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Remodeling of the dermal-epidermal junction in bilayered skin constructs after silencing the expression of the p.R2622Q and p.G2623C collagen VII mutants

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Abstract

The integrity of skin depends on a complex system of extracellular matrix molecules that form a biological scaffold. One of its elements is the dermal basement membrane that provides a link between the epidermis and the dermis. Mutations in collagen VII, a key component of the dermal membrane zone, are associated with dystrophic epidermolysis bullosa. Although it has been proposed that silencing the mutated *COL7A1* allele is a promising approach to restore the dermal basement membrane zone formed in the presence of collagen VII mutants, limitations exist to testing this proposal. Here, we employed a model that utilized skin-like constructs in which engineered collagen VII mutant chains harboring the R2622Q or G2623C substitution were expressed conditionally, but the wild type chains were expressed unconditionally. We demonstrated that switching off the production of the mutant collagen VII chains in skin constructs restores the organization of collagen VII and laminin 332 deposits in the dermal-epidermal junction to the level of control. We also demonstrated that remodeling of collagen IV deposits was not fully effective after silencing the expression of collagen VII mutants. Thus, our study suggests that while silencing mutant alleles of *COL7A1* may repair critical elements of the affected dermal basement membrane, it may not be sufficient to fully remodel its entire architecture initially formed in the presence of the mutant collagen VII chains.

Introduction

The integrity of the dermal-epidermal junction (DEJ) of skin depends on the complex structure of the dermal basement membrane formed by macromolecules produced by dermal fibroblasts and keratinocytes [1]. Key components of the dermal basement membrane include collagen IV, laminin 332, laminin 311, laminin 511, and nidogen [2-6]. These molecules engage in homotypic and heterotypic interactions to form an interlacing network which serves as a tightly-attached barrier between the epidermal and dermal layers. An additional element of the basement membrane zone (BMZ) critical for the dermal-epidermal

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bond is collagen VII [7, 8]. This protein consists of an extended triple-helical domain flanked by the N-terminal NC1 domain and the C-terminal NC2 domain [9]. Collagen VII self-assembles into anchoring fibrils whose function is to provide the link between the dermal basement membrane and the underlying stroma [7, 8]. It has been demonstrated that at the basement membrane level the NC1 domains of collagen VII bind with collagen IV and laminin 332 while on the site of the stroma the arcades of anchoring fibrils enlase with collagen fibrils [10-12]. This spatial arrangement of the anchoring fibrils constitutes a key structural characteristic of the DEJ.

A critical role of the anchoring fibrils in maintaining the stability of the DEJ was proposed by Briggaman *et al.* who suggested that the pathological features of the skin of certain patients with dystrophic epidermolysis bullosa (DEB), a rare blistering disease, are caused by the lack of anchoring fibrils [13]. Subsequently, it was determined that DEB is associated with mutations in *COL7A1* which can be inherited in an autosomal dominant (DDEB) or an autosomal recessive (RDEB) pattern [14, 15]. To date, a number of mutations in *COL7A1* were detected and experimental therapeutic approaches targeting the fundamental causes of DEB were proposed [16]. The majority of these approaches tested to date focus on DEB cases in which the amount of collagen VII is significantly reduced or in which this protein is totally missing [17]. A common feature of approaches to restore the function of the DEJ is to deliver the wild type collagen VII to affected sites by employing collagen VII-producing cells, by employing viral vectors to enable expression of the wild type collagen VII, or by applying a purified recombinant wild type collagen VII protein [16, 18, 19]. With regard to cases in which collagen VII mutants exert their pathological effects in a dominant fashion, it was suggested that the selective silencing of the mutant allele could be the right approach to reducing these negative effects [18]. To pursue this approach, it would be important to establish the extent to which the structure of the DEJ formed in the presence of mutant collagen VII chains remodels after silencing their expression. To address this problem, we have generated an experimental system exploiting the expression of the wild type and mutant chains of recombinant mouse collagen VII in skin-like constructs [20-22]. In this system the wild type chains are constantly and unconditionally expressed, while the expression of the mutant chains is tetracycline (Tet)-dependent. Utilizing this system, we generated skin-like constructs in which collagen VII molecules harboring mutant chains were initially expressed but whose expression was later switched off. This experimental system, representing the most effective silencing of the expression of a mutant but not a wild type allele, allowed us to determine the ability of the BMZ formed in the presence of selected collagen VII mutants to remodel upon switching off their production. The results of our study provide a few important observations on the consequences of such inhibition: (i) complete inhibition of the expression of the mutant chains effectively restores the organization of collagen VII in the DEJ of model skin-like constructs, (ii) upon switching off the expression of mutant collagen VII chains, patterns of the restoration of some BMZ components, illustrated here by laminin 332, parallel those of collagen VII, and (iii) after switching off the expression of mutant collagen VII chains, patterns of the restoration of certain BMZ components, represented here by collagen IV, may not parallel those of collagen VII.

Materials and Methods

Mutation nomenclature

Following the Human Genome Variation Society guidelines (www.hgvs.org), the mutations are numbered from the ATG start codon of the DNA sequence encoding the full-length human collagen VII and are listed with a “p” included in the mutation name.

DNA constructs for conditional expression of GFP-tagged procollagen VII mutant α chains and unconditional expression of the RFP-tagged wild type chains

DNA constructs encoding mouse mini-procollagen VII consisting of a truncated triple helical region flanked with intact N-terminal and C-terminal propeptides and harboring selected single amino acid substitutions (MTmProVII) are described elsewhere [20, 21]. The GFP-tagged versions of those mutant constructs (MTmProVII-GFP) cloned into the pAcGFP1-Hyg-N1 vector (Clontech Laboratories, Inc.) as well as DNA constructs encoding RFP-tagged wild type mini-procollagen VII α chain (WTmProVII-RFP) cloned into the pLenti6/V5-Dest (Invitrogen, Inc.) are also described elsewhere [21]. Here, to enable the conditional expression of the MTmProVII-GFP variants, relevant DNA constructs were cloned into the NheI/NotI sites of the pTRE2pur vector which includes a Tet-responsive promoter (Clontech Laboratories, Inc.). The fidelity of the Tet-regulated MTmProVII-GFP DNA constructs (MTmProVII-GFP^{Tet}) was determined by DNA sequencing.

