Col6a1 Null Mice as a Model to Study Skin Phenotypes in Patients with Collagen VI Related Myopathies: Expression of Classical and Novel Collagen VI Variants during Wound Healing

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Abstract

Patients suffering from collagen VI related myopathies caused by mutations in COL6A1, COL6A2 and COL6A3 often also display skin abnormalities, like formation of keloids or “cigarette paper” scars, dry skin, striae rubrae and keratosis pilaris (follicular keratosis). Here we evaluated if Col6a1 null mice, an established animal model for the muscle changes in collagen VI related myopathies, are also suitable for the study of mechanisms leading to the skin pathology. We performed a comprehensive study of the expression of all six collagen VI chains in unwounded and challenged skin of wild type and Col6a1 null mice. Expression of collagen VI chains is regulated in both skin wounds and bleomycin-induced fibrosis and the collagen VI γ3 chain is proteolytically processed in both wild type and Col6a1 null mice. Interestingly, we detected a decreased tensile strength of the skin and an altered collagen fibril and basement membrane architecture in Col6a1 null mice, the latter being features that are also found in collagen VI myopathy patients. Although Col6a1 null mice do not display an overt wound healing defect, these mice are a relevant animal model to study the skin pathology in collagen VI related disease.

Introduction

Mutations in COL6A1, COL6A2 and COL6A3 encoding collagen VI, cause Ullrich congenital muscular dystrophy (UCMD), Bethlem myopathy (BM) and myosclerosis myopathy [1–3]. In addition to the obvious muscular phenotype many patients also display skin abnormalities, including a predisposition for keratosis pilaris (follicular keratosis), abnormal scarring with formation of keloids or “cigarette paper” scars, dry skin, and striae rubrae [4–8].

Collagen VI forms a distinct microfibrillar network in most forms of extracellular matrix that anchors interstitial structures, such as nerves, blood vessels and larger collagen fibrils. In addition to being a collagen it belongs to the superfamly of proteins containing von Willebrand factor A (VWA) domains [9], globular protein modules that act by mediating protein-protein interactions. Collagen VI was long considered to consist of three genetically distinct α-chains (α1, α2 and α3). These chains form heterotrimeric monomers that assemble into dimers and tetramers already in the cell [10,11]. After secretion, polymers are formed by end-to-end interactions of the pre-assembled tetramers, yielding the characteristic beaded filaments seen by electron microscopy [12,13].

More recently, three novel collagen VI α-chains, α4, α5, and α6, encoded by the distinct genes Col6a4, Col6a5, and Col6a6 were identified [14,15]. These chains are composed of seven N-terminal VWA domains, a collagen triple helical region and a C-terminal non-collagenous domain containing two or three C-terminal VWA domains and one or two unique sequences. In addition, the α4 chain carries a Kunitz domain. Their triple helical regions are most similar to that of the α3 chain, and, in general, the recently identified chains resemble this chain. In contrast to the α3 chain, the α4, α5 and α6 chains have highly restricted distributions often associated with basement membranes [16].

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Collagen VI microfibril assembly is hampered in \textit{Col6a1}\(^{−/−}\) mice which display an early-onset muscle pathology that most closely resembles that of BM patients. These mice represent a valuable model for investigating the pathogenic mechanisms of collagen VI diseases at the molecular level and studies on \textit{Col6a1}\(^{−/−}\) null mice revealed that mitochondrial dysfunction and defective autophagy are involved in the pathogenesis of collagen VI myopathies \cite{17, 18}. We aimed to use the \textit{Col6a1}\(^{−/−}\) mouse strain as a model to study the role of collagen VI in the pathogenesis of skin abnormalities associated with collagen VI related myopathies. To evaluate if these mice adequately reflect the human skin phenotypes we performed a comprehensive study of the cutaneous expression of all six collagen VI chains, determined the skin morphology at the microscopic and ultrastructural levels, and studied wound healing. We compared our results to findings in wild type mice and related our observations to published data on the skin of collagen VI myopathy patients.

