Human mesenchymal stromal cells engineered to express collagen VII can restore anchoring fibrils in recessive dystrophic epidermolysis bullosa skin graft chimeras

A. Petrova, C. Georgiadis, R.A. Fleck, L. Allison, J.A. McGrath, F. Dazzi, W.L. Di, W. Qasim

PII: S0022-202X(19)32535-7
DOI: https://doi.org/10.1016/j.jid.2019.05.031
Reference: JID 2007

To appear in: The Journal of Investigative Dermatology

Received Date: 26 December 2018
Revised Date: 22 April 2019
Accepted Date: 15 May 2019


This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.
Human mesenchymal stromal cells engineered to express collagen VII can restore anchoring fibrils in recessive dystrophic epidermolysis bullosa skin graft chimeras.

Petrova A¹*, Georgiadis C¹, Fleck RA², Allison L², McGrath JA³, Dazzi F⁴, Di WL¹, Qasim W¹.

¹ Molecular and Cellular Immunology Unit, UCL GOS Institute of Child Health, 30 Guilford Street, London, WC1N 1EH, UK.
² Centre for Ultrastructural Imaging, King’s College London, London, SE1 1UL UK.
³ St John’s Institute of Dermatology, Kings College London (Guy’s Campus), Great Maze Pond, London, SE1 9RT, UK.
⁴ Department of Haemato-Oncology, Rayne Institute, Faculty of Life Sciences & Medicine, King’s College London, London, SE5 9NU, UK.
*Corresponding author e-mail: a.petrova@ucl.ac.uk

Conceptualization: AP, FD, JAM, WLD, WQ; Data curation: AP; Formal analysis: AP; Funding acquisition: WLD, WQ; Investigation: AP, CG; Methodology: AP, WLD, WQ; Project administration: AP, WQ; Resources: RAF, LA, FD, JAM; Supervision: WLD, WQ; Validation: AP, LA, WLD, WQ; Visualization: AP, CG; Writing-original draft: AP; Writing – review & editing: AP, CG, RAF, LA, FD, JAM, WLD, WQ.

ORCID
Petrova A 0000-0001-5294-399X
Georgiadis C 0000-0003-0031-8396
Fleck RA 0000-0003-1542-6218
Allison L 0000-0003-2954-9892
McGrath JA 0000-0002-3708-9964
Dazzi F 0000-0003-2407-236X
Di WL 0000-0002-4851-1649
Qasim W 0000-0001-8353-4494

Abbreviations: RDEB- recessive dystrophic epidermolysis bullosa, AF- anchoring fibril, DEJ-dermal-epidermal junction, MSC- mesenchymal stromal cell, SIN- LV-COL7- self-inactivating lentiviral vector encoding COL7A1 transgene, MOI- multiplicity of infection, VCN- vector copy number.
ABSTRACT

Recessive dystrophic epidermolysis bullosa (RDEB) is a debilitating genodermatosis caused by loss-of-function mutations in \textit{COL7A1} encoding type VII collagen (C7), the main component of anchoring fibrils (AFs) at the dermal-epidermal junction (DEJ). With no curative treatments presently available, retrovirally-transduced autologous epidermal grafts and intradermal lentivirally-engineered fibroblast injections are being investigated. Alternative approaches aim to infuse allogeneic mesenchymal stromal cells (MSCs) to provide a more generalised treatment for RDEB. We investigated whether healthy human MSCs could be engineered to overexpress C7 and correct RDEB in a human:mouse chimeric model. Initially, engineered MSCs incorporated \textit{ex-vivo} into RDEB grafts, their presence confirmed by FISH, revealed recovery of DEJ function with no signs of blister formation. Importantly, detection of AFs by transmission electron microscopy corroborated structural recovery. Next, MSCs co-transduced to express C7 and luciferase were delivered intradermally into grafted RDEB skin, resulting in localised MSC persistence with deposition of \textit{de novo} C7 at the site. Notably, C7 expression was sufficient to restore AF density to normal levels. In contrast, intravenously injected engineered MSCs were undetectable within grafts and lacked AF reconstitution. Our data suggest that while localised correction may be achievable using engineered MSCs, strategies for systemic administration require further modelling.
INTRODUCTION

Recessive dystrophic epidermolysis bullosa (RDEB) is a debilitating genodermatosis caused by loss-of-function mutations in the COL7A1 gene (Fine et al., 2014). COL7A1 encodes collagen alpha-1(VII) chain (C7) which is essential for anchoring fibril (AF) formation at the dermal-epidermal junction (DEJ). In RDEB, loss-of-function of C7 due to mutations in COL7A1 causes malformed, reduced or absent AFs (Hovnanian et al., 1997), which can compromise the integrity of the DEJ, leading to severe sublamina densa blistering and tissue cleavage.

Clinically, skin blistering can follow even minor mechanical stress causing skin erosions from birth in many subtypes of RDEB. Moreover, in severe forms of RDEB chronic erosions with secondary infections can progress to widespread, mutilating scars and joint contractures, and aggressive squamous cell carcinomas (Rodeck and Uitto, 2007). Furthermore, clinical manifestations extend beyond the skin and affect all internal mucosal surfaces (Fine and Mellerio, 2009). There are no curative therapies for RDEB, and supportive care, with daily dressings, meticulous wound care, nutritional support, and iron supplementation for chronic anemia are the mainstay of clinical management (Grocott et al., 2013, Mellerio et al., 2007). In this context, there is a pressing need to develop specific treatments for this disease to improve symptoms, quality of life and potentially to decrease the risk of squamous cell carcinoma.