Co-expression of the MTmProVII-GFP^{Tet} and the WTmProVII-RFP α chains in HEK293 cells

Co-expression of the MTmProVII-GFP^{Tet} and the WTmProVII-RFP chains was accomplished by employing HEK293 cells, as described [21]. In the study presented here, the conditional Tet-On expression of the MTmProVII-GFP^{Tet} variants was achieved by employing a Tet-responsive HEK293 cell line (Clontech Laboratories, Inc.). First, cells were transfected with constructs encoding p.R2622Q or p.G2623C MTmProVII-GFP^{Tet} variants. Subsequently, the puromycin-resistant clones were selected and analyzed for the Tet-dependent production of the MTmProVII-GFP^{Tet} chimeras, according to described methods [23]. In brief, the presence of GFP-positive clones was analyzed by fluorescence microscopy. In addition, the secretion of the MTmProVII-GFP^{Tet} variants into the extracellular space by cells cultured in the presence of doxycycline (Dox) added to cell culture media at 1 μ g/ml was demonstrated with Western blot assays. In these assays anti-GFP polyclonal antibodies (Santa Cruz Biotechnology Inc.) and anti-collagen VII antibodies (EMD Biosciences) were employed, as described [21].

Clones producing the p.R2622Q or p.G2623C MTmProVII-GFP^{Tet} variants were then transfected with a DNA construct encoding the WTmProVII-RFP α chain. Stable expression of the WTmProVII-RFP α chain in selected clones was confirmed by fluorescence microscopy and by Western blot assays with the use of anti-RFP antibodies (Invitrogen Inc.). When cultured in the presence of Dox, HEK293 cells selected in the above process produce the mProVII α chains able to assemble into homotypic triple helices and into heterotypic triple-helical molecules consisting of the wild type and mutant chains, as described [21]. In contrast, in the absence of Dox, these cells produce only homotypic WTmProVII-RFP molecules (Fig. 1).

Fibroblasts and keratinocytes from *COL7a1*^{-/-} mice

Immortalized fibroblasts and keratinocytes derived from the skin of mice with an inactivated *COL7a1* gene were a gift from Dr. John Klement, Department of Dermatology and Cutaneous Biology, Thomas Jefferson University, Philadelphia, PA [24].

Assays of the production of selected extracellular matrix proteins by mouse fibroblasts, keratinocytes, and HEK293 cells

The production of selected endogenous proteins critical for the structural integrity of the bilayered skin-like constructs (BSC) was analyzed by immunoblotting. Fibroblasts and HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 40 µg/ml of L-ascorbic acid phosphate magnesium salt n-hydrate (Waco Pure Chemical Co.). Mouse keratinocytes were cultured in a 1:1 mixture of F12 and DMEM (Mediatech Inc.) supplemented with 10% FBS, 40 µg/ml of L-ascorbic acid phosphate magnesium salt n-hydrate (Waco Pure Chemical Co.), and keratinocyte growth supplements (KGM-2, Lonza). Upon reaching confluency, proteins secreted to the cell culture media were collected by precipitation with 5% polyethylene glycol (8,000 Mw, Sigma-Aldrich Co.) and then analyzed by Western blot. Specifically, collagen I was detected with anti-collagen I antibodies (Santa Cruz Biotechnology Inc.), collagen III with anti-collagen III antibodies (Santa Cruz Biotechnology Inc.), collagen IV with anti-collagen IV antibodies (Millipore), and laminin 332 was detected with the anti-β3 antibody (Santa Cruz Biotechnology Inc.). The production of collagen VII by analyzed cells was evaluated with anti-collagen VII antibodies (EMD Biosciences).

Bilayered skin-like constructs (BSC)

BSC were prepared according to the published methods [25, 26]. Certain modifications were introduced to accommodate some unique requirements of the cell-specific experimental model employed here. In brief, fibroblasts from *COL7a1*^{-/-} mice and HEK293 cells co-expressing MTmProVII-GFP^{Tet} variants harboring p.R2622Q or p.G2623C substitutions and the WTmProVII-RFP chain were cultured in DMEM supplemented with 10% FBS. Subsequently, cells were harvested and counted. Next, 5×10⁶ of fibroblasts and 1×10⁶ of HEK293 cells were suspended in 0.5 ml of a mixture consisting of 3 mg/ml of neutralized collagen (BD Biosciences), 7 mg/ml of fibrinogen (Sigma-Aldrich), 20 mM of CaCl₂, and thrombin at 5U/ml (Sigma-Aldrich). Subsequently, 0.4-ml samples were distributed into wells of 12-mm diameter Transwell inserts (Corning Inc.). Collagen/fibrin gels with entrapped cells were allowed to set for 30 min at 37°C, and then the inserts were transferred to 10-cm diameter culture dishes (Sigma-Aldrich). Stainless steel mesh inserts were placed at the bottoms of these dishes to lift the Transwell inserts, thereby providing the optimal contact of their porous bottoms with cell culture media. Four inserts were placed into one dish, and then gel/cell constructs representing the dermal component of skin were covered with 75 ml of media. This relatively high culture media-BSC volume ratio was necessary to provide enough nutrients for the high-cell density constructs.

