of basement membrane integrity is a realistic outcome.

It may be possible to improve the efficacy and safety of allogeneic infusion protocols in the context of RDEB by utilizing a combined approach in which MSC migration to wounded tissue is enhanced and their transcription of COL7A1 is upregulated, thereby increasing cumulative C7 secretion within recipient tissue. In theory, this could allow for a reduced immunomyeloablative conditioning regimen by taking advantage of the immunosuppressive properties of MSCs, while also promoting an increased functional utility for MSCs via increased COL7A1 transcription.

With regards to an enhanced migratory capacity for MSCs, the CXCR4/stromal cell-derived factor 1-alpha (SDF-1α) axis, an interaction classically attributed to lymphocyte homing and development, has also been implicated in the recruitment of transplanted cells to injured tissue. Studies examining potential stem cell therapies for spinal cord injury [28] and myocardial infarction [29] have demonstrated the importance of the CXCR4/SDF-1α axis in this recruitment process. Furthermore, Jones and colleagues demonstrated that treating human fetal MSCs with SDF-1α in vitro resulted in a significant upregulation of CXCR4 transcription, as well as an increase in the number of cell surface CXCR4+ cells [30]. This strategy led to improved transplantation outcomes in a model of osteogenesis imperfecta, and holds promise as a technique to improve the number of exogenous MSCs recruited to injured tissue in various disease models.

Furthermore, a prime example of how the immunosuppressive properties of MSCs can coincide with their ability to improve wound healing is demonstrated by tumor necrosis factor alpha (TNFα)-stimulated protein 6 (TSG-6). Expression of TSG-6 by MSCs has been associated with both improved wound healing and down-regulation of macrophage proinflammatory signals at wounded tissue sites [31]. The role of TSG-6 in transplanted MSCs has also been highlighted by its anti-inflammatory properties and its ability to reduce infarct sizes in a model of myocardial infarction [32]. Transplantation therapies with the goal of healing wounded
tissue and/or providing anti-inflammatory effects could thus potentially benefit from increasing the degree of TSG-6 expression within the population of transplanted cells.

Lastly, previous studies have demonstrated the feasibility of upregulating COL7A1 transcription in fibroblasts [33,34] and keratinocytes [35] by incubating cells in the presence of cytokines such as TNFα and transforming growth factor beta (TGFβ). The upregulatory function that TGFβ has on COL7A1 expression has been characterized previously [36,37]. Additionally, Knaup and colleagues found that expression of COL7A1 in the RDEB cell lines was increased and attributable to elevated TGFβ levels in the local environment [38]. However, it remains to be seen whether these findings can be applied to MSCs; that is, whether MSCs can be induced to upregulate COL7A1 expression, and furthermore whether increased COL7A1 expression can be coupled with enhanced MSC migration and immunosuppression as a model for improved transplantation efficacy in RDEB. In the present study, we demonstrate that an in vitro cytokine preconditioning protocol can simultaneously upregulate Cxcr4, Tsg-6 and Col7a1 expression within murine MSCs. We also evaluate whether this approach can serve as a realistic addition to current stem cell infusion protocols aimed at treating RDEB patients.

Materials and methods
Isolation and culture of murine mesenchymal stem cells
MSCs were extracted from compact bone of healthy mice between the ages of 2 and 3 weeks using the protocol and characterizations described by Zhu and colleagues [39]. Cells were cultured in alpha minimum essential medium (αMEM) +10% fetal bovine serum +100 U/ml penicillin/streptomycin. Culture medium was changed every 2 or 3 days, and plastic-adherent cells were passaged at 70 to 80% confluence using 0.25% trypsin–ethylenediamine tetraacetic acid. Cells from passages 2 to 4 were used in all experiments. All animal studies were approved by the University of Minnesota Institutional Animal Care and Use Committee.

Reagents
Ligands, cytokines, and antagonists used within the preconditioning protocol and related experiments were purchased from R&D Systems (Minneapolis, MN, USA): recombinant mouse CXCL12/SDF-1α, recombinant mouse TGFβ2, recombinant mouse TNFα, and AMD3100.