Various therapeutic strategies are under development including intradermal (Remington et al., 2009, Woodley et al., 2004) and systemic (Woodley et al., 2013) injection of recombinant C7, intradermal injection of allogeneic fibroblasts (Petrof et al., 2013, Venugopal et al., 2013), hematopoietic stem cell (HCT) transplantation (Tolar and Wagner, 2012, Wagner et al., 2010), and ex vivo gene therapy approaches using vector modified fibroblasts or keratinocytes (2014, Droz-Georget Lathion et al., 2015, Georgiadis et al., 2016, Siprashvili et al., 2016). In
addition, infusion of allogeneic mesenchymal stromal cells (MSCs) is also being investigated (Conget et al., 2010, Petrof et al., 2015). MSCs have been proposed as a cell therapy with potential immunosuppressive properties (Bartholomew et al., 2002) or possible secretion of anti-apoptotic and anti-fibrotic growth factors at sites of injured tissue (Caplan, 2009, Tolar et al., 2009a). Their use has been advocated for a variety of conditions, across medical specialties and they are not without controversy (Sipp et al., 2018).

Recent in vitro model suggested that extracellular vesicles of bone marrow MSCs can help increase C7 levels by transporting the protein within the extracellular space by feeding RDEB fibroblasts with messenger RNA that codes for C7, resulting in COL7A1 translation and protein synthesis by RDEB fibroblasts (McBride et al., 2018). In vivo the potential therapeutic effect of MSCs on RDEB has previously been evaluated in syngeneic murine models of RDEB (Alexeev et al., 2011, Kuhl et al., 2015) where intradermal injections of wild type (WT) MSCs restored murine C7 expression to around 15% of WT with partial reconstitution of the damaged DEJ (Alexeev et al., 2011). Recently, Ganier et al. reported that intradermally delivered human bone marrow MSCs supported long term human C7 production and AF formation (Ganier et al., 2018). We hypothesized that engineering MSCs to overexpress C7 through lentiviral transduction could augment such effects. We have previously developed a self-inactivating- lentiviral (LV) platform combined with a human phosphoglycerate kinase (PGK) promoter and codon-optimized COL7A1 for the engineering of autologous RDEB fibroblasts and reported AF reconstruction at the DEJ in a human:mouse xenograft model (Georgiadis et al., 2016). Here we have evaluated the potential of human MSCs transduced with LVs to overexpress C7 alone or combined with luciferase, and investigated the rescue of disease phenotypes in vitro and in a humanized skin model in vivo. We evaluated the potential of both localized intradermal MSC injection and tail vein injection for systemic therapy.

RESULTS
**Generation and characterisation of C7 engineered MSCs**

Umbilical cord derived MSCs (UC MSC) isolated from a female donor were transduced with a third generation SIN-lentiviral vector encoding codon optimized C7 (SIN-LV-COL7) at a multiplicity of infection (MOI) of 5. The transduction efficiency was assessed 10 days post transduction by qPCR for proviral copy number (VCN), which averaged 1.5 copies per cell. The expression of C7 in transduced MSCs was verified by *in situ* immunofluorescence staining of the cells cultured on coverslips in the presence of 50 µg/ml ascorbic acid for 48hrs (Figure S1a). As measured by ImageJ, 33% of cells showed overexpression of C7 with a bright fluorescent signal. Repeat transduction experiments of UC MSCs confirmed C7 expression in cells and culture media by immunoblot (Figure S1b and c). Importantly, a full length ~290kDa C7 band was detected not only in the cell lysates but also in the cell culture supernatant indicating effective secretion of C7 protein from transduced MSCs. The mean C7 expression in UC MSCs from 5 different donors was quantified by flow cytometry and equated to 48.8% (range 25-85%) (Figure S1c and d). These results suggest that MSCs are readily amenable to lentiviral manipulation, and support expression and secretion of C7.

To investigate whether transduction influenced phenotypic or differentiation characteristics of MSCs, surface marker expression of MSCs 14 days after transduction was assessed. More than 97% of MSCs retained CD105, CD90 and CD73 markers and were negative for the haematopoietic marker, CD34 (Figure S2a). Furthermore, gene modified MSCs retained their plasticity and their ability to differentiate towards adipocyte and osteocyte lineages *in vitro* as demonstrated by *in situ* staining for Lipitox Neutral stain and osteopontin, respectively (Figure S2b).

**Ex-vivo generation of human RDEB skin grafts with human MSCs to support C7 restoration at the DEJ**
To determine whether engineered MSCs can contribute towards deposition and incorporation of C7 into the DEJ, a human:murine xenograft skin model as previously described was used (Georgiadis et al., 2016). To generate bio-engineered skin grafts *ex-vivo*, transduced MSCs were added to a supporting dermal compartment along with RDEB fibroblasts lacking C7 expression at a 1:1 ratio. RDEB keratinocytes were then seeded above the dermal compartment. Grafts incorporating RDEB keratinocytes and fibroblasts (but without engineered MSCs) were prepared under the same conditions as controls. Once established, these bio-engineered skin grafts were grafted onto dermal beds prepared on NOD-*scid* IL2Rgamma<sup>null</sup> mice (total n=5), and allowed to engraft over a period of 8-10 weeks. Additional control grafts generated using healthy donor keratinocytes and fibroblasts (n=2) were tracked in parallel.

