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Correction of Recessive Dystrophic Epidermolysis Bullosa by Transposon-Mediated Integration of *COL7A1* in Transplantable Patient-Derived Primary Keratinocytes

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Recessive dystrophic epidermolysis bullosa (RDEB) is caused by defects in type-VII collagen (C7), a protein encoded by the *COL7A1* gene and essential for anchoring fibril formation at the dermal-epidermal junction. Gene therapy of RDEB is based on transplantation of autologous epidermal grafts generated from gene-corrected keratinocytes sustaining C7 deposition at the dermal-epidermal junction. Transfer of the *COL7A1* gene is complicated by its very large size and repetitive sequence. This article reports a gene delivery approach based on the Sleeping beauty transposon, which allows integration of a full-length *COL7A1* cDNA and secretion of C7 at physiological levels in RDEB keratinocytes without rearrangements or detrimental effects on their clonogenic potential. Skin equivalents derived from gene-corrected RDEB keratinocytes were tested in a validated preclinical model of xenotransplantation on immunodeficient mice, where they showed normal deposition of C7 at the dermal-epidermal junction and restoration of skin adhesion properties. These results indicate the feasibility and efficacy of a transposon-based gene therapy approach to RDEB.

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INTRODUCTION

Recessive dystrophic epidermolysis bullosa (RDEB) is characterized by a functional deficit of type VII collagen (C7) protein due to loss of function mutations in the C7 gene (*COL7A1*). There are no curative therapies for RDEB, and the care of patients is palliative and restricted to the treatment of individual wounds (Ly and Su, 2008; Mellerio et al., 2007). Experimental therapies under development include recombinant C7 protein (Remington et al., 2008; Woodley et al., 2004; Woodley et al., 2013), infusion of allogeneic mesenchymal cells (Conget et al., 2010; Petrof et al., 2015), hematopoietic stem cell transplantation (Tolar and Wagner, 2012), and gene therapy (Murauer et al., 2015). Current ex vivo gene therapy approaches, based on autologous transplantation of genetically corrected epidermal grafts, aim at transferring the correct, full-length *COL7A1* cDNA (~9 kb) in keratinocyte stem cells by oncoretroviral or lentiviral vectors (Droz-Georget Lathion et al., 2015; Jackow et al., 2016; Sebastiano et al., 2014; Siprashvili et al., 2010; Titeux et al., 2010). These vectors are associated with a certain risk of insertional mutagenesis and have limited cargo capacity, resulting in genomic instability and rearrangements when used to transfer large C7 expression cassettes (Droz-Georget Lathion et al., 2015; Titeux et al., 2010). Recently, Georgiadis et al. (2015) codon-optimized the C7 cDNA cloned into a self-inactivating lentiviral vector and showed expression of a normally sized protein in 94% of transduced RDEB fibroblasts. Targeted exon skipping and nuclease-mediated surgical genomic correction of specific mutations in the C7 coding sequence is an emerging approach (Chamorro et al., 2016; Osborn et al., 2013; Turczynski et al., 2016). Although this finding represents a promising patient-specific therapy, it has to cope with the low efficiency of homologous recombination in primary cells (Coluccio et al., 2013; Duarte et al., 2014), which requires selection/enrichment of the genome-edited cells before graft generation and transplantation.

As an alternative approach, we investigated the feasibility of ex vivo *COL7A1* gene replacement by delivering a full-length *COL7A1* cDNA to graft-maintaining keratinocytes from RDEB patients by the Sleeping beauty (SB) transposon system. The system is based on the use of a previously described pT2

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Abbreviations: AdV, adenoviral; C7, type VII collagen; FLPe, enhanced flippase; FRT, flippase recognition target; HD, helper dependent; iRDEB, immortalized recessive dystrophic epidermolysis bullosa; RDEB, recessive dystrophic epidermolysis bullosa; RT, reverse transcriptase; SB, sleeping beauty

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transposon and an engineered hyperactive transposase (SB100X) (Mates et al., 2009), which can mediate precise cut-and-paste transposition of gene expression cassettes in a variety of human cells (Ivics and Izsvak, 2015) and in a clinical setting (Singh et al., 2014). Although the efficacy of transposase-mediated transgene insertion decreases with increasing cargo size (Turchiano et al., 2014), the hyperactive SB100X transposase has no strict size limits and can mediate the transposition of relatively large (>10 kb) DNA fragments without rearrangements (Escobar et al., 2016). The SB transposon integration pattern is almost random and potentially safer than that of retroviral vectors (Turchiano et al., 2014). We incorporated a *COL7A*1 expression cassette in the T2 transposon and provided the SB100X cassette in trans by plasmid transfection in an immortalized, patient-derived RDEB keratinocyte cell line (iRDEB) (Chamorro et al., 2013). Molecular characterization of single, transposed iRDEB clones showed the SB-mediated integration of the full-length *COL7A1* transposon and a complete restoration of full-size C7 protein expression. To transpose the *COL7A1* cassette in primary clonogenic RDEB keratinocytes, which are resistant to plasmid transfection, we developed an adenoviral (AdV) vector platform consisting in a helper-dependent (HD) AdV vector carrying the transposon cassette flanked by flippase recognition target (FRT) sites ($AdV^{HD}C7.F^{50}$), a second-generation vector (i.e., *E1*- and *E2A*-deleted) coding for enhanced flippase (FLPe) recombinase ($AdV^{\Delta 2}FLPe.F^{50}$), and a first-generation AdV vector (i.e., *E1*-deleted) coding for the SB100X transposase ($AdV^{\Delta 1}SB100X.F^{50}$). All AdVs are independent of the coxsackie and adenovirus receptor to enter target cells owing to a fiber modification in which the apical receptor-interacting motifs are derived from adenovirus serotype 50 (F^{50}) instead of prototypic serotype 5 (Janssen et al., 2013; Knaan-Shanzer et al., 2001). We used this combination of AdV platforms to infect primary RDEB keratinocytes carrying the recurrent mutation 497insA in the *COL7A1* gene, which generates premature stop codon in exon 5 and no C7 expression (Gardella et al., 1999). Infection with the three different types of vectors did not affect the clonogenic potential of RDEB keratinocytes and restored C7 expression in vitro. The infected keratinocyte bulk population was used to generate skin equivalents that were grafted on immunodeficient mice, which showed C7 secretion, functional reconstruction of the dermal-epidermal junction, and restoration of adhesion properties in a validated preclinical model of human epidermis in vivo.