RNA extraction, reverse transcription, and quantitative polymerase chain reaction
RNA was extracted using an RNeasy Mini Kit and RNase-Free DNase Set (Qiagen, Venlo, the Netherlands) according to the manufacturer’s protocol. RNA concentrations were quantified using a NanoDrop system (Thermo Fisher Scientific, Waltham, MA, USA). All samples used in downstream experiments had an absorbance260/280 ratio exceeding 2.00, and ribonucleic acid concentrations were diluted to 50 to 100 ng/μl prior to cDNA synthesis. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative polymerase chain reaction (qPCR) was carried out using a StepOnePlus Real-Time PCR system (Applied Biosystems). SYBR Green Master Mix reagent (Life Technologies, Grand Island, NY, USA) was used for a fluorescent probe according to the manufacturer’s guidelines. Primers for Col7a1 consisted of 5′-TGGTAAACACCTCGGCA-CAG-3′ (forward) and 5′-AAGTCTGGGCTCACGAA TG-3′ (reverse). Primers for Tsg-6 consisted of 5′-GCTCA CGGATGGGGATTCAA-3′ (forward) and 5′-TGTTAG GTTTCGAGACGACC-3′ (reverse). Primers for Cxcr4 consisted of 5′-GGGCTGCTAGAGCGGATGTGG-3′ (forward) and 5′-CATCAACTGCCAGAAAGGGG-3′ (reverse). Primers for GAPDH consisted of 5′-CCAG CAAGGACACTGACGAA-3′ (forward) and 5′-CCCTA GGCCTCCCCTCTGTAT-3′ (reverse). All qPCR reactions were carried out in triplicate in a total reaction volume of 20 μl (8 μl RNase-free water, 10 μl of 2× SYBR Green Master Mix, 0.5 μl each forward and reverse primers, and 1 μl cDNA). Reaction times and temperatures for all qPCR reactions were as follows: initial 10-minute hold for enzyme activation (95°C) followed by 40 cycles of 15-second denaturing (95°C), 30-second annealing (53°C), and 30-second extension (60°C). qPCR data were analyzed using ExpressionSuite Software (Applied Biosystems) according to the comparative cycle threshold (2-ΔΔCT) method. PCR for purposes of gel electrophoresis was carried out using AmpliTaq DNA polymerase reagents (Applied Biosystems) according to the manufacturer’s instructions.

Enzyme-linked immunosorbent assays
Sandwich enzyme-linked immunosorbent assays (ELISAs) for detection of secreted C7 ELISA kit (CUSABIO, Wuhan, China) according to the manufacturer’s protocol. For each experiment, 2 x 10⁵ cells were split evenly into two flasks and incubated in αMEM. Treated cells were exposed to 15 ng/ml TGFβ +30 ng/ml TNFα. At 48 hours, medium was removed and frozen at −80°C until ELISAs were carried out.

In vitro chemotaxis assay
In vitro chemotaxis assays were performed using a 12-well chemotaxis chamber (Neuro Probe Inc., Gaithersburg, MD, USA). GFP-expressing cells were lifted using 0.25% trypsin–ethylenediamine tetraacetic acid and allowed to settle in a 1 ml suspension of αMEM for 1 hour prior to chemotaxis experiments. Cells were suspended at a concentration of 5 x 10⁷/ml, such that approximately 50,000 cells were placed into the 100 μl top compartments. Bottom wells were filled with αMEM + varying concentrations of SDF-1α. For blocking controls,
cells were incubated for 1 hour in 100 μg/ml AMD3100, a potent CXCR4 receptor antagonist. Following the assays, the nonmigrated surfaces of the 25 × 80 mm polycarbonate filters were washed in phosphate-buffered saline, and the migrated surfaces were fixed in 10% neutral buffered formalin. Cells were visualized under a fluorescent microscope using a FITC filter and counted three times per well at 200× (total magnification).

Flow cytometry
Flow cytometry experiments were carried out on a FACS-Canto system (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo (Tree Star Inc., Ashland, OR, USA) and FCX Express 4 Research Edition (De Novo Software, Los Angeles, CA, USA). Prior to extracellular staining, cells were lifted using 0.25% trypsin–ethylenediamine tetraacetic acid and allowed to settle in a 1 ml suspension of αMEM for 1 hour. Extracellular CXCR4 was detected using 1 μg/100 μl APC-tagged rat monoclonal anti-mouse CXCR4 antibody with 1 μg/100 μl APC-tagged rat IgG2b-k antibody used as an isotype control (BD Biosciences). All extracellular staining included an initial Fc block using a purified rat monoclonal anti-mouse CD16/CD32 antibody at 0.5 μg/100 μl (eBioscience, San Diego, CA, USA).