After two days of culture, the cell layer representing the epidermal part of the skin was created by seeding 4×10⁴ keratinocytes derived from *COL7a1*^{-/-} mice on the upper surface

of each of the dermal constructs. Subsequently, BSC were maintained for 4-6 weeks in cell culture media consisting of a 1:1 mixture of F12 and DMEM (Mediatech Inc.) supplemented with 10% FBS, 40 µg/ml of L-ascorbic acid phosphate magnesium salt n-hydrate (Waco Pure Chemical Co.), and keratinocyte growth supplements (KGM-2, Lonza). This cell culture regime was established after running a series of pilot experiments. In these experiments, attention was paid to ensure that at the end of culture, the BSC was characterized by an intact structure of the dermal layer and a cohesive layer of keratinocytes.

Because of the opacity of the BSC, keratinocytes present on the top of these constructs were not readily visible via a microscope. To monitor the keratinocytes' morphology throughout the duration of experiments, parallel pilot samples were also prepared. These pilot samples were constructed by setting a cell-free thin layer of collagen/fibrin gels in tissue culture plates. Keratinocytes seeded on polymerized pilot gels were maintained in conditions identical to those used for the BSC.

Microscopic assays of cultured BSC

Throughout the duration of culture, dermal and epidermal layers of the BSC were monitored by microscopy. For evaluation of cells present in the dermal layer, the analyzed skin constructs were placed in a cell culture dish fitted with a glass bottom (MatTek Co.). Subsequently, employing an inverted fluorescence microscope, the presence of HEK293 cells expressing fluorescently-tagged mProVII variants in the BSC was visualized (Eclipse Ti, Nikon Instruments Inc.). For evaluation of the epidermal component of the BSC, keratinocytes seeded on thin layers of the pilot collagen/fibril gels were observed with the use of an upright microscope (Eclipse E600, Nikon Instruments Inc.). Due to the relatively low contrast between the unstained cells and the gel layer, the morphology of keratinocytes was observed using the differential interference contrast (DIC) technique.

Conditional expression of the p.R2622Q or p.G2623C MTmProVII-GFP^{Tet} variants in BSC

BSC were employed to study the effects of switching off the production of the MTmProVII-GFP^{Tet} α chains on the remodeling of the epidermal-dermal interface initially formed in the presence of the mutant chains. In the main experimental 2ON/2OFF group, BSC formed by cells from *COL7a1* ^{-/-} mice and by those expressing the WTmProVII-RFP and MTmProVII-GFP^{Tet} variants were initially cultured for two weeks in the presence of 1 µg/ml of Dox; this 2ON/2OFF regime was established based on a series of pilot assays in which we found that two weeks is enough for the DEJ to develop into a well defined structure, an observation consistent with earlier reports [27]. In these culture conditions both wild type and mutant mProVII α chains are produced and fold into homotypic and heterotypic molecules consisting of only wild type, only mutant, and both types of chains, respectively [21]. As suggested by Marinkovich *et al.*, molecules produced in the dermal layer of skin equivalents diffuse throughout them and, together with proteins synthesized by keratinocytes, interact to form elements of the extracellular matrix of skin [28]. Next, after two weeks of culture, Dox was omitted from cell culture media and the culture of BSC proceeded for an additional two weeks. In these conditions the expression of the WTmProVII-RFP chain continued while the expression of the MTmProVII-GFP^{Tet} variants ceased (Fig. 1). In addition to the 2ON/2OFF group the following control groups were also

studied: (i) 2ON group in which BSC were cultured in the presence of Dox for two weeks, (ii) 4ON group in which BSC were cultured in the presence of Dox for four weeks, and (iii) 4OFF group in which BSC were cultured in the absence of Dox for four weeks (see Fig. 1). Each of these groups consisted of multiple discs that were later analyzed in biochemical and microscopic assays. Moreover, a control group of BSC in which transfected HEK293 cells were replaced with untransfected ones was also prepared. To exclude any potential influence of Dox on the morphology of BSC, we also prepared yet another control group which included the previously described HEK293 cells transfected only with a DNA construct encoding the WTmProVII-RFP variant whose expression is Tet-independent [21]. These control BSC constructs expressing only the WTmProVII-RFP were cultured in the presence of Dox for control purposes. Subsequently, the morphology of their DEJ was evaluated, as described below. Yet another control group consisted of BSC formed by untransfected HEK293 cells co-cultured with normal murine keratinocytes and fibroblasts (CELLnTEC Advanced Cell Systems).

Qualitative Western blot and reverse transcription polymerase chain reaction (RT-PCR) assays of expression of selected proteins in BSC

At selected time points BSC from analyzed groups were collected and processed for protein and RT-PCR assays. In brief, for protein assays collected BSC were frozen in liquid nitrogen and then crushed. Pulverized samples were suspended in a lysis buffer consisting of 1% SDS, 1% sodium deoxycholate, 0.1% Triton X-100, 10 mM EDTA, 3% β -mercaptoethanol, and a mixture of protease inhibitors (Thermo Scientific). Subsequently, the samples were briefly sonicated and then any insoluble material was removed by centrifugation.

Because of the high-cell density conditions present in the BSC it is expected that the majority of chromophore-tagged C propeptides are enzymatically processed, the total mProVII was assayed by Western blot with the use of the anti-collagen VII antibodies (EMD Biosciences) instead of those for GFP and RFP. Moreover, the production of the intact intracellular pool of WTmProVII-RFP and the MTmProVII-GFP subpopulations was monitored directly by an inverted fluorescence microscope as described above. In addition to mProVII variants, BSC collagen IV and laminin 332 were analyzed by Western blot with the anti-collagen IV (Millipore) and anti- β 3 laminin chain antibodies (Santa Cruz Biotechnology Inc.), respectively.

For the qualitative RT-PCR collected BSC were processed for the isolation of total mRNA (RNeasy kit, Qiagen). Subsequently, the expression of selected markers was analyzed. Table 1 presents the list of analyzed markers and corresponding primers employed in the RT-PCR assays (RT-PCR Master Mix, Affimetrix, Inc.).