Upon harvesting, the grafts were processed for paraffin embedding, cryosectioning and transmission electron microscopy (TEM). All grafts exhibited appropriate differentiated epidermal architecture by H&E staining and Keratin 10 staining (Figure 1a and b). Human derivation of the grafted area was confirmed by species-specific staining for mitochondrial marker (complex IV subunit II) and showed clearly demarcated human:murine borders (Figure 1c). Severe blistering was observed in the grafts generated using RDEB cells alone which closely resembled the human disease phenotype (Figure 1c). This was due to lack of functional C7 in RDEB causing splitting of basal keratinocytes from the underlying dermis (n=5) (Figure 1a and c). On the contrary, there was no blistering observed in the grafts containing engineered MSCs (n=5), consistent with restoration of integrity of the DEJ in the graft (Figure 1a). This was further supported by robust expression of human C7 in grafts incorporating engineered MSCs, indicating deposition of C7 throughout the DEJ (Figure 2a). The level of C7 was significantly higher in the grafts containing engineered MSCs compared to RDEB grafts measured by the corrected total area fluorescence (CTAF 6.51 +/- 4.99 x10<sup>6</sup>,...
n=5 versus 1.31 +/- 0.265 x10^6 SD n=5, p<0.05) (Figure 2b). Weak background staining was detected in the control RDEB grafts, probably due to the antibody recognising truncated residual C7 in the RDEB keratinocytes.

To confirm whether MSCs were still present in the graft after 8 - 10 weeks engraftment on mice, human X and Y chromosomes were sought in the grafts by FISH. The keratinocytes and fibroblasts had been isolated from a male RDEB patient whereas MSCs were obtained from a female donor. XX chromosomes were detected in the grafts containing MSCs, and all cells were of XY origin in the grafts generated using RDEB cells alone (Figure 2c). In addition, despite only a small number of transduced MSCs present within the graft, C7 levels were comparable to those seen in WT grafts (CTAF 6.51 +/- 4.99 x10^6, n=5 versus 3.58 +/- 1.13 x10^6, n=3 p=0.36) (Figure 2b). Collectively, these results provide supportive evidence that engineered MSCs can secrete C7 and mediate deposition of the protein at the DEJ.

Next, to assess whether the C7 expressed in the grafts incorporating engineered MSCs had resulted in de novo formation of AFs, ultrathin sections of each graft were imaged by TEM (Figure 3a). The micrographs of the grafts containing RDEB cells alone had a blistering phenotype, and an extensive splitting of sub-lamina densa leading to complete separation of the epidermis from the underlying dermis with only rudimentary AFs seen. On the contrary, the micrographs of MSC-containing grafts revealed an abundance of sub-lamina densa fibrillar structures that bore the ultrastructural characteristics of normal AFs exhibiting cross-banding and extending ~200 nm into the dermis, looping around interstitial collagens. There was no blistering or tissue cleavage at the DEJ with a robust lamina densa throughout, consistent with functional correction of the DEJ with restoration of dermal-epidermal adhesion by AFs.

AFs formation was further quantified by quantitative ultrastructural techniques in which AFs were counted along a 50 micron continuous stretch of the DEJ in a blinded fashion. The
density of AFs per 10 micron of the DEJ was then calculated. The grafts containing engineered MSCs showed a significantly (n=5 p<0.001) higher number (>100) AFs compared to RDEB grafts lacking MSCs (<40) (Figure 3b). The density of AFs in the grafts containing engineered MSCs averaged 30 +/-3 SD AFs per 10 micron (n=5) which was very similar to that in WT grafts, whereas in the RDEB grafts an average of 8 +/- 1.5 SD AFs per 10 micron was detected (n=5 p<0.001). MSCs engineered to over express C7 conferred in vivo AF correction through restoration of C7 expression.

**Intradermal injection of engineered human MSCs into human RDEB skin grafts**

To determine whether engineered MSCs can be delivered for direct in vivo benefit, cells were injected intradermally into RDEB grafts pre-established on immunodeficient mice. To allow monitoring of the injected MSCs, the cells were also co-transduced with a LV-Luciferase-eGFP vector and enriched to 99.5% by flow cytometry on the basis of eGFP expression (Figure S3b). In vivo bioluminescent imaging using IVIS to detect luciferase expression showed persistence of MSCs over a period of 30 days (Figure S3a). At this point the animals were euthanized and grafts processed for DNA, paraffin embedding, cryosectioning and TEM. Quantitative PCR analysis confirmed VCN in the injected animals, indicating the presence of gene modified MSCs.

Human origin of the grafts was confirmed by mitochondrial marker (complex IV subunit II), and a clearly demarcated human:murine border of the grafts was evident by H&E staining (Figure 4a and b), where small blisters could be seen in the PBS-injected animal but not in the animals that received MSC injection.

The persistence of gene engineered MSCs was further confirmed by the presence of eGFP in cells in the dermal compartment of the grafts (Figure 4c). Notably, Ki67 antibody, which reacts with nuclei of actively proliferating cells, showed immunopositivity not only in the
basal epidermal layer, where keratinocyte stem cell reside, but also in the dermis, indicating that injected MSCs persisted and continued to proliferate in the dermal compartment. (Figure 4d).