Data analysis
Differences between measured variables were conducted using a two-tailed Student’s t test, with P < 0.05 considered significant.

Results
Effect of preconditioning duration on Col7a1 and Tsg-6 mRNA expression
To investigate whether murine MSCs are capable of upregulating transcription of Col7a1 and Tsg-6, cells were treated with 10 ng/ml TGFβ +20 ng/ml TNFα in αMEM, incubated for 24, 48, or 72 hours, and compared with untreated controls. Following the designated incubation periods, RNA was extracted, reverse transcribed, and subjected to qPCR. The observed relative quantification values across two experiments are displayed in Figure 1b. As shown, increased transcription of both Col7a1 and Tsg-6 was observed across all three time points, demonstrating that MSCs can upregulate transcription of these two genes via exposure to cytokine preconditioning. With regards to a time-dependent effect of preconditioning, Col7a1 transcription was significantly higher at 48 hours (5.7-fold increase ±0.20) relative to 24 and 72 hours, while Col7a1 transcription at 72 hours was also significantly higher than at 24 hours. Tsg-6 transcription was highest at 24 hours (4.5-fold increase ±0.79) and significantly higher than at 72 hours but not at 48 hours.

Effect of preconditioning dosage on Col7a1 and Tsg-6 mRNA expression
To determine whether a dose–response effect exists with regards to strength of cytokine exposure and subsequent changes in Col7a1 and Tsg-6 transcription, cells were treated for 48 hours in the presence of varying cytokine concentrations and compared with untreated controls (Figure 1c). Cells treated with 15 ng/ml TGFβ +30 ng/ml TNFα showed the greatest increase in both Col7a1 (8.4-fold increase ±0.12) and Tsg-6 (3.8-fold increase ±0.19) transcription, and these changes were significantly higher than in the other three treatment groups. There was no significant difference in Col7a1 transcription between the 5 ng/ml TGFβ +10 ng/ml TNFα and the 10 ng/ml TGFβ +20 ng/ml TNFα groups, while a significant increase and decrease was seen below and above the 15 ng/ml TGFβ +30 ng/ml TNFα group, respectively. These results, taken together with those shown in Figure 1b, demonstrate that treating cells with 15 ng/ml TGFβ +30 ng/ml TNFα for 48 hours elicits the greatest fold increase in Col7a1 transcription, and this protocol was used in all subsequent experiments.

Preconditioning effects after removal of cytokines
To determine how persistent the preconditioning effects are with regards to Col7a1 and Tsg-6 mRNA expression, cells were treated with 15 ng/ml TGFβ +30 ng/ml TNFα for 48 hours, washed with phosphate-buffered saline, and placed in αMEM for 48 hours as a cytokine-free environment. Figure 1d shows that after being removed from the preconditioning environment for 48 hours, there was still a twofold increase (±0.17) in Col7a1 mRNA levels relative to untreated cells. Interestingly, Tsg-6 mRNA expression appeared to be downregulated once removed from the preconditioning environment. These results suggest that while Col7a1 upregulation can be maintained for at least 48 hours following removal of cytokine stimuli, the effects on Tsg-6 upregulation are more transient and revert to a downregulated state within 48 hours of cytokine removal. It should be noted that, as seen in Figure 1b, both Col7a1 and Tsg-6 transcription could be held in the upregulated state for at least 72 hours as long as the preconditioning environment was present, but, as the results in Figure 1d demonstrate, once cells were removed from the preconditioning environment the upregulatory effects on Tsg-6 transcription appeared to be reversed in the absence of extracellular cytokines.