Microscopic assays of the BMZ formed between epidermal and dermal layers of BSC

The effects of the presence of the p.R2622Q or p.G2623C MTmProVII variants and the effects of switching off their expression on the formation and remodeling of the dermal-epidermal interface were analyzed by fluorescence microscopy. Specifically, BSC collected at various time points were fixed in a zinc fixative (BD Biosciences), and then the BSC

samples were embedded in a gel consisting of 2% bactoagar (BD Biosciences) and 2.5% gelatin (Sigma-Aldrich), as described [29]. Next, the gel-embedded samples were gradually dehydrated in ethanol. After dehydration, the gel-embedded BSC discs were cut along their diameter followed by embedding in paraffin. Subsequently, the histological specimens were prepared and stained with hematoxylin-eosin (H&E) for visualization of the general morphology.

The morphology of the interface between the layers of keratinocytes and gel-embedded fibroblasts and HEK293 cells was analyzed by immunostaining. In brief, samples were rehydrated and washed with phosphate buffered saline (PBS) containing 0.05% Tween-20 (PBST). Next, the samples were treated for 30 min at room temperature (RT) with Chondroitinase ABC (Sigma-Aldrich) solubilized in 50 mM Tris, 60 mM CH₃COONa, pH 8.0. After washing with PBST, the samples were treated with normal sheep serum (Santa Cruz Biotechnology Inc.) followed by blocking with casein blocking solution (Thermo Scientific). The primary polyclonal anti-collagen IV antibody (Millipore) at 1:50 dilution, anti- β 3 laminin chain antibodies (Santa Cruz Biotechnology Inc.) at 1:20 dilution or the NC1AF7 polyclonal anti-collagen VII antibody [20] at 1:100 dilution were added to the specimens in 5% normal goat serum (Santa Cruz Biotechnology Inc.) for 1h at RT. Subsequently, the secondary polyclonal goat anti-rabbit IgG antibodies conjugated with AlexaFluor 594 (Invitrogen Inc.) were applied in 5% normal goat serum. Control groups in which the primary antibodies were omitted or substituted with normal rabbit IgG (Santa Cruz Biotechnology Inc.) added at 1:50 dilution were also prepared. Finally, the nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI), and then the analyzed specimens were mounted with the use of the ProLong Gold antifade reagent (Invitrogen Inc.). Control native mouse skin samples were processed in a similar way.

Immunostained specimens were visualized with the use of a fluorescence microscope (Eclipse E600, Nikon Instruments Inc.) equipped with a digital camera (DS-Qi1Mc and DS-Fi1, Nikon Instruments Inc.). To ensure objective downstream measurements of the pixel intensities of analyzed interface regions, the digital images of all analyzed samples were taken with the identical camera settings. The pixel intensities of interfaces immunostained for collagen IV, laminin 332, and collagen VII were analyzed with the NIS-Elements image analysis program (Advanced Research version, Nikon Instruments Inc.). Data from a number of independent experiments, defined as those started with the preparation of BSC from cultured cells, were collected, and then mean pixel intensity values for each analyzed experimental group *i.e.* 2ON, 4ON, 2ON/2OFF, and 4OFF were calculated. Since in the 4OFF groups no mutant chains were produced, this group served as the control for the 2ON, 4ON, and 2ON2OFF groups. The final results were plotted as 2ON/4OFF, 4ON/4OFF, and 2ON2OFF/4OFF ratios.

Results

Production of selected macromolecules by BSC cells

In preparation for creating BSC, the involved cells were tested for production of critical macromolecules. Employing Western blot assays, we demonstrated that collagen I and collagen III were produced only by fibroblasts, while laminin 332 was secreted primarily by

keratinocytes; collagen IV was secreted by all analyzed cells. Western blot assays with anti-collagen VII antibodies demonstrated that this protein was only produced by transfected HEK293 cells (Fig. 2).

Expression of mProVII variants

The ability of the WTmProVII-RFP chains and the MTmProVII-GFP mutant chains to form stable triple-helical molecules was thoroughly characterized [21]. In these earlier studies we demonstrated a similar amount of MTmProVII-GFP mutants produced by transfected HEK293 cells. Moreover, the ability of procollagen C proteinase (PCP) to catalyze the critical cleavage of the C-terminal propeptide of recombinant procollagen VII was also demonstrated [22].

The expression system employed here utilizes the Tet-dependent expression of the MTmProVII-GFP^{Tet} chains and unconditional production of the WTmProVII-RFP. The Tet-dependent comparable production of the MTmProVII-GFP^{Tet} variants was confirmed by Western blot and microscopic assays; as demonstrated in Fig. 3, the GFP-positive MTmProVII-GFP^{Tet} variants were detected only in the presence of Dox. In contrast, continuous expression of the WTmProVII-RFP chains was Dox-independent (Fig. 3).

BSC cultures

Fibroblasts and keratinocytes isolated from the skin of *COL7a1*^{-/-} mice were employed to assemble BSC. In addition, HEK293 cells engineered to express WTmProVII-RFP and MTmProVII-GFP^{Tet} chains were also employed. The decision to employ HEK293 cells as a delivery vehicle for collagen VII instead of *COL7a1*^{-/-} fibroblasts and/or keratinocytes was dictated by two major factors: (i) our attempts to transfect or infect those cells with a set of required DNA constructs and to select clones stably expressing mProVII variants were largely unsuccessful, and (ii) we were interested in studying the utility of engineered cells, other than fibroblasts and keratinocytes, as a delivery vehicle of collagen VII to the DEJ site. At present, we cannot conclude why the *COL7a1*^{-/-} fibroblasts and keratinocytes failed to stably express sets of DNA constructs needed to achieve long-term Tet-dependent expression needed to establish a BMZ in the BSC. Of note, however, is our observation that those cells were extremely sensitive to the sets of antibiotics (see Material and Methods) needed to select positive clones expressing the designed mProVII variants in a Tet-dependent and Tet-independent fashion. In contrast, the HEK293 cells fulfilled all requirements imposed by our specific experimental design of the Tet-dependent production of the MTmProVII-GFP^{Tet} chains and the Tet-independent production of the WTmProVII-RFP chain, thereby rendering them an ideal candidate for the employed model.