**Results**

**Collagen VI deficiency does not impact skin morphology**

Visual examination of the skin of collagen VI deficient mice revealed no obvious abnormalities and light microscope analysis showed a similar appearance of wild type and \textit{Col6a1}\(^{−/−}\) null skin (Fig. S1a). This was surprising as not only the \(α1\) chain is lacking in these mice, but the assembly and secretion of the other collagen VI chains is also severely affected (for details, see 3.3). Apparently collagen VI is not necessary to maintain the morphology of mouse skin under physiological conditions.

**Wound morphology in collagen VI deficient mice is not changed**

Wound healing experiments were performed in skin of wild type and \textit{Col6a1}\(^{−/−}\) null mice in which full thickness excisional defects had been created \cite{19, 20}. Light microscope analysis of the wounds did not reveal any obvious differences between wild type and \textit{Col6a1}\(^{−/−}\) null mice at day 4, 7, 10 and 14 after wounding (Fig. S1b). The distance between the severed ends of the panniculus carnosus and the area of the granulation tissue were unchanged (Fig. S2).

**Expression of collagen VI chains is regulated in skin wounds and fibrosis**

To detect consequences of the lack of the collagen VI \(α1\) chain on the expression of the classical \(α2\) and \(α3\) chains and the newly identified \(α4\), \(α5\), and \(α6\) chains, we performed a comprehensive study of the distribution of the six collagen VI chains in wounds of wild type and \textit{Col6a1}\(^{−/−}\) null mice using chain-specific affinity purified antibodies. During wound healing the \(α1\), \(α2\) and \(α3\)
chains were strongly expressed in wild type skin and could be detected already at day 4 (Fig. 1.; Fig. S1c). Throughout the healing process α1, α2 and α3 chains were mainly found just below the newly formed epidermis. The α2 and the α3 chains were detected by immunofluorescence labelling also in skin and wounds of Col6a1 null mice, although at a lower level (Fig.1; Fig. S1c). The staining appeared patchy and irregular compared to that of wild type mice. This pattern was further investigated by immunofluorescence staining of primary mouse fibroblast cultures from wild type mice. This staining appeared patchy and irregular compared to that of α2 chain. By contrast, wild type fibroblasts secreted collagen VI and formed an extended extracellular network. Analysis of cell culture media from Col6a1 null primary fibroblasts showed that some α3 chain was present as a single chain, indicating that a fraction the collagen VI α3 chains were secreted as individual molecules without forming heterotrimers (Fig. 2a).

The novel collagen VI α4, α5 and α6 chains displayed a more restricted expression. In unwounded wild type skin the α5 chain was localised around blood vessels [16] and its expression was increased during wound healing (Fig. S3). The α5 chain was mainly present below the granulation tissue. Interestingly, in the wound area the α5 chain was localised in the epineurium of newly formed nerves, but not around blood vessels (Fig. S4). The expression of the α6 chain was up-regulated during wound healing and was also detected below the granulation tissue (Fig. S5). Whereas labelling for the α5 chain started at day 7 of wound healing, the α6 chain was detected already at day 4. At later stages of wound healing, the staining for the α5 and α6 chains decreased. The α4 chain was not detected in mouse skin. Col6a1 null skin was negative for all novel chains.

As dysregulation of the tissue remodelling phase of wound healing results in fibrosis, we also studied the expression of the collagen VI chains in fibrotic skin induced by local bleomycin injection. Here, as in wound healing, the α3 chain was strongly expressed in the fibrotic dermis. However, in contrast to wounds where the α5 chain was absent from blood vessels, this chain was up-regulated in the blood vessels in the fibrotic dermis. As in wounds, the expression of the α6 chain was also up-regulated in the blood vessels in the fibrotic dermis (Fig.3).