Effects of preconditioning on type VII collagen protein secretion
To demonstrate whether the transcriptional upregulation of Col7a1 seen following cytokine preconditioning corresponds to increased secretion of C7, a sandwich ELISA was performed to compare the culture medium of untreated cells with cells treated for 48 hours with 15 ng/ml
Prior to the 48-hour incubation period, 2 x 10^5 cells were split evenly into two flasks for each of the two experimental and control groups. As shown in Figure 2, treated cells showed a significantly higher level of C7 secretion relative to untreated cells and an approximate 70% increase above baseline (14.4 ± 1.6 vs. 8.3 ± 0.17 ng/ml; *P* <0.005).

**Simultaneous upregulation of Cxcr4, Col7a1, and Tsg-6**

The importance of the CXCR4/SDF-1α chemokine axis within the context of stem cell transplantation and migration to injured tissue has been demonstrated across several disease models [28-30]. Having shown simultaneous upregulation of Col7a1 and Tsg-6 mRNA expression, we next aimed to incorporate Cxcr4 upregulation as part of the preconditioning protocol. To achieve this, cells were treated with 15 ng/ml TGFβ +30 ng/ml TNFα for 48 hours as described above, after which 30 ng/ml SDF-1α was introduced for 1 hour. Figure 3a demonstrates that, under this protocol, upregulation of all three genes could be achieved simultaneously, and that Cxcr4 mRNA levels were 2.2-fold higher than in untreated cells. While Col7a1 expression here was not significantly different from that in cells treated with 15 ng/ml TGFβ +30 ng/ml TNFα for 48 hours without the 1-hour SDF-1α treatment (Figure 1c), Tsg-6 expression was significantly less (3.8-fold vs. 2.0-fold, *P* <0.05) in the presence of SDF-1α treatment, although still twofold higher than in untreated cells and comparable with levels seen in the other treatment gradients shown in Figure 1c.

To assess the physiologically relevant utility of this protocol – that is, whether preconditioning could also upregulate cell surface CXCR4 protein – treated cells were examined using flow cytometry (Figure 3b) and were found to exhibit a 28.5% increase in the cell surface CXCR4 signal relative to untreated cells. Next, a chemotaxis chamber was used to determine whether this increase in cell surface CXCR4 would result in improved migratory capabilities toward an SDF-1α gradient. As displayed in Figure 3c, treated cells showed a significantly greater migratory response toward SDF-1α gradients of 60 and 90 ng/ml, but not 30 ng/ml. Importantly, it is worth noting that, similar to the report by Potapova and colleagues [40], our attempts at characterizing cell surface CXCR4 expression of cells brought directly from monolayer conditions to flow cytometry experiments generally yielded an undetectable CXCR4 signal (data not shown), while cells that were subjected to a 1-hour resetting period in a cell suspension environment during SDF-1α treatment yielded the results described herein.
Discussion

The results presented here demonstrate for the first time, to the best of our knowledge, upregulation of Col7a1 mRNA and C7 expression in MSCs using an exogenous preconditioning protocol. Additionally, we present the feasibility of a three-tiered preconditioning model to improve the efficacy of transplanted MSCs in the context of RDEB therapy. This model incorporates: an improved preconditioning protocol. Additionally, we present the feasibility and broad applicability of preconditioning protocols, whereby transplanted cells are rendered more functionally suitable

in vitro for the specific disease of interest prior to transplantation.

To appreciate the physiologic significance of a sixfold to eightfold increase in Col7a1 expression by MSCs, it is necessary to compare expression levels across various cell types. We previously provided a characterization of baseline Col7a1 expression across murine bone marrow and stem cell lineages, and found MSCs to exhibit an approximate 15-fold greater expression profile than whole bone marrow cells as well as CD150⁺/48⁺ and Lin⁻ subsets of the bone marrow population [27]. Based on these previous characterizations, a sixfold to eightfold increase in baseline Col7a1 expression of MSCs, as demonstrated throughout the present study, would place them at one-third the expression level of multipotent adult progenitor cells, and at 18% of the relative Col7a1 expression found in wild-type skin. At the protein level, Alexeev and colleagues found that intradermal injections of wild-type MSCs into a mouse model of RDEB resulted in C7 expression at 15% that of wild-type levels [26]. Incorporating the approximate 70% increase in C7 secretion we observed under our current preconditioning regimen, this would bring C7 levels toward the 30% of the amount of wild-type C7 that is believed to be adequate for preventing blistering in the context of RDEB [41]. Thus, it is reasonable to suggest that preconditioned MSCs would be capable of supplying the necessary C7 to facilitate significant restoration of the DEJ following transplantation.