The dermal part of the BSC consisted of cells embedded into a collagen/fibrin gel, while the epidermal part was formed by keratinocytes seeded on the top of gel constructs (Fig. 4). The addition of fibrin was dictated by our observation that the keratinocytes we employed did not spread well on pure collagen gels (Fig. 4), an observation consistent with an earlier report [30]. In contrast, in the presence of fibrin, these keratinocytes attached and spread well throughout the duration of BSC culture (Fig. 4). As demonstrated in Fig. 4, the BSC

cultured for four weeks formed stable structures with well-defined epidermal and dermal layers.

Qualitative RT-PCR assays of selected markers in BSC cultured in the presence and in the absence of Dox

The continuous production of macromolecules critical for the structure of the analyzed BSC was studied by monitoring the expression of selected markers listed in Table 1. As indicated in Fig. 5, all analyzed markers were expressed in a way consistent with the applied cell culture conditions. For instance, the presence of the RT-PCR product spanning the MTmProVII/GFP junction site in the MTmProVII-GFP^{Tet} variants seen in Tet-On conditions and its absence in Tet-Off conditions indicates a Tet-dependent expression of the analyzed mutants. Similarly, the continuous expression of the WTmProVII-RFP variant represented by a specific RT-PCR product further indicates the predicted behavior of the designed experimental system. The presence of the RT-PCR product corresponding to the NC1 domain of collagen VII provides further evidence of the expression of intact mProVII variants in the employed experimental system. Furthermore, the constant expression of genes encoding the $\alpha 1$ chain of collagen IV, the $\alpha 3$ and $\gamma 2$ chains of laminin 332, and nidogen 1 and nidogen 2 provides strong evidence for the robust production of macromolecules relevant to the formation of the BMZ in the model BSC employed here. Moreover, the continuous expression of caspase 14, a member of the caspase family whose expression is restricted to epidermal keratinocytes, and integrin $\alpha 6$, as well as the robust expression of markers for collagen I and collagen III chains, ensures the viability of keratinocytes and fibroblasts through the entire culture of the BSC (Fig. 5) [31-33]. The generally unchanged expression of the analyzed cell-specific markers observed throughout the period of the described experiments indicates that the initial ratios of cells seeded into the BSC were largely preserved at the end of the experiment. PCR data seen in Fig. 5 together with protein data presented in Fig. 3 indicate overall comparable levels of the expression of the MTmProVII-GFP^{Tet} and WTmProVII-RFP chains in HEK293 cells.

Qualitative Western blot and microscopic assays of proteins produced in BSC in the presence and in the absence of Dox

In addition to the broad RT-PCR-based screening of the expression of selected markers, a marked accumulation of analyzed proteins in the BSC was demonstrated by immunoblot assays. Figure 6 shows the accumulation of mProVII variants and collagen IV. Similar accumulation was also observed for laminin 332 (not shown). In addition to collagen VII-specific bands corresponding to the individual mProVII chains, cross-linked or aggregated forms of those chains were also apparent (Fig. 6); such an aggregation is expected in the high-cell density of tissue-like BSC.

The unconditional character of the expression of the WTmProVII-RFP α chains and the Tet-dependent expression of the MTmProVII-GFP^{Tet} α chains was followed not only by Western blot assays but also by direct observation of the expression of those variants with the use of fluorescence microscopy. These assays clearly demonstrate that the WTmProVII-RFP variant was expressed continuously, while the expression of the MTmProVII-GFP variants was Tet-dependent (Fig. 6).

Formation of DEJ in BSC cultures

To analyze the BMZ formed only in the presence of the wild type mProVII, a control group, consisting of the BSC in which HEK293 cells were transfected only with a DNA construct for the WTmProVII-RFP expressed in the Tet-independent fashion, was analyzed (Fig. 7). A microscopic assessment of the organization of the BMZ was carried out by visualization of collagen IV, laminin 332, and collagen VII deposits. Based on these assays, we determined that the analyzed molecules were well organized within the clearly-defined DEJ (Fig. 7). Since this control group was cultured in the presence of Dox added for control purposes, the above observation indicates that this antibiotic had no apparent effects on the formation of the DEJ in the studied skin constructs. The staining patterns of the analyzed collagens deposited in the DEJ of the BSC were similar to those seen in the native mouse skin (Fig. 7). In contrast, no localized collagen VII-specific staining at the DEJ was observed in the control BSC formed in the absence of cells expressing mProVII variants. Moreover, in this group, the collagen IV-specific and laminin 332-specific staining were characterized by diffused patterns (Fig. 7). Well-organized DEJ was also seen in yet another control group in which BSC were formed by wild type murine keratinocytes and dermal fibroblasts cultured in the presence of untransfected HEK293 cells (Fig. 8).

Analysis of remodeling of DEJ formed in the presence of the MTmProVII-GFP^{Tet} variants

Although Western blot assays and RT-PCR assays of BSC cultured in the presence or in the absence of Dox give the overall assessment of the expression of BMZ-relevant macromolecules, they do not provide any information about the site-specific formation of the BMZ as a function of the presence or of the absence of the p.R2622Q or p.G2623C mutants. Thus, employing fluorescence microscopy we measured the pixel intensity of the BMZ regions of the BSC immunostained for collagen IV, laminin 332, and collagen VII (Fig. 9). The results of these measurements indicate that the highest accumulation of both collagen IV and collagen VII in the DEJ was observed in the 4OFF group in which there was no expression of the p.R2622Q or p.G2623C mutants (Fig. 5 and Fig. 6). In contrast, in the 2ON and 4ON conditions in which the mutant chains were present, the accumulation of collagen IV, laminin 332, and collagen VII in the BMZ was poor (Fig. 9). In the 2ON/2OFF group in which mutant variants were expressed for two weeks and then their expression was shut off, the accumulation of laminin 332, collagen VII was comparable to that seen in the 4OFF control (Fig. 9). The accumulation of collagen IV in the 2ON/2OFF group did not reach the level of the 4OFF control (Fig. 9). Figure 10 depicts graphic representation of the above morphological observations.