The collagen VI α3 chain in skin is proteolytically processed

The α1, α2 and α3 chains were detected by immunoblot of extracts from both unwounded skin and the wound area (Fig. 4). In Col6a1 null mice, not only the α1 chain but also the α2 chain was not detected in extracts of unwounded skin, however, a weak α2 chain signal was seen in extracts of wounds of these mice at day 7. The lacking signal for the α2 chain in unwounded skin is in contrast to the immunohistochemical analysis, probably because

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**Figure 2. Analysis of the collagen VI α3 chain in primary fibroblast cultures from wild type and Col6a1 null mouse skin.** Cells were isolated from newborn wild type and Col6a1 null mice and cultured for 4 days. (a) Immunostaining for the collagen VI α3 chain (green) and the endoplasmic reticulum marker PDI (red). Nuclei were stained with DAPI (blue). Bar, 100 μm. (b) Immunoblot analysis of collagen VI assembly in cell lysates (C) and supernatants (S). Cells were lysed with SDS-PAGE sample buffer, samples treated with 2 M urea and separated under non-reducing conditions on an agarose-polyacrylamide (0.5%/2.4%) composite gel. Immunoblots were developed with an antibody against the collagen VI α3 chain. (*) indicates the mobility of the single α3 chain, (β) indicates α3 chain dimers. (c) Higher magnification from (a). Bar, 50 μm.

**Figure 3. Collagen VI distribution in fibrotic skin lesions of wild type mice.** Mice were treated for 4 weeks with bleomycin as described in Methods. NaCl injection served as control. Frozen sections were incubated with affinity purified antibodies against the collagen VI α3 (green), α5 (green) or α6 (green) chains. The sections stained for the α5 and α6 chains were co-stained with an antibody against the endothelial marker CD31 (red). Nuclei were stained with DAPI (blue). Bar, 50 μm.

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the amount of this chain extracted for immunoblots was below the
detection limit. The α2 chains from wild type and Col6a1 null
mice migrated with the same expected mobility. In contrast, the
α3 chain was detected in both uninjured skin and wounds at day 7,
but was extensively degraded in unwounded skin of wild type mice
and even more so in wounds of wild type and Col6a1 null mice.
Immunoblotting revealed a ladder of bands ranging from the full-
length protein to 35 kDa fragments (Fig. 4). Interestingly, wound
extracts contained more α3 chain and α3 chain fragments than
extracts of unwounded skin, indicating an increased synthesis or
greater solubility of collagen VI in wounds. The extracted material
may represent tetramers that have not yet been assembled into
fibrils or molecules that are being degraded due to high protein
turnover. Also wound extracts from Col6a1 null mice contained
more α3 chain, than extracts of unwounded skin. This material
probably represents a soluble intracellular pool of α3 chain in the
Col6a1 null fibroblasts (Fig. 4).

The full-length α5 and α6 chains gave only weak bands in
immunoblots of extracts of unwounded skin of wild type mice. In
wound extracts at day 7 the signals for the full-length chains were
stronger, but absent in extracts from Col6a1 null mice except for a
weak band for the α5 chain (Fig. 4).

Altered tensile strength and collagen fibril and basement
membrane architecture in Col6a1 null mice
Collagen VI microfibrils are connected to large collagen fibrils
[21] and are thought to regulate their formation [22]. We
therefore stained wounds of wild type and Col6a1 null mice with
antibodies against collagen I, but the overall distribution of this
collagen was similar between genotypes (not shown). Picrosirius
red staining and polarization microscopy can reveal changes in the
properties of collagen fibrils. Indeed, a reduced birefringence
was observed at day 7 in wounds of Col6a1 null mice (Fig. 5a). This

Figure 4. Collagen VI in extracts of unwounded skin and wounds derived from wild type and Col6a1 null mice. Extracts from
unwounded skin and wounds were subjected to SDS-PAGE on 4–12% polyacrylamide gradient gels under reducing conditions, proteins transferred
to a membrane and detected with affinity purified antibodies against the collagen VI α1, α2, α3, α5 and α6 chains. Boxed areas a and b on the right
show a longer exposure. Arrows indicate the position of the full length proteins, asterisks indicate artefact bands.
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reduction was not apparent anymore at day 10 of wound healing (not shown).