RESULTS

SB-mediated transposition of a full-length human *COL7A1* cDNA in iRDEB keratinocytes

The SB100X and the T2-based transposon were exploited to investigate the restoration of C7 expression in patient-derived, iRDEBs carrying null mutations in the *COL7A1* gene. The transposon cassette carried the full-length *COL7A1* cDNA (pLRZScol7) (Baldeschi et al., 2003) under the phosphoglycerokinase promoter (pT2C7) (Figure 1a). We transfected iRDEB with pT2C7 in the presence or absence of the SB100X transposase-expressing plasmid. Transfection efficiency was ~20% in both samples 2 days after transfection, as assayed by immunofluorescence for C7 expression (Supplementary Figure S1a online). Twenty-one days after transfection, the culture transfected with pT2C7+pSB100X contained approximately 7% $C7^+$ cells, whereas control cells were negative for C7 expression (Figure 1b and Supplementary Figure S1a). Transposed gene expression was tested in the bulk cell population by reverse transcriptase–PCR: iRDEB cells express background levels of *COL7A1* mRNA that do not result in mature protein synthesis (Chamorro et al., 2013), whereas a significant *COL7A1* mRNA expression over the background was detected in cells transfected with pT2C7+pSB100X (Supplementary Figure S1b online). We then cloned by limiting dilution the transposed iRDEB bulk culture and found 14 single clones positive for C7 cDNA (Supplementary Figure S2 online). Then we assessed by RT-PCR the expression of phosphoglycerokinase -driven C7 cDNA in randomly selected 8 of 14 clones (Supplementary Figure S3 online). Vector copy number and the integrity of the transposed cassette were tested by Southern blot analysis, after digesting genomic DNAs with *Bgl*II and *Dra*III or *Hind*III, respectively. Hybridization with a C7-specific probe released bands longer than 2.3 kb at the time of *Bgl*II digestion and 1.9kb at the time of *Dra*III digestion, indicating an average vector copy number of 1.5 (Figure 1c). The 10-kb band present at the time of *Hind*III digestion showed that transposition occurred without rearrangement in the 3′ (Figure 1d) and 5′ end of collagen VII cDNA (Supplementary Figure S4 online). To unequivocally prove that the *COL7A1* integrants resulted from SB–mediated transposition events, we mapped the insertion sites using a modified version of the linker-mediated PCR technique able to preferentially map the junction between the left-inverted repeat and the human genome (Turchiano et al., 2014) (Supplementary Table S1 online; Supplementary Figure S5 online). Bioinformatics analysis of modified linker-mediated PCR amplicons (Supplementary Figures S6 and S7 online) showed only one bidirectional mapping of the single transposon copy hosted by clone 7. To confirm that in all other clones the left-inverted repeat–genome junction was preferentially amplified by the modified linker-mediated PCR, we designed specific primers on the genomic regions flanking the right-inverted repeats. In all hits but one (9.2) we found the preference for the amplification of left-inverted repeat–genome junction obtained by modified linker-mediated PCR technique (Supplementary Figure S8 online). Most importantly, all the 10 transposon hits belonging to 8 clones showed the TA di-nucleotides signature of SB-mediated transposition without genomic rearrangements, deletions, or insertions in the target sites (Supplementary Figure S5).

Finally, we verified the production of intracellular and secreted C7 mature protein by Western blot analysis (Figure 1e, upper and lower panels). All clones tested expressed a C7 protein of normal size. All together, these data show that the SB-mediated transposition allows integration of intact copies of the large *COL7A1* cDNA at low numbers in C7-deficient keratinocytes and the expression and secretion of a normal C7 protein.

Restoration of C7 expression in primary recessive dystrophic epidermolysis bullosa keratinocytes

Despite the significant potential of the SB-mediated transposition system, plasmid transfection of the transposon/