Discussion

Since silencing a mutant allele presents a number of challenging problems such as incomplete inhibition of a target or nonspecific interference with functions of unaffected genes, no clear data on the utility of a potential silencer to repair DEJ formed in the presence of collagen VII mutants exists. To address this problem, We analyzed the effects of the selective silencing of the expression of the mProVII α chains harboring the p.R2622Q or p.G2623C single amino acid substitutions associated with RDEB and DDEB, respectively [34, 35]. Our experimental model utilizes Tet-dependent production of the p.R2622Q or

p.G2623C mutants coupled with Tet-independent production of the wild type chains. Employing fibroblasts and keratinocytes from *COL7a1* ^{-/-} mice to form skin equivalents represented by BSC ensured the biological relevance of the selected model. The presence of these cells guaranteed the production of macromolecules critical for the formation of the dermal BMZ. Thus, the described model provides a robust system adequate to study the extracellular effects of the efficient silencing of a mutant allele on the remodeling of the BMZ formed in the presence of collagen VII mutant chains. At the same time, however, the presented model has certain limitations which should be considered when evaluating its biological relevance: (i) the employed model is based on immortalized cells that may not fully represent all biological characteristics of the corresponding native mouse cells, (ii) mProVII variants are a truncated version of the full-length mouse collagen VII, a characteristic that may not replicate all features of the native collagen VII, (iii) BSC formed in cell culture conditions do not fully reproduce all characteristics of the native tissue, and (iv) unlike in native skin where collagen VII is delivered to the BMZ from epidermal and dermal layers, the mProVII variants are produced by engineered HEK293 cells present exclusively in the dermal layer of the BSC. Considering the second point, the NC1 domain is preserved in the mProVII variants, thereby ensuring critical NC1-mediated stabilization of the BMZ in the BSC [12]. Moreover, the NC2 domain critical for the self-assembly of collagen VII molecules is also preserved and able to engage in key collagen VII-collagen VII interaction [20]. Furthermore, the utility of a similar truncated collagen VII variant to fulfill functions of its full-length counterpart was demonstrated by Chen *et al.* who showed that expression of such a construct in keratinocytes from an RDEB patient restores the biological functions of those cells [36]. We also postulate that the dermal origin of the mProVII variants in the studied BSC does not limit their experimental utility. This notion is supported by studies on therapeutic approaches to RDEB which showed that the dermal component of skin is an adequate delivery platform of collagen VII to the dermal BMZ [37].

Considering the very problem of the effects of selective silencing of the expression of mutant collagen VII chains on the dermal BMZ analyzed here, the applied model provides some important observations. First, in the presence of the p.R2622Q or p.G2623C α chains produced in Tet-On conditions, the staining patterns of collagen IV, laminin 332, and collagen VII deposits are diffused, thereby indicating disorganization of the dermal BMZ formed in the presence of these mutants. These results are in agreement with the observation of the limited ability of these mutants to form critical collagen VII-collagen VII dimers and to participate in the formation of the anchoring fibrils [20, 34, 35]. The alterations in the organization of collagen IV, laminin 332, and collagen VII networks present in the analyzed BSC formed in Tet-On conditions indicates that collagen VII mutants do not only alter the structure of the anchoring fibrils, but may also impact the networks formed by other macromolecules of the dermal BMZ. This observation, together with the presence of well-defined DEJ formed in the BSC cultured in Tet-Off conditions, points to the strict dependence of the proper architecture of the BMZ on the correct structure of its specific elements.

We have also demonstrated that the alterations of the morphology of the collagen VII and laminin 332 deposits formed in the presence of the p.R2622Q and p.G2623C mutants

reverse after completely switching off their expression. This result indicates that the silencing of a mutant allele may be an effective approach to restore the function of the DEJ in the DEB skin. At the same time, however, our results suggest that the dynamics of remodeling of collagen IV do not parallel those seen for collagen VII and laminin 332, thereby suggesting that the reconstruction of the BMZ should be evaluated at the level of its individual components. As presented, our studies do not identify mechanisms responsible for BMZ remodeling in BSC cultured in 2ON/2OFF conditions. It is predicted, however, that the restoration of the collagen VII and laminin 332 layers during a 2OFF period is an effect of both the partial degradation of the mProVII mutant molecules produced during the 2ON period and the continuous accumulation of the wild type molecules produced in the 2OFF phase. As a result of this possible mechanism, the wild type-to-mutant ratio increases, thereby improving the collagen VII-dependent organization of the DEJ.

Since our experimental protocol of switching off the Tet-regulated promoter causes complete silencing of the expression of the mutant allele without altering the functions of the wild type one, our current study does not allow determining whether the incomplete inhibition of the expression of the mutant chains would restore the cohesive organization of collagen VII in the BMZ. Based on the work on collagen VII from selected DDEB cases, Fritsch *et al.* demonstrated that increasing the wild type to mutant chain ratio from 1:1 to 9:1 was necessary to markedly improve the thermostability of collagen VII molecules [38]. This study suggests that even relatively small quantities of the mutant chains may alter the structural functions of collagen VII, thereby demonstrating the need for total silencing of the expression of mutant chains for therapeutic approaches to succeed.