**Intradermally injected engineered MSCs deposit C7 at the DEJ leading to de novo anchoring fibril formation**

Importantly, C7 deposition was seen at the DEJ of the grafts after injection of engineered MSCs, whereas no C7 immunopositivity was detected in PBS injected control grafts (Figure 5a). Although this deposition was not continuous along the DEJ, the areas of C7 immunopositivity were observed.

To assess whether the C7 expressed in the grafts which received intradermal MSC injections had resulted in *de novo* formation of AFs, electron microscopy was employed (Figure 5b). Ultrastructurally, the grafts that received PBS injection had a clear blistering phenotype, and an extensive splitting of sub-lamina densa leading to complete separation of the epidermis from the underlying dermis with only wispy, hypoplastic AFs detected. Consistent with C7 immunofluorescent observation, AF formation was not continuous along the lamina densa of MSC-injected grafts, and areas of decreased density of AFs were observed. However, selected areas of MSC-injected grafts exhibited significantly increased numbers of sub-lamina densa fibrillary structures that bore the ultrastructural characteristics of well-defined, banded AFs (n=3, p<0.001). When blinded quantitative ultrastructural technique described previously was employed (Tidman and Eady, 1985), the average density of AFs in the grafts that received intradermal MSCs injection was 31 +/-2 SD AFs per 10 microns (n=3), similar to the levels seen in WT grafts. In contrast, the density of AFs in PBS injected grafts remained at baseline level of, 8 AFs per 10 microns.
Collectively, even though intradermal injections of engineered MSCs did not result in continuous deposition of C7 along the DEJ, the cells were able to form sufficient mature AFs to restore adhesion and improve functional integrity.

**Systemically injected engineered MSCs fail to reconstitute C7 in RDEB grafts**

Next we investigated whether human luciferase modified MSCs could be delivered systemically via tail vein injections and then migrate to sites of human RDEB skin grafts to deliver C7. Serial *in vivo* bioluminescent imaging starting at 1 hour following MSC injection in three animals, found predominant bioluminescent signal concentrated in the lungs with the only other notable signals detected being close to the tail vein injection site or hind limbs (Figure S4a). Signal dissipated by day 14 and upon necroscopy, human MSCs were not identified by flow cytometry in blood or bone marrow (data not shown), and no vector signatures were detected in skin, bone marrow, liver, blood, spleen or lungs. There was no difference in either morphology (Figure S4b) or C7 expression (Figure S4c) of the RDEB skin grafts between PBS and engineered tail vein injected MSC animals, and *de novo* AFs were not detected by TEM (data not shown). Overall, there was no evidence that systemically injected engineered MSC migrated to sites of skin grafts, and the majority appeared to be rapidly sequestered during passage through the lungs.

**DISCUSSION**

RDEB therapy aims to achieve restoration of AFs necessary for skin stability. This study demonstrates that human MSCs engineered to overexpress C7 can rescue RDEB phenotype through formation of *de novo* mature AFs. However, successful reconstitution was only achieved when C7 overexpressing MSCs were used in the construction of human skin grafts or injected intradermally to pre-grafted tissue in humanised murine chimeras. Unlike previous animal studies evaluating the potential of MSC for the treatment of RDEB, we employed
NOD-scid IL2Rgamma\textsuperscript{null} mice instead of NMRI strain nude mice for grafting experiments because of their superior tolerance of xenografts. NMRI nude mice have a mutation in \textit{Foxn1} gene which affects thymic development but they retain NK immunity. Furthermore, they are known to develop extra-thymic T cell function with age. NOD-scid IL2Rgamma\textsuperscript{null} mice, on the other hand are T,B and NK deficient and we have found they readily support long engraftment of human skin grafts.

MSCs have long been proposed as a cell based therapy for RDEB. It has been postulated that MSCs may harbour immune-modulating properties that could reduce inflammation in RDEB skin (Kuhl et al., 2015), and the effects of MSCs in wound healing have been reported in a variety of settings (Qi et al., 2014, Sasaki et al., 2008). MSCs share many characteristics with fibroblasts (Haniffa et al., 2009) and murine MSCs have been shown to differentiate into fibroblast-like cells and produce ECM components that regulate collagen assembly into fibrils in an \textit{in vitro} 2D co-culture system (Alexeev et al., 2011). However, demonstration of clinical efficacy in humans has yet to be achieved in controlled trials and there are ongoing controversies around nomenclature, description and characterisation of MSCs. Reports of endogenous C7 expression in MSCs have indicated low level expression with notable donor-to-donor variation in studies of bone marrow-derived MSCs, (Ganier et al., 2018, Tolar et al., 2009b). There may be considerable differences in the transcriptome and secretome of MSCs derived from different sources (Assuncao-Silva et al., 2018, Hass et al., 2011). In our experiments, no significant C7 expression was detected in untreated MSCs isolated from umbilical cord tissue (UC MSCs), but we found UC MSCs were readily amenable to lentiviral transduction and could be engineered to overexpress and secrete C7 without impairing their differentiation potential.