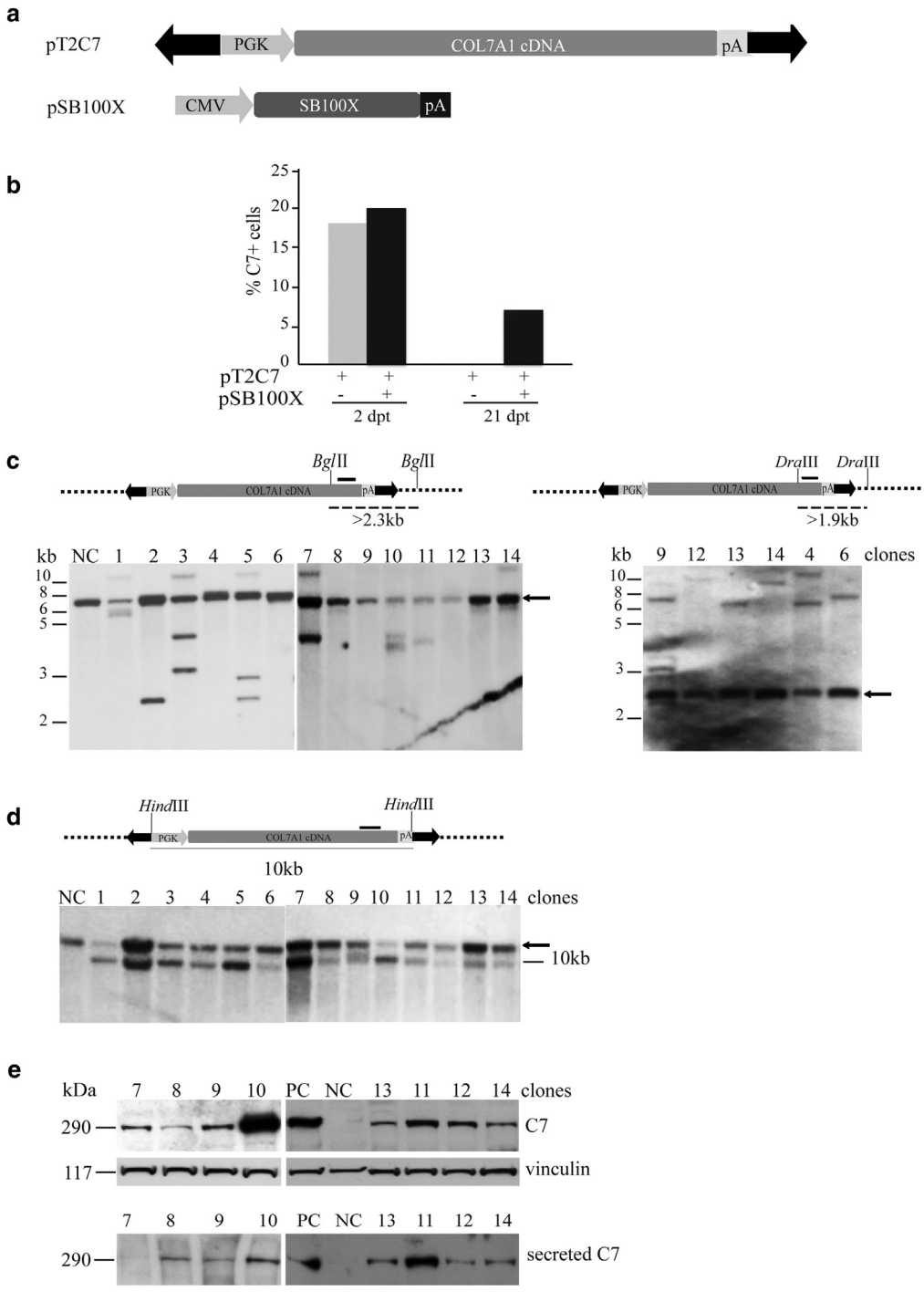

Figure 1. C7 transposition in iRDEB keratinocytes. (**a**) Schematic outline of pT2C7 and pSB100X plasmids. The black arrows represent the IR of the transposon. (**b**) pT2C7-transfected iRDEB in presence or in absence of pSB100X. $C7^+$ cells scored by immunofluorescence 2 and 21 days after transfection. (**c**) Southern blot on iRDEB clones. *Bgl*II (left panel) and *Dra*III (right panel) digested DNA hybridized to a C7-specific probe. Dotted lines represent human genome. (**d**) Southern blot on iRDEB clones. *Hind*III digested DNA probed with a C7-specific probe. Black bars indicate C7-specific probe. Black arrows indicate endogenous signal. (**e**) Western blot analysis on total extracts (upper panel) and on culture supernatants (lower panel) of iRDEB-transposed clones. Vinculin represents internal loading control. CMV, cytomegalovirus; iRDEB, immortalized recessive dystrophic epidermolysis bullosa; NC, not treated; PC, wild-type keratinocytes; PGK, phosphoglycerate kinase.

transposase components in primary RDEB keratinocytes is inefficient and toxic (Supplementary Figure S9 online) and remains an obstacle to its practical application in gene therapy. To overcome this limitation, we delivered the SB100X transposase and the pT2C7 transposon via the infection of target cells with $AdV^{\Delta 1}$SB100X.F^{50} and AdV^{HD}C7.F^{50}, respectively. To ameliorate the transposition efficiency we avail the FRT/FLPe recombinase (Zhang et al., 2013), flanking the C7 expression cassette by the FRT sites and providing the FLPe recombinase by infecting target cells with $AdV^{\Delta 2}$FLPe.F^{50} (Figure 2a). RDEB keratinocytes were isolated from a small skin biopsy obtained from a 23-year-old patient carrying a homozygous mutation (497insA) in the *COL7A1* gene, which generates a premature stop codon in exon 5 and no C7 expression at protein level. Positive staining for p63 indicates the presence of highly clonogenic, progenitor/stem cells (Figure 2b) (Gardella et al., 1999; Pellegrini et al., 2001; Senoo et al., 2007). Moreover, the clonogenic ability of RDEB keratinocytes (not infected and AdVs-infected) and healthy epidermal cultures are similar (Figure 2c).