Unlike the remodeling of laminin 332 and collagen VII deposits, the diffused immunostaining pattern suggests that remodeling of collagen IV assemblies in the 2ON/2OFF group was not fully effective. Considering that collagen IV was continuously produced by the employed cells, at present, it is not clear why the organization of collagen IV in BSC has not been fully restored during the 2OFF period in the absence of the MTmProVII-GFP^{Tet} chains. Since collagen IV and collagen VII interact with each other in a site-specific manner, this observation may suggest that the restoration of the collagen IV network may depend on the existence of properly formed collagen VII assemblies [12]. It is also possible that, within the time constraints imposed by our model, the restoration of the collagen IV network could not be fully achieved. Thus, our study suggests that the kinetics of the remodeling of individual elements of the DEJ may not be identical, and that the restoration of specific networks may require established templates formed by various components of the dermal BMZ.

Based on our study presented here, it is apparent that the effects of expressing and switching off the production of the p.R2622Q and p.G2623C mutants on the morphology of DEJ of the analyzed BSC are similar. We postulate that these observed similarities could be attributed to the proximity of the p.R2622 and p.G2623 sites and the similar effects of the p.R2622Q and p.G2623C substitutions on the collagen VII-collagen VII interaction [20]. This notion is supported by the fact that both substitutions are located close to p.C2634, a key site critical for the formation of pivotal collagen VII-collagen VII dimers. Furthermore, in our earlier studies we demonstrated that the p.R2622Q and p.G2623C substitutions alter heterotypic

binding of the mutant molecules to the wild type counterparts and homotypic mutant-mutant interactions, and we postulated that this alteration may affect the formation of anchoring fibrils [20].

Our study presented here provides evidence that inhibiting the expression of mutant chains of collagen VII restores certain structural elements of the DEJ formed in the presence of those chains. Although our studies provide information on the remodeling of the BMZ after completely blocking the expression of mutant chains of collagen VII, future studies will investigate the effects of partial suppression of those chains, a situation more probable *in vivo*.

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Figure 1.

A schematic of the experimental model for switching off the expression of collagen VII chains harboring single amino acid substitutions. This schematic illustrates specific mProVII molecules produced in the BSC in 4OFF, 2ON, 4ON, and 2ON/2OFF conditions. GFP-tagged mutant chains and RFP-tagged wild type chains of mProVII variants are indicated. Symbols: +/- indicate the presence or the absence of Dox, Col-VII in DBMZ; mProVII variants present in the dermal basement membrane zone, E/D; epidermal and dermal layers of the BSC.

Figure 2.

Western blot assays of the secretion of selected proteins by fibroblasts and keratinocytes from *COL7a1* ^{-/-} mice and HEK293 cells. Symbols: Col-I, Col-III, Col-IV; collagen I, collagen III, and collagen IV, respectively, Lam-β3; β3 chain of laminin 332, mCol-VII; marker for mProVII isolated from cell culture media of cells that produce it [20-22], HEK-293 and tr-HEK-293; untransfected cells and cells transfected with constructs encoding the mProVII variants, respectively. Specific antibodies employed in Western blot assays are indicated.

Figure 3.

Tetracycline-dependent expression of the MTmProVII-GFP^{Tet} variants. A microscopic visualization of the lack of the GFP-positive signal in the absence of tetracycline (-Dox) and the production of the chains in the MTmProVII-GFP^{Tet} chains in the presence of tetracycline (+Dox) (A). Note that WTmProVII-RFP chains are produced constantly regardless of the presence or the absence of tetracycline. Western blot assays of the Tet-dependent production of the MTmProVII-GFP^{Tet} variants harboring R2622Q or G2623C substitutions and the Tet-independent expression of the WTmProVII-RFP chains (B).

Figure 4.

Microscopic assays of the morphology of BSC. A, DIC morphology of keratinocytes seeded on thin layers formed by a collagen gel and a collagen/fibrin gel. Note that on the collagen gel keratinocytes do not spread and proliferate poorly. In contrast, in the presence of fibrin, these cells form a cohesive layer. B, H&E staining of a BSC in which the epidermal layer (E) and the dermal layer (D) are indicated. The insert depicts the overall morphology of the BSC cultured for four weeks.

Figure 5.

A representation of qualitative RT-PCR assays for detecting the expression of selected markers in the BSC cultured in 2ON, 4ON, 4OFF, and 2ON/2OFF conditions (A). Qualitative RT-PCR assays of the expression of caspase 14 in BSC harboring the R2622Q and G2623C substitutions and those expressing only the WTmProVII-RFP chains (WT) (B). Description of mouse genes and relevant protein fragments: *Col1a1*; $\alpha 1$ chain of collagen I, *Col3a1*; $\alpha 1$ chain of collagen III, *Col4a1-3'* and *Col4a1-5'*; C and N termini of $\alpha 1$ chain of collagen IV, *COL7a1-5'*; NC1 of collagen VII, *COL7a1-GFP*; NC2/GFP junction site of the MTmProVII-GFP^{Tet} variant, *COL7a1-RFP*; NC2/RFP junction site of the WTmProVII-RFP variant, *Lamc2*; laminin $\gamma 2$ chain, *Lama3*; laminin $\alpha 3$ chain, *Itga6*; integrin $\alpha 6$, *Nid1* and *Nid2*; nidogen 1 and nidogen2, respectively, *Gapdh*; glyceraldehyde 3-phosphate dehydrogenase, Casp14; caspase 14.

Figure 6.

Analysis of collagen IV and collagen VII in BSC cultured in Tet-On and Tet-Off conditions. Western blot assays of collagen IV and collagen VII extracted from BSC harboring only WTmProVII-RFP chains (WT) and those that harbor both the R2622Q and G2623C MTmProVII-GFP^{Tet} plus WTmProVII-RFP chains (A). Note that some of the collagen VII-positive bands migrate higher than expected most likely due to the formation of stable homotypic and heterotypic complexes in the tissue-like environment of the BSC. B, Microscopic assays of BSC demonstrating the Tet-dependent expression of the MTmProVII-GFP^{Tet} chains and the Tet-independent expression of the WTmProVII-RFP chains (B). Symbols: Col-IV; collagen IV-positive bands, mCol-VII; mProVII chains.