We found that engineered human MSCs could replace the C7 secretory function of fibroblasts in the dermal compartment of the bioengineered skin equivalent and restored integrity of the
DEJ through AF formation. Robust expression of human-specific C7 was detected in grafts incorporating engineered MSCs with fluorescence intensity reaching levels seen in WT grafts. Importantly, there was no blistering at the ultrastructural level with intact sub-lamina densa and abundant AFs, whose density reached the WT levels of 30 AFs per 10 microns. Furthermore, we found that direct intradermal injection of engineered MSCs can restore C7 at the DEJ, and although this deposition was not continuous, it translated to formation of de novo AFs which reached overall density of the WT grafts.

A previous C7-hypomorphic mouse model study reported that most human bone marrow MSCs underwent apoptosis by day 28 after administration (Kuhl et al., 2015), but in our humanised model we found human MSCs persisted and could be readily detected by serial imaging for 30 days and post-mortem by eGFP signal. Importantly, the MSCs appeared to proliferate and expand in the dermal compartment of the grafts as evident by Ki67 immunopositivity. Similar persistence of human cells has been reported when bone marrow MSCs were injected into human skin grafts (Ganier et al., 2018), although it should be noted that these experiments employ immunodeficient mice, thereby avoiding host mediated rejection effects. It is worth noting that Ganier et al. injected a much higher dose of $2 \times 10^6$ cells as a single injection in grafts with surface area over half the size of that used in this study, where $0.5 \times 10^6$ cells were delivered in two separate injections to prevent aggregation or clumping.

To address the systemic manifestations of RDEB, we tested intravenous systemic delivery of MSCs. However, when engineered MSCs were delivered by tail vein injection in mice with established RDEB human skin equivalents, we were not able to detect luciferase signals in grafts. A major hurdle of IV delivery is entrapment of systemically injected MSCs in the lungs (Lee et al., 2009) and our experience confirms this observation. Beyond entrapment effects in the lungs (or other organs), successful MSC therapy requires homing to marrow
niches and sites of wound healing or blistering. Low levels of engraftment at wound sites is a major issue (Sasaki et al., 2008), even though it has been speculated that the hypoxic environment in blistered skin may support accumulation of MSCs (Iinuma et al., 2015). One of the limitations of the animal model described in this study is the likely absence of adequate chemoattractant signals from ectopic grafts generated from human skin. Such signals may be critical for successful MSC homing to sites of tissue injury. It has been speculated that marrow engraftment is a prerequisite for onward MSC migration and homing to sites of blistering. Efforts to improve engraftment of MSCs by preconditioning (Perdoni et al., 2014) may improve donor MSC engraftment in bone marrow by stromal niche clearance and by reducing host mediated rejection effects, although the intensity of chemotherapy required to secure such effects would likely limit such a strategy to the context of allogeneic haematopoietic stem cell transplantation (Tolar and Wagner, 2013, Wagner et al., 2010).

In summary our data suggests that therapeutic benefits at sites of localised blistering might be achievable using directly injected MSCs engineered to overexpress C7. Banks of engineered MSCs that can be used in multiple recipients could have cost and convenience advantages over strategies that require engineering of autologous fibroblasts. Finally, the systemic burden of RDEB mandates further modelling and evaluation of strategies for more generalised delivery and enhanced homing to sites of tissue disease.

MATERIALS AND METHODS

**RDEB primary keratinocytes and fibroblasts isolation and culture**

A 6-mm RDEB skin biopsy was obtained with authorization from the National Research Ethics Services, Westminster (07/H0802/104), and with written informed consent from patients with RDEB-1 ((+/-) c.1732C>T p.Arg578*)/((+/-) c.2710+2T>C IVS20+2T>C) and
RDEB-2 (+/+) c.425A>G p.Lys142Arg)). The cells were isolated and cultured as previously described (Georgiadis et al., 2016).

**MSCs culture and transduction**

MSCs were cultured in alpha MEM (Thermofisher, Waltham, MA USA) supplemented with 5% human platelet lysate (Stem Cell Technologies, Cambridge, UK). Cells were transduced at MOI 5 with SIN-LV-COL7, and the medium was replaced 24hrs later. The cells were expanded until sufficient number of cells was obtained for downstream analysis and application. For *in vivo* experiments, SIN-LV-COL7 transduced MSCs were treated with LV-Luc-GFP vector at MOI 5 and sorted for GFP+ population by FACS.

**Bioengineered skin preparation and grafting on immunodeficient mice**

The method for preparing and grafting bioengineered skin on immunodeficient NOD-scid IL2Rgamma<sup>null</sup> mice have been described previously (Georgiadis et al., 2016) and is illustrated in Figure S5. This model was adapted from original studies using immunodeficient nude mice (Di et al., 2011, Di et al., 2012, Larcher et al., 2007). In brief, to prepare the dermal compartment fibrinogen solution (cryoprecipitate derived from a porcine plasma source) containing 1.5 ×10<sup>5</sup> WT fibroblasts, RDEB patient ((+/+) c.425A>G, p.Lys142Arg)) fibroblasts or a 1:1 mix of 0.6x10<sup>5</sup> RDEB patient ((+/+) c.425A>G, p.Lys142Arg )) fibroblasts and 0.6x10<sup>5</sup> SIN-LV-COL7-transduced MSCs was combined with 0.025mmol/l CaCl2 (Sigma-Aldrich, Gillingham, UK) and 11 IU of bovine thrombin (Sigma-Aldrich). The mixture was poured in two 35-mm wells and allowed to solidify at 37 °C for 1hr. WT or RDEB patient human keratinocytes (1.2 × 10<sup>6</sup> cells per well) were then seeded on the fibrin matrix to form the epidermal layer of the bioengineered skin. Bioengineered skins were allowed to reach confluency and then manually detached from tissue culture wells and grafted onto immunodeficient mice. Grafting was performed under sterile conditions using 6-week-
old female immunodeficient mice housed under protective conditions. Six to eight weeks after grafting, bioengineered human skins were either harvested, received 2 intradermal injections of engineered MSCs in 50ul PBS each or injected with 2x10^6 engineered MSCs in 150ul PBS via tail vein. In vivo imaging of bioluminescence was done using an IVIS Lumina III In Vivo Imaging System (PerkinElmer, MA, USA, live image version 4.5.18147) All animal studies were approved by the University College London Biological Services Ethical Review Committee and licensed under the Animals (Scientific Procedures) Act 1986 (Home Office, London, United Kingdom).