Confirming this result, analysis of the collagen I fibril architecture by electron microscopy revealed changes in the extracellular matrix between the fibrils in wounds of the Col6a1 null mice at day 7 (Fig. 5b–d). At central areas of the wound, the amount of fine microfibrillar structures interwoven between the collagen I fibrils was reduced and the collagen I fibrils were more densely packed (Fig. 5c, h). In contrast, the differences in fibril diameter distribution were marginal (Fig. 5i). In addition, in more peripheral areas of the wound where the collagen I fibrils had larger diameters, an irregular fusion of fibrils was often seen (Fig. 5d). No obvious changes in collagen fibril architecture were detected in unwounded skin from mice of the two genotypes (not shown).

However, to investigate if the lack of collagen VI alters the tensile strength of unwounded skin, we performed mechanical tests on skin of wild type and collagen VI null mice. When the skin was stretched, the ultimate load and stress were significantly lower in collagen VI null mice (Fig. 5j, k). Moreover, in addition to abnormalities in collagen I fibril architecture in wounds, electron micrographs of unwounded skin revealed that the basement membranes of blood vessels and nerves as well as of adjacent muscles were sometimes duplicated in Col6a1 null mice (Fig. 5e–g).
Discussion

Collagen VI is thought to contribute to tissue remodelling [23,24] and in addition to the obvious muscular pathologies, mutations in human collagen VI genes also often lead to keloidal formation and other skin related phenotypes. Col6a1 null mice serve as a well-established model for the muscle phenotypes, but have not been studied with regard to skin changes. In a first step we characterized the expression of collagen VI chains in mouse skin. We then performed wound healing experiments in skin of wild type and Col6a1 null mice to assess whether this mouse model is also useful to assess the relevance of the classical collagen VI for skin development and for tissue reconstitution following injury. Since absence of the collagen VI α1 chain results in the failure to form the classical α1α2α3 trimer of collagen VI, we moreover sought to assess compensatory expression of the recently identified α4, α5 and α6 chains of collagen VI.

In human wounds, the classical α1 α2 and α3 chains were studied at the mRNA level revealing increased expression in fibroblast-like cells and in endothelial cells of newly formed vessels [25]. Collagen VI gene expression was not detected in smooth muscle cells or in myoepithelial cells of eccrine glands. We could show that in granulation tissue of wounds of wild type mice the classical α1, α2 and α3 chain-containing collagen VI was more strongly expressed than in unwounded skin. The widespread deposition of the protein in dermis indicates that collagen VI is abundantly secreted by fibroblasts. In addition, in wounded skin of wild type mice the α5 chain was up-regulated in the epidermis of newly formed nerves and the α6 chain in the tissue below the wound, but not within the granulation tissue. These results indicate that collagen VI is involved in the wound healing process and that the novel chains could play a more specific role than the broadly expressed classical ones. Up-regulation of collagen VI containing the classical chains was also detected in spontaneously fibrotic skin of tight skin (Tsk+/−) mice [26] and in bleomycin induced lung fibrosis [27]. Indeed, using the bleomycin model of skin fibrosis, we also found up-regulation of the α2 chain in the dermis and of the α5 and α6 chains in blood vessels, indicating that collagen VI is generally up-regulated in fibrotic tissue. Similarly, the α6 chain is expressed at higher levels in fibrotic muscle of Duchenne muscular dystrophy patients [28]. Interestingly, it was recently shown that a proteolytic fragment of collagen VI α1 chain is significantly elevated in the serum of patients with chronic obstructive pulmonary disease or idiopathic pulmonary fibrosis [29] and in a rat model of liver fibrosis [30]. However, in contrast to the hypertrophic scars or keloids occurring in patients with a collagen VI myopathies, we did not observe such disturbed wound healing in the Col6a1 null mice. Most likely this can be explained by the fact that mice have a lesser tendency to overshooting wound healing than humans [31].