Figure 7.

Microscopic analysis of collagen IV, laminin 332, and collagen VII in the DEJ. An upper row depicts collagen IV-positive, laminin 332-positive, and collagen VII-positive DEJ of mouse skin. Due to the relatively strong staining of laminin in the pericellular zone, the insert in the central panel shows the detailed staining of laminin 332 present in the DEJ. The middle row shows DEJ in BSC harboring cells expressing only WTmProVII-RFP chains (WT). These constructs were cultured for four weeks in the presence of Dox to demonstrate the lack of influence of this antibiotic on the formation of BSC. The bottom row (Ctrl) demonstrates the lack of clear DEJ in BSC harboring untransfected HEK293 cells. DEJ in analyzed BSC are indicated with asterisks. Symbols: E, D; epidermal layer and dermal layer of BSC, respectively.

Figure 8.

Microscopic analysis of collagen IV, laminin 332, and collagen VII in the DEJ of BSC formed by normal mouse keratinocytes, normal mouse fibroblasts, and untransfected HEK293 cells. DEJ in the analyzed BSC are indicated with asterisks. Symbols: E, D; epidermal layer and dermal layer of BSC, respectively.

Figure 9.

Remodeling of DEJ formed in the presence of the MTmProVII-GFP^{Tet} chains harboring the R2622Q or G2623C substitutions. Representative images show a diffuse pattern of collagen IV-positive, laminin 332-positive, and collagen VII-positive staining in BSC cultured in 2ON and 4ON conditions in which the MTmProVII-GFP^{Tet} chains harboring the R2622Q or G2623C substitutions are expressed. In contrast, in 4OFF conditions in which mutant chains are not expressed, the staining pattern of the analyzed collagens is limited to the DEJ. In 2ON/2OFF conditions in which mutants were initially expressed but after two weeks their expression was switched off, the organization of laminin 332 and collagen VII deposits in the DEJ was restored, but collagen IV organization remained largely diffused. DEJ in the analyzed BSC are indicated with asterisks. Symbols: E, D; epidermal layer and dermal layer of BSC, respectively.

Figure 10.

Graphic representation of measurements of pixel densities of collagen IV-positive laminin 332-positive, and collagen VII-positive deposits in the DEJ areas seen in Figure 9. A number of images were analyzed and then data were combined and presented as 2ON/4OFF, 4ON/4OFF, 2ON2OFF/4OFF ratios of mean pixel intensity values. The presented results reflect microscopic observations of the remodeling of collagen IV, laminin 332, and collagen VII deposits in the DEJ of BSC seen upon switching off the expression of the MTmProVII-GFP^{Tet} chains harboring the R2622Q or G2623C substitutions.

Table 1

PCR primers employed in qualitative assays of expression of proteins in BSC.

Target ^a	Forward primer 5'-3'	Reverse primer 5'-3'
<i>Col1a1</i>	CGCTACTACCGGGCCGATGATGC	CAGGCGGGAGGTCTTGGTGGTTTT
<i>Col3a1</i>	CCCTGTGTCCTGTGTGGT	TAGTCTCATTGCCTTGCGTGTGTTG
<i>Col4a1-3'</i>	CGCCTCCAGGAACGACTACTCTTA	AGGTGGATGGCGTGGGCTTCTTG
<i>Col4a1-5'</i>	TCCCGGAACACTGCTGAAAG	GGGCCGGGCTCTCCTCT
<i>COL7A1-5'</i>	GCCCAGCCCAGAGATAGAGTG	CGGGGCCAGACGGAGTGT
<i>COL7A1-GFP</i>	GGTGCCCCCTGAAGATGAC	TCGCCGATGGGGGTATT
<i>COL7A1-RFP</i>	CAGCCTGTCATCCCTTTGTCTAT	CTCCCAGCCGGCAGTCTTCT
<i>Lamc2</i>	AGCGCCATCGGGACGTGTTAGTT	GCCCCAATCTTGCCGAATCTCTTT
<i>Lama3</i>	GCCCAGGAAGGCAGTTTGCCTGGAA	TCGGTGGCTTCTGTGATGGGGACAG
<i>Itga6</i>	CTGGACACCCGCGAGGACAAC	TCAACCGCCATCGCAGAAACT
<i>Nid1</i>	CAGGTCCCCGTGGTGTGTTGAGAA	CCCGGGTGGAGGAGGAAGTGAT
<i>Nid2</i>	CAGGGGCGGAACGTGACCAGACT	CCCCGGAAGGCCACAGCAGAAT
<i>Casp14</i>	ACAGAGACCCCGTGAGGAACACTAC	CCGGAGGGTGCTTTGGACTT
<i>Gapdh</i>	GGGGAGCCAAAAGGGTCATCATCT	GACGCCTGCTTACCACCTTCTTG

^aDescription of mouse genes and proteins they encode: *Col1a1*; α1 chain of collagen I, *Col3a1*; α1 chain of collagen III, *Col4a1-3'* and *Col4a1-5'*; C and N termini of α1 chain of collagen IV, *COL7A1-5'*; NC1 of collagen VII, *COL7a1-GFP*; NC2/GFP junction site of the MTmProVII-GFP^{Tet} variant, *COL7a1-RFP*; NC2/RFP junction site of the WTmProVII-RFP variant, *Lamc2*; laminin γ2 chain, *Lama3*; laminin α3 chain, *Itga6*; integrin α6, *Nid1* and *Nid2*; nidogen 1 and nidogen2, respectively, *Gapdh*; glyceraldehyde 3-phosphate dehydrogenase, *Casp14*; caspase 14.