**Analysis of bioengineered grafted tissue**

Immunofluorescence staining of frozen graft tissue sections was performed with the following antibodies: hC7 LH7.2 (Sigma-Aldrich), keratin 10 (Abcam, Cambridge, UK), complex IV subunit II MT-CO2 (Abcam), Ki67 (Abcam) or GFP (Abcam). Followed by incubation with secondary antibodies: Alexa Fluor goat anti-mouse 488 (Invitrogen, Paisley, UK), goat anti-rabbit Cy3 (ThermoFisher), and counterstained with DAPI. Paraffin embedded sections were stained by a hematoxylin and eosin histochemical technique. Staining was visualized and imaged using a Leica DMLB upright microscope (Leica Microsystems CMS, Wetzlar, Germany) and a Zeiss Axiophot 2 (Zeiss, Oberkochen, Germany) and processed using Image-Pro 6.2 (MediaCybernetics, Rockville, MD). Post-processing and quantification was carried out using ImageJ. For fluorescence quantification, 7 images were taken at the same exposure and area along the DEJ selected using ImageJ software. ImageJ was then used to measure the area, integrated density and mean grey values of the selected region. The same measurements were acquired in regions of no fluorescent signal for background readings. The corrected total area fluorescence (CTAF) was calculated as follows. CTAF= Integrated Density – (Area of the DEJ x mean fluorescence of background reading). The measurement was done in 3 images for each biological replicate (n=5 for RDEB grafts and n=3 for WT
grafts). Student’s t-test was used for statistical analysis. Fluorescent in situ hybridization (FISH) was performed by Clinical Cytogenetics Unit, Camelia Botnar Laboratories, Great Ormond Street Hospital.

**TEM**

Sample processing for TEM was performed as previously described (Georgiadis et al., 2016). Images were acquired with JEOL JEM 1400 Plus TEM with a JEOL Ruby CCD camera (JEOL, Welwyn Garden City, UK). Consecutive 40 overlapping images covering 50 microns of a well-defined lamina densa and lamina lucida were taken at x12k magnification in a blinded fashion. Subsequent AF scoring was performed using established quantitative ultrastuctural techniques (Tidman and Eady, 1985). Student’s t-test was used to carry out the statistical analysis.

**DATA AVAILABILITY STATEMENT**

No datasets were generated or analysed during the current study.

**CONFLICT OF INTERESTS**

The authors state no conflict of interest.

**ACKNOWLEDGEMENTS**

This work was supported by CureEB, NIHR, GOSH-ICH BRC. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR, or the Department of Health. The authors would like to thank Dr Su Lwin for her clinical expertise in RDEB and stimulating discussions, and Dr James R. McMillan for sharing his expertise in electron microscopy for scoring anchoring fibrils. The authors acknowledge Microscopy and Flow Cytometry Core Facilities at UCL GOSH ICH and the staff at Biological Services Animal Facility.
REFERENCES


Figure legends

Figure 1. Histological and immunofluorescent analysis of the cytoarchitecture and human origin of the grafts.

a. H&E staining of normal morphology of human skin. Blistering shown by the black arrow. b. Immunofluorescent staining for Keratin 10 showed normal differentiation pattern of the grafts with suprabasal cells expressing keratin 10. c. Human origin of the graft was confirmed by human specific MTCO2. White dotted line demarcate the border between mouse and human tissue. Stars show blisters. Scale bar=25µm. WT- wild type grafts, RDEB- RDEB grafts, MSC C7- RDEB grafts containing engineered MSCs, hu- human, ms- mouse.

Figure 2. In vivo restoration of type VII collagen expression in the grafts containing MSC C7.

a. Immunofluorescent analysis of C7 (red) expression at the dermal-epidermal junction (DEJ). Blister denoted by black arrows in RDEB graft. b. Quantification of fluorescent intensity at the DEJ area confirms C7 expression was significantly increased in the grafts with MSC C7 when compared to RDEB grafts. CTAF is corrected total area fluorescence. Statistical analysis was carried out using Student’s t-test. Error bars represent standard deviation (SD) from 3 images in biological n=5 for each RDEB and MSC C7 grafts and n=3 for WT grafts. Representative images are shown on the right. Scale bar=25µm. c. Fluorescent in situ hybridization (FISH) analysis revealed the presence of female MSCs within the grafts. Red- X chromosome, green- Y chromosome. WT- wild type grafts, RDEB- RDEB grafts, MSC C7- RDEB grafts containing engineered MSCs.