RDEB keratinocytes were cultivated onto a feeder of irradiated 3T3-J2 cells, according to standard procedures

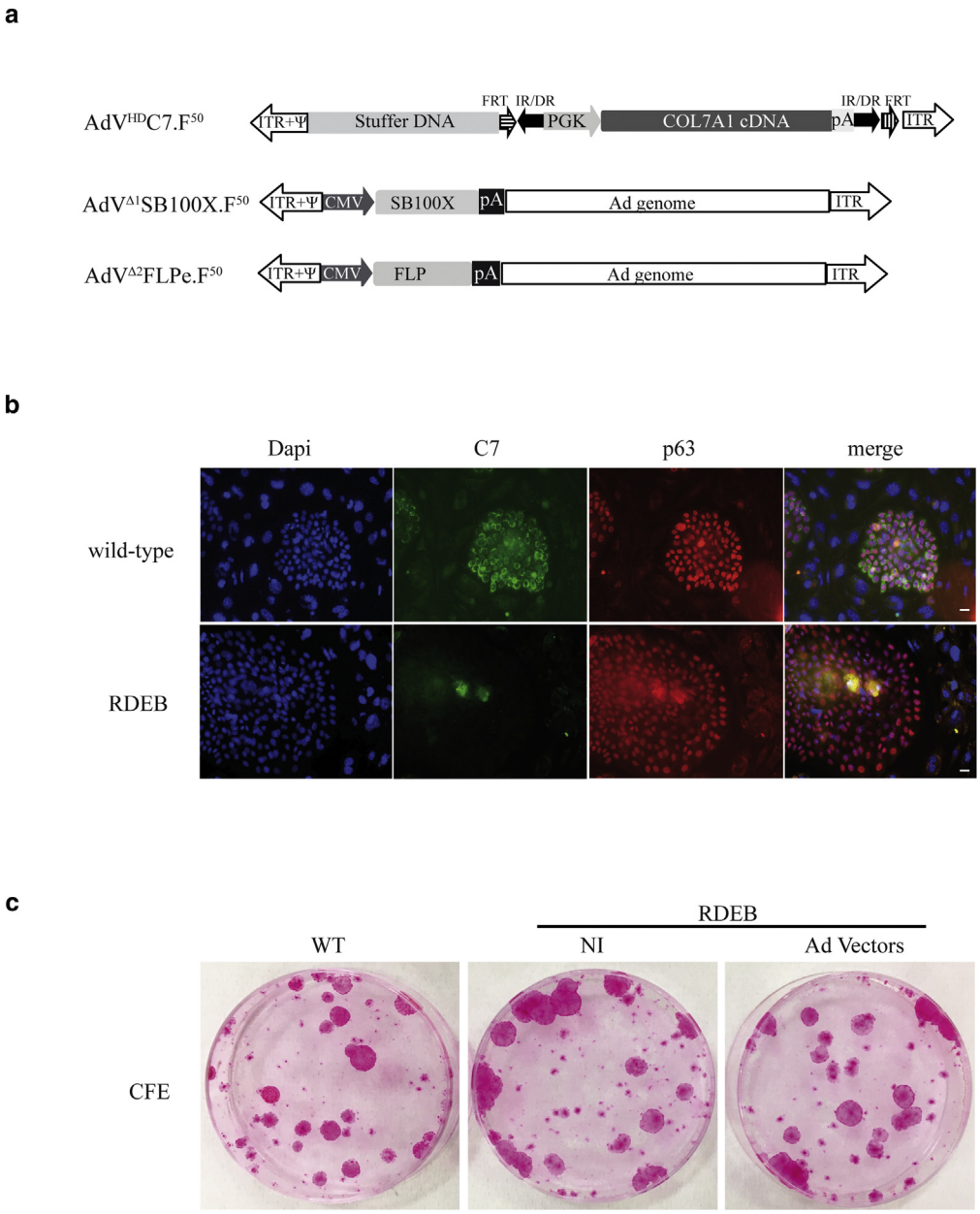

Figure 2. Characterization of adenoviral vectors and human primary keratinocytes from RDEB patient. (**a**) Schematic representation of the adenoviral vectors used. AdV^{HD}C7.F^{50} represents the HD adenoviral vector bearing the T2C7 transposon flanked by FRT sites. AdV$^{\Delta 1}$SB100X.F^{50} and AdV$^{\Delta 2}$FLPe.F^{50} represent the first- and second-generation adenoviral vectors carrying the transposase SB100X and FLPe recombinase, respectively. (**b**) Immunofluorescence for C7 (green) and p63 (red) expression in wild-type and RDEB keratinocytes. Scale bar = 20 μm. (**c**) CFE assays performed on WT, NI, and AdVs-infected RDEB keratinocytes (Ad Vectors) two passages after infection. CFE, colony-forming efficiency; FRT, flippase recognition target; HD, helper dependent; NI, not infected; RDEB, recessive dystrophic epidermolysis bullosa; WT, wild type.

used for cell therapy of third-degree burn wounds (Pellegrini et al., 1999; Ronfard et al., 2000). RDEB keratinocytes were infected as a passage 3 mass culture with a suspension of Ad vectors encoding C7, SB100X, and FLPe at MOI 250, 1000, 1000, respectively (Supplementary Figure S10 online). Immunofluorescence analysis of infected RDEB keratinocytes found a robust infection with the AdV^{HD}C7.F^{50} and AdV$^{\Delta 1}$SB100X.F^{50} vectors (Figure 3a). To demonstrate that the infection by Ad vectors did not affect the clonogenic potential of RDEB keratinocytes, we cultivated serially infected, uninfected cells and wild-type keratinocytes and calculated the number of cell doublings. As shown in Figure 3b, uninfected (black line) and genetically modified (red lines) RDEB keratinocytes exhibit similar population doublings and growth potentials, which were comparable to those of two healthy controls (grey lines). Accordingly, clonal conversion and progressive appearance of aborted colonies were similar in RDEB (uninfected and infected) and healthy epidermal cultures (data not shown).