To address the transiency of preconditioning effects observed in the present study, we demonstrate that Col7a1 expression can be held in the upregulated state for at least 72 hours in the presence of cytokines. We also demonstrate that an upregulated state of Col7a1 expression can be held for at least 48 hours following removal of cytokine stimuli, albeit at lower levels than seen in the presence of preconditioning. A legitimate question thus arises regarding whether the increase in Col7a1 would be too transient to establish any significant change following transplantation. Here we wish to emphasize the Trojan horse aspect of preconditioning with regards to delivery of C7 to cutaneous sites, where the increase in Col7a1 expression seen pre-transplant would provide an initial restorative benefit, after which MSCs would be expected to resume baseline C7 secretion, which Alexeev and colleagues have shown to be approximately 15% that of wild-type DEJ [26]. Based on our current results, the transition from an upregulated state to a baseline state would be expected to occur after at least 48 hours, and perhaps even longer depending on the cytokine milieu present in RDEB skin [42]. Importantly, RDEB cells have been shown to exhibit increased expression of TGFβ and COL7A1 [38], albeit dysfunctional COL7A1, and thus it is logical that preconditioned wild-type MSCs would continue to display elevated C7.
secretion once in the RDEB environment via elevated TGFβ in vivo. Cytokine preconditioning of MSCs may thus provide an added initial influx of C7 to cutaneous sites, followed by a probably lessened but continual secretion of C7. Future in vivo studies will be necessary to examine whether systemic infusions of preconditioned MSCs are capable of bringing C7 levels at the DEJ in RDEB skin to levels sufficient for long-term cutaneous repair.

It is worth highlighting the downregulation in Tsg-6 expression (3.8-fold vs. 2.0-fold) we observed when going from the 48-hour treatment of 15 ng/ml TGFβ +30 ng/ml TNFa (Figure 1c) to the addition of 30 ng/ml SDF-1α for 1 hour (Figure 3a). It is conceivable that SDF-1α, a chemokine implicated in the migration of proinflammatory cells, may exert feedback inhibition on anti-inflammatory signals such as TSG-6. Although both Cxcr4 and Tsg-6 genes could simultaneously be brought to the upregulated state, it appears that in the context of a pre-transplantation protocol, the addition of SDF-1α to the preconditioning regimen would be at the expense of partial loss of Tsg-6 expression. Given the improved migrational performance seen with the addition of SDF-1α, however, increased cell surface CXCR4 protein at the expense of partially dampened Tsg-6 expression may be worthwhile during the transplantation window, as wounded cutaneous sites (for example, RDEB skin) experienced by MSCs following transplantation would provide an additional stimulus for prolonged Tsg-6 upregulation in vivo [31].

Furthermore, it is worth noting that although we were able to observe a 2.2-fold increase in Cxcr4 expression using the protocol described by Jones and colleagues [30], we were unable to attain the degree of upregulation described in their original findings (approximately fivefold). A probable explanation for this difference is the source of cells used, as human fetal MSCs were used by Jones and colleagues while MSCs from mice aged 2 to 3 weeks were used in the present study. Potapova and colleagues also described an internalization of CXCR4 protein in response
to SDF-1α exposure in human MSCs [40], and here we report an increase in cell surface CXCR4. However, the difference in responses is probably due to the vastly greater SDF-1α concentrations used by Potapova and colleagues (1 μg/ml vs. 30 ng/ml in the present study) and the differing effects of such concentrations on sensitization of cell surface CXCR4 and shunting of the chemokine receptor toward intracellular pools.

Recently, the work by Lin and colleagues has provided an exciting demonstration of the clinical possibilities afforded by manipulating the CXCR4/SDF-1α axis [43]. The administration of AMD3100 (a CXCR4 antagonist) and low-dose tacrolimus resulted in liberation of bone marrow cells into the circulation and improved wound healing at cutaneous sites. While these results could have implications across many clinical contexts, they also highlight the importance of SDF-1α expression at wounded tissue sites and its role in recruiting CXCR4-expressing bone marrow-derived cells, as blocking SDF-1α using intradermal antibody injections resulted in a loss of wound healing benefits. Looking beyond the CXCR4/SDF-1α axis, future attempts at exogenously upregulating the CCR10 expression of MSCs prior to transplantation may also prove to be a valuable approach, as the CCR10/CCL27 axis has been implicated in improved targeting of MSCs to cutaneous sites [42].