Figure 3. In vivo functional correction through LV-COL7-mediated restoration of type VII collagen anchoring fibrils (AFs).

a. TEM analysis of the skin grafts shows the formation of de novo anchoring fibrils (AFs). (x5000, scale bar=2µm) and higher (x12000, scale bar=1µm) Red arrows point at AFs. Black stars show blister. Inset shows de novo AFs at higher magnification. b. Quantitative analysis revealed restoration of normal density of AFs in grafts containing engineered MSC C7. Statistical analysis carried out using Student’s t-test. Error bars represent standard deviation (SD) n=5 for each RDEB and MSC C7 grafts and n=3 for WT grafts. DEJ- dermal-epidermal junction, KC- keratinocytes, WT- wild type grafts, EB- EB grafts, MSC C7- EB grafts containing engineered MSCs.

Figure 4. Engineered MSCs persist and proliferate in the human RDEB grafts.

a. Human origin of the graft was confirmed by human specific MTCO2. White dotted line demarcates the border between mouse and human tissue. b. H&E staining of normal morphology of human skin. Blistering shown by the black arrow. c. Immunofluorescent staining for GFP detected the presence of engineered MSCs. MTCO2 (red) confirms the human origin of all the grafts. d. Ki67 (red) identifies highly proliferative cells in the basal epidermal layer but also detected the presence of MSCs within the dermal compartment. Keratin 10 (green) showed normal differentiation pattern of the grafts. Scale bar=25µm. PBS-PBS injected RDEB graft, ID- RDEB grafts injected intradermally with engineered MSCs, hu- human, ms- mouse.
Figure 5. *In vivo* restoration of collagen type VII expression and functional correction through *de novo* anchoring fibril (AF) formation.

**a.** Immunofluorescent analysis of C7 (red) expression at the dermal-epidermal junction (DEJ). Red arrows show focal C7 expression. Inserts show C7 expression at higher magnification. **b.** TEM analysis of the skin grafts shows the formation of *de novo* anchoring fibrils (AFs). The images are shown at low (x3000, scale bar=5µm) and high (x12000, scale bar=1µm) magnification. Red arrows point at AFs. Green arrowheads point at hemidesmosomes. Black stars show blister. Insert shows *de novo* AFs at higher magnification. Scale bar=25µm. PBS-PBS injected RDEB graft, ID- RDEB grafts injected intradermally with engineered MSCs, KC- keratinocytes.
a

Adipocyte differentiation  Osteocyte differentiation  Undifferentiated MSCs

LipiTOX neutral lipid stain  Osteopontin  Collagen type VII
Fibrin matrix in a 35-mm well

35-mm full-thickness wound is made, followed by de-vitalization of mouse skin

Graft is placed on the wound and secured with de-vitalized mouse skin as a biological bandage
SUPPLEMENTARY MATERIALS AND METHODS

Production of third-generation COL7A1-expressing-LV vector, titration and vector copy number measurement

A third generation pCCL self-inactivating-LV vector encoding a full-length codon-optimized COL7A1 transgene was produced as previously described (Georgiadis et al., 2016). Quantitative polymerase chain reaction for HIV Psi packaging element (Ψ) and housekeeping gene albumin was used to determine the titre and proviral copy number.

Flow cytometry

For phenotypic analysis of MSCs, the following human antibodies were used: CD 73-APC, CD 105-PE, CD 90-FITC and CD 34-Vio770 (all Miltenyi Biotec, Surrey, UK) according to manufacturer’s instructions. For C7 expression analysis following transduction, cells were fixed in Fix & Perm® Medium A (ThermoFisher) for 20mins at RT in the dark. The cells were then washed with 2-3 % FBS/PBS, in 2 ml, twice; spun down at 400g for 4mins at RT. The cell pellets were resuspended in Fix & Perm® Medium B with C7 antibody at a 1:25 dilution (LH7.2, Santa Cruz Biotechnologies, Heidelberg, Germany), and incubated for 30mins at 4°C in the dark. The cells were washed with 2 %FBS/ PBS, twice and pelleted at 16,000 rpm for 4mins. The cell pellets were resuspended in Fix & Perm® Medium B with Alexa 488 goat anti-mouse 2⁰ antibody at a 1:200 dilution (ThermoFisher), and incubated for 30mins at 4°C in the dark. The cells were washed with 2% FBS/PBS twice, resuspended in 0.5ml 2-3% FBS/PBS and analysed using flow cytometry.

Cyto-immunofluorescence staining

1 x 10⁵ MSCs, SIN-LV-COL7 transduced and untransduced, were plated on coverslips and cultured in the presence of 50 µg/mL ascorbic acid for 2 days. The cells were then fixed with 4% neutral buffered formalin, permeabilized and unspecific binding blocked with 0.1%
Triton X-100, 3% FBS in PBS, and incubated overnight at 4°C with C7 primary antibody at a 1:500 dilution (LH7.2, Sigma-Aldrich, Dorset, UK). After washing, cells were incubated with Alexa-488 goat-anti-mouse secondary antibody at a 1:500 dilution, (ThermoFisher) and DAPI (ThermoFisher). The slides were mounted with Prolong gold (ThermoFisher). Micrographs were taken using a Leica DMLB upright microscope (Leica Microsystems CMS GmbH, Wetzlar, Germany). Quantification used ImageJ counting tool and accompanying macro.