In AdVs infected cells, the ratio between clonogenic and abortive colonies was not altered (Figure 3c). Colony-forming efficiency and C7-specific immunofluorescence analysis performed on AdVs-infected keratinocytes indicated the presence of clonogenic C7$^+$ colonies (Figure 3d). Six cell doublings (almost 15 days) after AdVs infection, immunofluorescence analysis found that transposition could be attained not only in p63-positive cells but in all types of clonogenic cells (Pellegrini et al., 2001; Senoo et al., 2007) (Figure 3e). The frequency of p63$^+$C7$^+$ colonies (29 of 34) scored in 4 different experiments was 85% and correlates with the frequency of p63$^+$ colonies in uninfected RDEB keratinocytes (41 of 50; 82%). The presence of C7 expression was followed up to 17 days after infection by quantitative real-time reverse transcriptase PCR and C7 cDNA was followed over time up to P9 (42 days after infection) by PCR

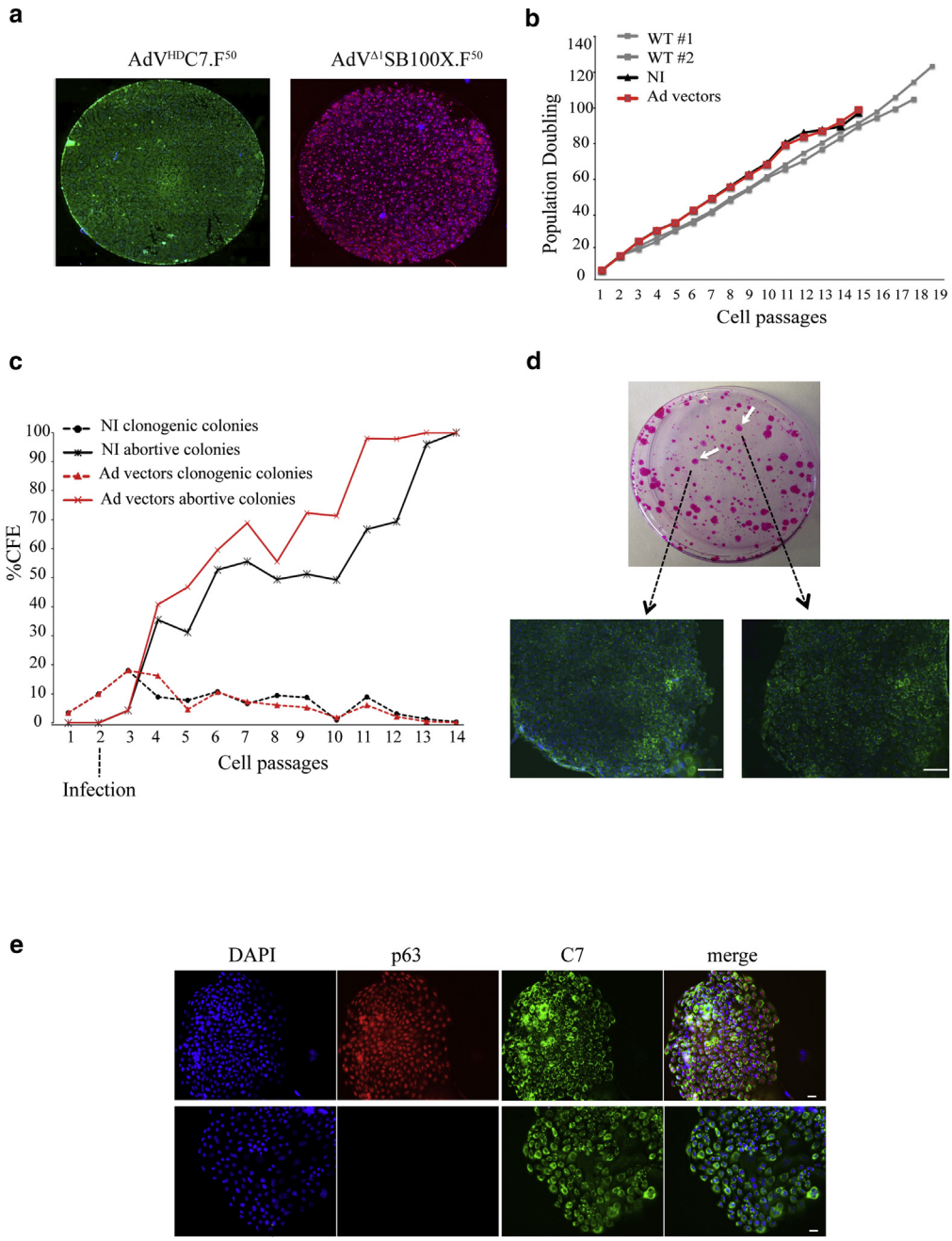

Figure 3. Restoration of full-length C7 protein expression in keratinocytes from RDEB patient. (**a**) Images of immunofluorescence on C7 and SB100X in RDEB keratinocytes upon AdV infection. (**b**) Population doublings underwent by two normal donors (WT 1 and 2; grey line), RDEB keratinocytes infected with AdVs (red line), and NI cells (black line). (**c**) Number of clonogenic (dashed line) and abortive colonies over the total colonies are reported in AdVs-infected cells (red line) and in NI cells (black line). (**d**) Digital image shows colonies of AdVs-infected keratinocytes. C7 expression in two transposed RDEB keratinocyte colonies are indicated by white arrows. Scale bar = 200 μm (**e**) C7 (green) and p63 (red) expression in two C7-transposed colonies. Scale bar = 20 μm. NI, not infected; RDEB, recessive dystrophic epidermolysis bullosa; WT, wild type.

(Supplementary Figure S11 online). To calculate the proportion of transposed clones, we serially diluted the AdV-treated (i.e., $AdV^{HD}C7.F^{50}$ + $AdV^{\Delta 1}SB100X.F^{50}$ + $AdV^{\Delta 2}FLPe.F^{50}$) bulk population 7 days after infection (3 cell doublings) to minimize the residual level of unintegrated $AdV^{HD}C7.F^{50}$ vector, and expanded 48 clones that underwent more than 20 cell doublings. Three clones (6%) resulted positive for the integration of C7 cDNA and negative for AdV backbone by PCR analysis (Supplementary Figure S12a and b online).