**Transforming growth factor beta: more than just fibrosis**

Like many cytokines, TGFβ has numerous attributed roles in a variety of contexts. One of its most widely known functions is as an anti-inflammatory and profibrotic stimulus. Specifically, TGFβ released from macrophages during an inflammatory response is known to promote myofibroblast differentiation as part of the wound repair and profibrotic process [31]. Additionally, culturing MSCs in the presence of TGFβ has previously been shown to upregulate levels of α-smooth muscle actin [44], a marker attributed to but not specific for myofibroblasts. If these two properties are loosely connected, an association between TGFβ-treated MSCs adopting a myofibroblast-like phenotype and a subsequent progression to a profibrotic state may be drawn. However, increased α-smooth muscle actin and associated rates of contractile activity are not limited to fibrotic processes, and in fact are thought to be an important mechanism in tissues that are actively employing new extracellular matrix and/or attempting to increase tissue strain [45]. While this has been demonstrated in settings such as ligament repair [46], it may also be involved at the DEJ in RDEB skin following incorporation of new C7 into the tissue architecture. Additionally, preconditioning of MSCs using a cytokine cocktail that included TGFβ was shown to be beneficial in restoring cardiac function in a murine model of myocardial infarction [47]. Previous reports of TGFβ-mediated increases of α-smooth muscle actin and contractility in MSCs should thus not be used synonymously with a profibrotic process, but instead should be looked at as a mechanism that can occur in a variety of physiologic contexts. Of course, future in vivo studies are needed to assess whether the contextual benefits of TGFβ preconditioning outweigh any profibrotic changes that may accumulate prior to the MSCs reverting back to the baseline state, where they have previously been shown to have a beneficial effect on the cutaneous environment in the RDEB phenotype [26].

Prolonged TGFβ signaling has also been implicated in the context-dependent procarcinogenic transformation of MSCs in certain cancer pathologies. For example, MSCs cultured for 21 days in the presence of TGFβ as part of tumor-conditioned medium were shown to increase expression of procarcinogenic factors [48]. Culturing MSCs with tumor-conditioned medium for 16 days was also shown to promote transition of the cells into tumor-associated fibroblasts, which are associated with various protumorigenic and epithelial-to-mesenchymal functions [49]. Conversely, inhibition of TGFβ signaling in MSCs exposed to tumor-secreted factors led to increased proinflammatory responses of MSCs to the tumor microenvironment [50]. Thus, while TGFβ is certainly an important cytokine for interactions between dysplasias and local MSCs, its role is probably context dependent and cell line dependent, and is thought to serve both tumor suppressive and pro-epithelial-to-mesenchymal functions in different settings [51].

Patients with RDEB experience drastically increased rates of squamous cell carcinoma (SCC), and this process may involve the known increased TGFβ signaling found in RDEB SCC skin [38]. However, since TGFβ is also elevated in non-SCC RDEB skin, Knaup and colleagues reflect that the increased TGFβ in this setting may also be an attempt to call for increased COL7A1 expression rather than as part of a strict carcinogenic process. They also point out that increased TGFβ by itself is unlikely to cause malignant consequences, while concomitant mutations and stage of tumorigenesis during exposure to elevated TGFβ signaling seems to be more significant [38,52]. Of course, cellular therapies for RDEB involving any aspect of TGFβ and other cytokine signaling should seriously consider whether an added risk for SCC may develop. Given that the involved pathways probably require prolonged time intervals and multifaceted signals to develop, the 48-hour preconditioning protocol presently used that involves isolated TGFβ and TNFα signals would not be expected to increase risk for SCC following transplantation. Additionally, the chronic inflammatory state and dysfunctional cutaneous environment associated with RDEB are thought to largely drive the increased risk for SCC [38], and the presented therapy would seek to limit chronic inflammation via stabilization of the DEJ and thus reduce overall cancer progression. However, it is unclear...
what the negative consequences may be following the introduction of cytokine-treated MSCs into RDEB patients, specifically those in which epithelial dysplasia has previously been established. Future studies will be necessary to elucidate whether this therapeutic approach may elevate risk for SCC in recipients with or without prior epithelial dysplasia.