**Immunoblotting**

Transduced and untransduced cell pellets of UC MSCs, or 48 hr cultured serum-free supernatant supplemented with 50µg/ml ascorbic acid and concentrated using ProteoExtract® Protein Precipitation kit (Merck Millipore, Hertfordshire, UK), were resuspended in cell lysis buffer for total protein isolation. The total protein concentration in the supernatant was determined using a Bio-Rad protein assay kit (BIO-RAD, Hertfordshire, UK) and equal quantities (50µg) of total protein were loaded on SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes (PVDF) and incubated with anti-C7 antibody overnight at RT with shaking, followed by incubation with secondary antibody conjugated with HRP. Signal detection was performed using the ECLplus system (GE Healthcare, Hatfield, UK). Wild-type human fibroblasts were used to identify the full-length C7 band. Anti-Vinculin mAb (cl. V284, Sigma-Aldrich, Dorset, UK) was used as a total protein loading control for cell lysate samples. Total protein amounts in the samples from concentrated culture medium loaded on the SDS-PAGE were checked by Ponceau S (Sigma-Aldrich, Dorset, UK) staining of the PVDF membrane following the protein transfer.

**In vitro differentiation assay**

MSCs were subjected to directed differentiation using StemPro Adipogenesis and Osteogenesis kits (ThermoFisher, Waltham) according to manufacturer’s instructions. The
differentiation was assessed by staining using HCS LipidTOX™ Red neutral lipid stain (ThermoFisher) according to manufacturer’s instructions and cyto-immunofluorescent staining with Osteopontin monoclonal antibody (2F10, ThermoFisher).

Supplementary Figure legends

Figure S1. MSCs can be engineered to express and secrete C7 using a SIN-LV-COL7A1 vector.

a. In situ immunocytochemistry of MSCs cultured on coverslips in the presence of 50 μg/ml ascorbic acid for 48hrs. C7 (red) immunopositivity could be seen in approximately 30% of cells transduced with SIN-LV-COL7A1 at MOI 5 and not in untransduced cells. Nuclei were counterstained with 4’.6-diamidino-2-phenylindole (blue). Scale bar=25µm. b. Immunoblot analysis showed the presence of a band at 290kDa corresponding to full length C7 protein in UC MSCs transduced with lentiviral vector (from two separate transduction experiments) but not in the untransduced sample. Vinculin was used as a loading control. Wild-type fibroblasts were used a positive control for the full-length C7. c. Immunoblot performed on supernatant of cultured cells showed restoration of secreted C7 in engineered MSCs. Ponceau S staining was used to confirm the total protein amounts in the loaded samples. d. Flow cytometric assessment of transduction efficiency in MSCs from 5 different donors. e. Average expression of C7 by flow cytometry in SIN-LV-COL7-transduced MSCs at MOI 5. Error bars represent SD from 5 different donors. UT- untransduced, MOI- multiplicity of infection, MSC-C7 MSCs transduced with SIN-LV-COL7, the numbers indicate repeat transduction experiments, WT Fb- wild-type fibroblasts, s/n- supernatant.

Figure S2. SIN-LV-COL7 MSCs retain their plasticity following lentiviral transduction.

a. Lentiviral transduction did not affect MSC phenotype and cells remained positive for CD90, CD105 and CD73 and negative of haematopoietic CD34 marker by flow cytometry. b. MSCs retained their plasticity following lentiviral transduction and were able to differentiate into adipocytes and osteocytes. Scale bar=25µm. UT- untransduced, MOI- multiplicity of infection.

Figure S3. In vivo tracking of engineered MSCs delivered intradermally.

a. Strong luciferase signal could be seen in all three animals that received intradermal injections with little decrease up until day 30. b. Flow analysis of injected MSCs showed that 85% of cells overexpressed C7 and were all positive for Luc-GFP expression.

Figure S4. In vivo tracking of engineered MSCs delivered systemically.

a. Predominant bioluminescent signal concentrated in the lungs with the only other notable signals detected being close to the tail vein injection site or hind limbs. The signal dissipated by day 14. b. Histological assessment showed no difference between MSC and PBS injected grafts. Blistering shown by the black arrow. Black dotted line demarcates the border between mouse and human tissue. c. Immunofluorescent analysis of C7 (red) expression indicates
absence of de novo protein. Scale bar=25µm. PBS- PBS injected RDEB graft, MSC_Luc_C7 IV- RDEB graft injected intravenously with engineered MSCs, hu- human, ms- mouse.

**Figure S5. Schematic representation of bioengineered skin preparation and grafting on immunodeficient mice**

To generate a dermal compartment, fibrinogen solution was populated with either primary RDEB fibroblasts alone or mixed with SIN-LV-COL7-transduced MSCs at a 1:1 ratio, and the mixture was poured into two 35-mm wells. Primary RDEB keratinocytes were then seeded on top and allowed to reach confluency. Equivalent size full-thickness wounds were created on the back of NOD-scid IL2Rgamma<sup>null</sup> mice under sterile conditions, followed by de-vitalization of the mouse skin. Manually detached skin equivalents were then placed onto the wounds, and de-vitalized mouse skin was used as a biological bandage until the grafts take.