Functional correction of recessive dystrophic epidermolysis bullosa in vivo by transposed COL7A1 keratinocytes

To determine whether secreted C7 produced by *COL7A1*-transposed keratinocytes can restore the dermal-epidermal junction in vivo, we generated skin equivalents with a bulk culture of transposed RDEB keratinocytes, and grafted them on the dorsal region of immunodeficient (nu/nu) mice, as previously described (Larcher et al., 2007; Gache et al., 2004). Primary RDEB keratinocytes were infected with AdVs as describe above. Ten days after infection, the culture was plated on fibrin matrix to prepare 11 transplantable human skin equivalents to be grafted on 11 immunodeficient mice. Six of 11 mice that received the genetically modified skin equivalents were successfully grafted. Two mice died for reasons not related to the xenograft. The grafts from 4 mice were analyzed macroscopically, histologically, and molecularly 6, 10, and 12 weeks after transplantation, a time by which approximately 1.5 to 3 epidermal turnover cycles had occurred (Duarte et al., 2014). In Figure 4 we reported the results of 4 mice from 6 to 12 weeks after transplant.

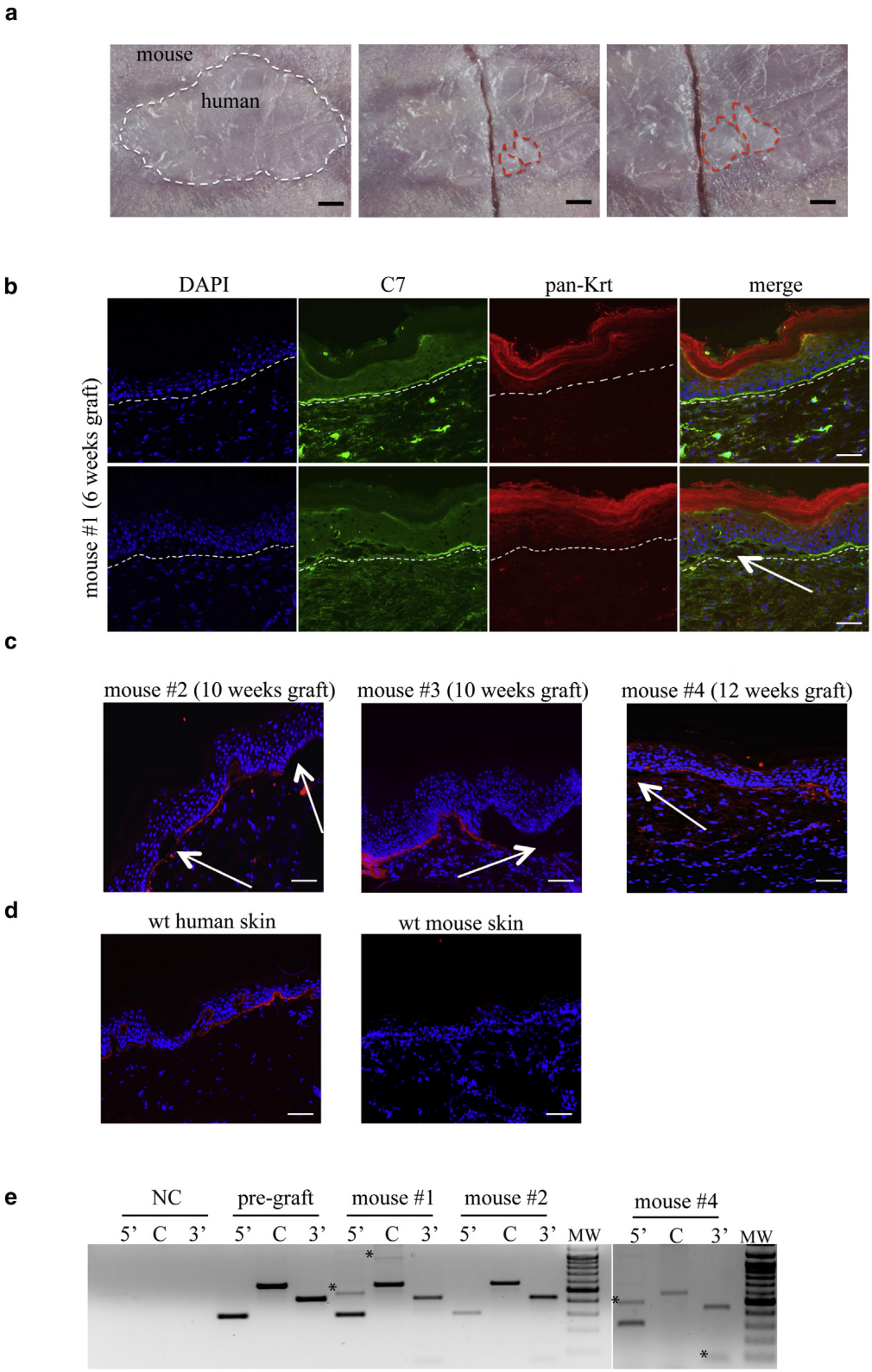

Figure 4. Transplantation of C7-transposed human RDEB keratinocytes onto mice. (**a**) Engrafted mouse shows human regenerated skin (white dashed area, left panel; scale bar = 1.6 mm) 6 weeks after grafting. Red dashed squares (right panel; scale bar = 1.1mm) indicate two blisters upon dissection (central panel; scale bar = 1.6mm). (**b**) Immunofluorescence analysis for the detection of human C7 and Pan keratin expression in sections of 6-week-old graft (mouse #1). Dotted line borders the mouse and the engrafted human skin. (**c**) Immunofluorescence analysis for the detection of human C7 in sections of 10-week-old graft (mouse #2 and #3) and 12-week-old graft (mouse #4). White arrows indicate blister areas. (**d**) WT human and mouse skin are positive and negative controls, respectively. Scale bar = 50 µm. (**e**) PCR analysis on 5′, 3′, and C of the C7 cDNA in NC RDEB keratinocytes, in pregraft C7-transposed bulk population, and in biopsies from mice #1, #2, and #4. *Unspecific signals. C, central region; NC, not treated; RDEB, recessive dystrophic epidermolysis bullosa; WT, wild type.