Emerging potential of preconditioning

The concept of preconditioning MSCs prior to transplant has up to this point been largely focused on the settings of myocardial infarction (see review by Li and colleagues [53]) and ischemic stroke (see review by Yu and colleagues [54]). Although the degree of transplanted cell death in these ischemic environments has represented a barrier to their therapeutic potential, in vitro hypoxic preconditioning has been used as a method to enhance MSC graft survival post transplant [55]. In addition to promoting cell survival, preconditioning in ischemic disease has also been shown to prove functionally useful. For instance, in vitro treatment of MSCs using oxidative stress signals led to up-regulation of various cardiogenic factors [56], and this method may show promise for future myocardial infarction infusion protocols. Herrmann and colleagues found that preconditioning with TGFβ led to an enhanced cardioprotective role for MSCs [57], and MSCs preconditioned with a cytokine cocktail, including TGFβ, were shown to be beneficial for restoring cardiac function in a model of myocardial infarction [47]. Furthermore, enabling MSCs to be better migrators toward injured tissue is another application of preconditioning, as shown previously by Jones and colleagues via upregulation of CXCR4 expression in a model of osteogenesis imperfecta [30]. Several aspects of MSC functionality – whether graft survival, migration, or disease modification – have thus been demonstrated to improve under preconditioning protocols. Considering that our overall knowledge of MSCs is still in its relative infancy, even more so is our understanding of their potential applications toward therapy. As researchers continue to target MSCs as candidates for cell-based therapies in the future, the concept of preconditioning is something that should be considered for investigation. Since some form of in vitro expansion is required as an intermediate step between harvesting and transplant due to the relative low frequency of MSCs at extraction sites, the addition of preconditioning protocols does not require extensive time or effort, and the advantages gained from this application could have extraordinary potential.

Clinical strategies for approaching recessive dystrophic epidermolysis bullosa therapy

There are several promising approaches on the horizon for attaining improved outcomes in RDEB patients. First, there exist considerable efforts to further modify stem cell transplantation techniques that have previously been shown to ameliorate the RDEB phenotype [15]. While the exact mechanism as to how HCT is capable of producing these results has yet to be fully elucidated, it is thought that nonhematopoietic cells within the graft, including MSCs, may be largely responsible [17]. This hypothesis is supported in part by findings that bone marrow-derived MSCs can give rise to epithelial progenitors that promote regeneration and restoration of C7 within grafted C7-null skin [58], and also by evidence that MSCs are directly capable of restoring partial DEJ function in RDEB skin [26]. The ability to exogenously upregulate COL7A1 and C7 expression in MSCs in the pre-transplant period, as demonstrated here, thus supports a larger and more defined role for MSCs within the overall transplantation approach toward RDEB therapy in the future. The use of stem cell transplantation is not without its hazards, however, as the intensive immunosuppressive regimen required for such a procedure is an additional stressor to RDEB patients. An additional benefit of expanding the role for MSCs in this context may thus allow for a less intensive immunomodulatory protocol in the pre-transplant and post-transplant periods by taking advantage of the inherent immunosuppressive properties of MSCs. With regards to how this preconditioning method may impact screening and harvesting protocols for allogeneic transplants, existing methods such as haplotyping, extraction, expansion, and fluorescence-activated cell sorting would largely go unchanged (see review by Ikebe and Suzuki [59] for overview of MSC collection and expansion protocols). Of course, the incorporation of a preconditioning regimen would require an additional step within the expansion phase of cell preparation, but would otherwise not be expected to complicate existing protocols. Whether additional safety concerns would be introduced during the infusion window by way of applying exogenous cytokines during cell culture expansions is something that will need to be addressed in future in vivo animal and human studies.

Second, the use of intradermal fibroblast injections as a method for treating RDEB has transitioned into the setting of human studies [10,60]. These methods have been shown to improve wound healing in ulcerated areas of patients’ skin and to promote increased presence of C7 at the DEJ. The current understanding of how injected fibroblasts exert these beneficial effects is via upregulating endogenous production of mutant C7 [61]. Thus, while these techniques may prove useful in RDEB patients with some degree of functional baseline C7 production, they may not attain benefits in patients with complete absence of COL7A1 expression. Taking into consideration that fibroblasts have previously been shown to upregulate COL7A1 expression via in vitro cytokine treatments [33,34], the idea of preconditioning cells prior to transplantation, as demonstrated in
the present study with regards to MSCs, is something that warrants investigation in other transplantation modalities such as intradermal fibroblast injections. As with other therapeutic strategies in the context of RDEB, however, intradermal fibroblast injections are not without their limitations. The need for multiple injections across different areas of skin and the questions surrounding the half-life of efficacy for each injection are variables that will need to be addressed in the future, and that also highlight the benefits of transitioning to systemic allogeneic fibroblast infusions as a potential related therapeutic modality.

Third, there has been considerable attention placed on the idea of using C7 as a therapeutic strategy for treating RDEB patients. This approach began with the use of intradermal recombinant C7 injections [12,13], which were shown to reverse the RDEB phenotype in grafted skin as well as in an RDEB mouse model. Given that the use of intradermal injections could be limited by the diffusing capacity of C7 and the large surface area of RDEB lesions, as well as the inability to reach mucosal lesions (for example, of the esophagus), the use of systemic intravenous infusions of soluble C7 have now come into focus [14]. Initial reports of this approach demonstrated an incorporation of injected C7 into the DEJ of RDEB skin grafts and improved dermal–epidermal integrity. While it is likely that this approach will one day translate into improved outcomes in human RDEB patients, the use of systemic C7 injections, much like stem cell transplantation and intradermal fibroblast injections, is also not without its limitations. For instance, although C7 exhibits a relatively long half-life of several months [62], in the absence of an endogenous producer of functional C7 it is conceivable that an individual with RDEB would require lifelong rounds of injection for a sustainable therapy to manifest itself. Additionally, it will be important to determine whether certain recipients may be at risk for developing immunity against injected C7. Recently, it has been demonstrated that anti-C7 antibodies may be relatively common among RDEB patients and that most may be nonpathogenic [63]. However, in the event that an antibody response does occur following injections, this approach may also warrant some degree of immunosuppressive modulation.

Each of the strategies for approaching RDEB therapy described above have several advantages and numerous obstacles. The effect of preconditioning on cells prior to transplant, specifically in terms of COL7A1 upregulation as described here, would not only be beneficial in the context of bone marrow and cord blood transplantations for RDEB therapy, but could also prove valuable with regards to stromal cell (mesenchymal and fibroblast) therapies.

Conclusions
To our knowledge, we demonstrate for the first time an up-regulation of Col7a1 mRNA and C7 expression in MSCs using an exogenous preconditioning protocol. By using a regimen of TGFB, TNFa, and SDF-1α, MSCs are capable of simultaneously upregulating Col7a1, Tsg-6, and Cxcr4 expression. This three-tiered approach renders MSCs more functionally equipped for treating RDEB via increased C7 secretion, more potent immunosuppressants and wound-healers via upregulated Tsg-6, and better migrants toward injured tissue via enhanced cell surface CXCR4 expression. HCT has previously been shown to ameliorate the RDEB phenotype in pediatric patients, and this response is thought to be partially attributable to MSCs within the graft [17]. Additionally, MSCs have been shown to restore C7 at the DEJ in a mouse model of RDEB [26]. These previous findings, along with our current presented data, suggest that preconditioned MSCs represent a feasible methodology for approaching systemic RDEB therapy. Ongoing and future in vivo studies and clinical trials involving allogeneic transplants for RDEB may benefit from analyzing the utility of such preconditioning protocols, and whether they provide an improvement over the effects seen with unconditioned cells.

Abbreviations

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
CP designed the study, conducted experiments, interpreted data, and prepared the manuscript. JAM interpreted data and prepared the manuscript. JT designed the study, interpreted data, and prepared the manuscript. All authors read and approved the final version of this manuscript.

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