Macroscopic inspection was performed on mouse 1 (Figure 4a, left panel), and skin biopsy samples from engrafted skin were isolated for histologic examination and for PCR analysis to confirm C7 transgene integration. The macroscopic analysis found regeneration of a human skin with typical histologic architecture. Upon surgical dissection (Figure 4a, central panel), we observed derma-epidermal blister formations in two areas (Figure 4a, right panel), indicating the absence of C7 deposition in those areas. This finding was confirmed by immunofluorescence analysis with C7 and pan-keratin species-specific antibodies that confirm the human derivation of the grafted area and the

nonhomogenous C7 deposition in the derma-epidermal layer. As reported in Figure 4b, deposition of C7 in mouse 1, 6 weeks after graft, stained all along the section with the exception of some small area probably corresponding to the blisters. Human $C7^+$ staining was also observed in sections from mouse 2, 3, and 4, 10 and 12 weeks after grafting (Figure 4c), similarly to what obtained with wild-type human skin and differently from the mouse skin (Figure 4d). Blister areas were observed in all mice independently from the time points after graft. Molecular analysis of the human skin from mouse 1, 2, and 4 from 6 to 12 weeks after grafting, respectively, showed PCR-amplified bands accounting for the full-length C7 transgene (Figure 4e) indicating absence of gene rearrangement events in vivo. Qualitative and quantitative PCR signals from the HD-Ad vector backbone (Supplementary Figure S13 online) ruled out long-term persistence of the $AdV^{HD}C7.F^{50}$ vector in vivo. Collectively, these data provide evidence that human C7 expression from a polyclonal population of COL7A1-transposed keratinocytes can be achieved in vivo, contributing to restore the dermal-epidermal junction and adherence of the skin to the dermis.

DISCUSSION

Dystrophic epidermolysis bullosa is a severe monogenic genodermatosis caused by mutations in the *COL7A1* gene. The phenotypic variability of dystrophic epidermolysis bullosa depends on the types of mutations in *COL7A1* gene as well as on their position along the gene. The full spectrum of gene mutations in dystrophic epidermolysis bullosa remains elusive; thus, patient-tailored gene correction therapy represents a difficult task. Among the therapeutic strategies for RDEB, autologous transplantation of genetically corrected keratinocytes is a promising approach, which has been successfully exploited in the case of another skin adhesion defect, the LAMB3-deficient junctional epidermolysis bullosa (De Rosa et al., 2014; Mavilio et al., 2006). Gene correction of autologous RDEB keratinocytes has been obtained by stably integrating the full-length *COL7A1* cDNA by murine leukemia virus or HIV-derived retroviral vectors, which, however, led to significant transgene rearrangement and expression of C7 of abnormal size, which could not be completely controlled even in the best cases (Georgiadis et al., 2015). The presence of numerous repeated sequences in the ~9-kb-long *COL7A1* cDNA may trigger rearrangements during the retrotranscription process and the integration of truncated or incomplete proviruses into the host cell genome. An alternative approach is the use of DNA-based integration systems, such as transposition of DNA expression cassettes by the SB transposon and the engineered, hyperactive SB100X transposase, which was successfully used in the past to integrate relatively large expression cassettes in a variety of human cells (Mates et al., 2009; Escobar et al., 2016). The SB-mediated approach was also used in 2003 (Ortiz-Urda et al., 2003) to correct LAMB3-deficient junctional epidermolysis bullosa in vitro and in vivo upon xenotransplantation of gene-corrected human skin on immunodeficient mice. With respect to SB components used by Ortiz-Urda et al. (2003), we used the hyperactive SB100X transposase (Mates et al., 2009) and a new version of transposon IR, called T2 (Cui et al., 2002; Geurts et al., 2003) improve stable gene transfer of the large collagen VII cDNA in human RDEB keratinocytes without drug selection.

In this study, we generated a T2-based transposon plasmid carrying the full-length *COL7A1* cDNA and cotransfected it with an SB100X transposase plasmid in iRDEB keratinocytes, leading to about 30% transposition of at least 1 copy per cell of the *COL7A1* transposon. Molecular analysis of individual keratinocyte clones showed transposase-mediated integration of intact-size *COL7A1* cDNA, which led to intracellular and secreted C7 protein synthesis at levels comparable to that of keratinocytes from healthy donors. Southern blot analysis for the integrity of collagen VII cDNA did not exclude micro-rearrangements in the coding sequence, although we detected by western blot analysis a collagen VII protein of comparable size to that observed in wild-type keratinocytes. To reproduce the autologous skin transplantation strategy previously used to correct LAMB3 deficiency in junctional epidermolysis bullosa patients (Mavilio et al., 2006) in a preclinical model, we attempted to genetically correct primary, early passage keratinocytes from a RDEB patient with the SB transposon-based system. Because human primary keratinocytes are refractory to physico-chemical transfection, we vectorized the C7 transposon carrying the *COL7A1* gene and flanked by FRT sites into helper-dependent, also known as high-capacity, AdV (i.e., $AdV^{HD}C7.F50$), and the SB100X transposase into a separate, first-generation AdV (i.e., $AdV^{\Delta1}SB100X.F^{50}$). As previously reported, AdVs represent the system of choice to transiently and efficiently deliver genetic material into human keratinocytes without affecting their clonogenic potential (Coluccio et al., 2013; Chamorro et al., 2016). Because the mechanism of transposition identified the circular transposon forms as optimal substrate for SB-mediated integration (Hausl et al., 2010; Yant et al., 2002), we developed a second-generation AdV carrying a *FLPe* recombinase expression unit (i.e., $AdV^{\Delta2}FLPe.F^{50}$) to mediate circularization of the FRT-flanked transposon from the $AdV^{HD}C7.F^{50}$ linear genome that can subsequently serve as a substrate for the SB100X transposase. RDEB keratinocytes infected with this AdV-based gene delivery platform grew in culture and had a cloning efficiency similar to that of untreated cells, demonstrating the nontoxicity of the delivery system. Transposition events restored the synthesis of C7 protein expression and secretion in treated iRDEB and resulted in a transposition of C7 cDNA in 6% of the clonogenic primary RDEB keratinocytes infected with AdVs. We detected p63α transcription factor in the $C7^+$, transposed colonies, indicating that transposition events may occur in a progenitor/stem cell compartment.

To show the efficacy of transposition in restoring normal functional properties in skin derived from transposed RDEB keratinocytes, we used a validated preclinical model to study human skin in vivo, based on xenotransplantation of skin equivalents on immunodeficient mice (Del Rio et al., 2002; Gache et al., 2004; Larcher et al., 2007; Coluccio et al., 2013; Duarte et al, 2014). Despite the relatively low number (<10%) of successfully transposed keratinocytes in the culture obtained to derive the skin equivalents, we observed that the grafted skin, clearly demarcated by human-murine junctional boundaries, contained C7-corrected keratinocytes able to produce and deposit C7 protein at the dermal-epidermal junction.

This finding shows that transposition occurred in bona fide, graft maintaining epithelial progenitor/stem cells and that an in vivo selection effect, most likely caused by competition of the adhering versus nonadhering keratinocytes, allows restoration of skin adhesion properties even starting from a relatively low number of gene-corrected clonogenic cells. Transposase-mediated integration of full-length *COL7A1* cDNA was demonstrated by PCR analysis on genomic DNA derived from pregraft and postgraft keratinocytes.

This study shows the feasibility of correcting a recurrent RDEB mutation in human keratinocytes by the SB transposon integration system safely delivered to keratinocyte progenitor or stem cells by nonintegrating Ad vectors. This molecular tool allows the ex vivo manipulation of keratinocytes obtained from RDEB patients' skin biopsies to generate transplantable human skin for therapeutic purposes. Although the level of gene correction in clonogenic RDEB keratinocyte is far from being optimized, the C7 deposition along the dermo-epidermal junction obtained in our in vivo model was sufficient to sustain skin adhesion. Overall, transposition efficiency in primary stem cells remains a challenging task. Efforts should be dedicated to ameliorating the delivery of circular transposon forms from the adenoviral shuttle by the FLPe/FRT recombinase or similar systems and in general the co-infection by different AdVs. Increasing transposition rate in keratinocyte cultures is necessary to achieve robust levels of transgene integration in graft-maintaining epithelial progenitor or stem cells, a mandatory prerequisite for clinical translation of this technology.

MATERIALS AND METHODS

Cell cultures

The cell cultures used in this study are immortalized RDEB keratinocytes (Chamorro et al., 2013), mouse 3T3-J2 fibroblasts (Todaro and Green, 1963), and wild-type and RDEB human keratinocytes. Details of culture conditions are reported in Supplementary Methodology.

Immunofluorescence analysis

Immunofluorescence analysis for C7 expression was performed on iRDEB, normal donor keratinocytes, RDEB keratinocytes, and skin biopsies from mice 2−4 using mouse monoclonal LH7.2 antibody (Sigma-Aldrich, Milan, Italy). Instead for skin biopsy derived from mouse 1, we used rabbit polyclonal anticollagen VII antibody (Abcam; Cambridge, United Kingdom) and mouse pan-keratin antibody (cell signalling). For p63 staining, we used rabbit polyclonal p63 antibody (custom made). Immunofluorescence for SB transposase was performed with the goat SB transposase antibody (RD Systems; Space Import Export, Milan, Italy). Details of secondary antibody and immunofluorescence's protocol are reported in Supplementary Methodology.

Bioengineered skin preparation and grafting on immunodeficient mice

C7-transposed primary keratinocytes were seeded on RDEB fibroblast-containing fibrin dermal equivalents prepared as previously described (Larcher et al., 2007; Gache et al., 2004). Briefly, each 9-cm^2 dermal equivalent containing RDEB fibroblasts was seeded with 5 x 10^5 keratinocytes. The size of engrafted skin is variable, although an average area is around 3−4 cm^2. Bioengineered skin equivalents were grafted onto the back of immunodeficient nu/nu mice ($n = 11$) according to Del Rio et al. (2002). Successful engraftments occur in 6 of 11 mice. Two mice died for reasons not related to the graft. Six, 10, and 12 weeks after grafting, four mice were killed and grafts harvested for skin immunohistochemistry and molecular analysis. Animal studies were approved by our institutional animal care and use committee according to all legal regulations.

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CONFLICT OF INTEREST

Michele De Luca is a member of the board and cofounder of Holostem Terapie Avanzate srl.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at http://dx.doi.org/10.1016/j.jid.2016.11.038.

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