Interestingly, a patchy immunofluorescence staining for the α2 and α3 chains was observed in Col6a1 null mice, and by immunoblot analysis we detected a more pronounced α3 chain degradation in wound extracts of wild type and Col6a1 null mice. This is in contrast to the expectation that assembly and secretion of all collagen VI chains is abolished in Col6a1 null mice, based on the fact that the α1 chain is absolutely required for the assembly of triple helical collagen VI molecules [32]. The distribution of the α3 chain is reminiscent of the reduced and patchy collagen VI α3 chain staining seen in a patient with UCMD carrying a mutation in COL6A1 [28]. Furthermore, the intracellular accumulation of the α3 chain in Col6a1 null fibroblasts correlates with the increased intracellular collagen VI labelling that was seen in cultures from UCMD patients [5] and the accumulation of the α1 chain in collagen VI α3 chain mutant mouse fibroblasts [33], indicating that synthesis of individual collagen VI chains is independent. Co-immunolabelling with an antibody against the endoplasmic reticulum (ER) protein PDI indicated that the α3 chain was retained in this subcellular compartment, as has been described for UCMD patients [34]. By immunoblot we could show that the formation of collagen VI tetramers is abolished in Col6a1 null mice, thereby blocking collagen VI microfibril assembly. However, intracellular and secreted proteolytic fragments of the other collagen VI α chains are present and may contribute to pathogenic mechanisms by a deleterious action inside or outside of the cell. Indeed, recently a cleavage product of the collagen VI α3 chain, named endotrophin, was shown to augment fibrosis, angiogenesis, and inflammation through recruitment of macrophages and endothelial cells and was associated with aggressive mammary tumor growth and metastasis. These effects were partially mediated through enhanced TGF-β signalling, which contributes to tissue fibrosis [35]. In our model, more collagen VI and its fragments are extracted from granulation tissue than from unwounded skin, indicating either an increased synthesis or a decreased anchorage in the newly formed tissue. This may allow an increased diffusion into neighbouring tissues, thereby promoting TGF-β signalling.

Although collagen VI, predominantly in its classical form, is strongly expressed in wounds, the consequences of its absence are not overt. Immunofluorescence staining for wound healing markers such as α-smooth muscle actin, desmin, the F4/80 epitope or CD31 and for several extracellular matrix proteins and collagen VI binding partners did not show marked differences between wild type and Col6a1 null mice (not shown). Only when collagen fibrils at day 7 of wound healing were stained with picrosirus red and visualized by polarization microscopy, a clear difference between wild type and Col6a1 null mice was seen. The reason for this difference became obvious when the collagen I fibrils in day 7 wounds were visualized in greater detail by electron microscopy. A larger proportion of the fibrils were closely spaced in the Col6a1 null mice than in wild type mice, indicating that collagen VI deficiency alters matrix architecture and possibly biomechanical properties. Similar ultrastructural alterations were also seen in tendons of mice deficient for either collagen VI α1 or α3 chains [33,36]. In Col6a1 null tendons the diameter distribution of collagen I fibrils was significantly shifted towards thinner fibrils. An analysis of fibril density (number/area unit) demonstrated a ~2.5 fold increase in the Col6a1 null versus wild type tendons and Col6a1 null tendons displayed reduced biomechanical strength and stiffness [36], which corresponds to the reduced ultimate load and stress of Col6a1 null skin in stretching experiments shown here (Fig. 5). Interestingly, ultrastructurally abnormal collagen I fibrils were observed in tendon, but not in cornea, of Col6a1 null mice, indicating a tissue-specific action of collagen VI on collagen I fibrillogenesis [33]. Possibly the role of collagen VI is more pronounced in tissues which are exposed to mechanical stress. Nevertheless, a recent ultrastructural analysis of the skin of a patient with BM carrying a mutation in the collagen VI α2 chain revealed variations in size of collagen I fibrils, flower-like cross sections of collagen I fibrils, as well as thickening and duplication of vascular and nerve basement membranes in the skin [37] strikingly similar to the changes that we detected in Col6a1 null mice. Indeed, this peculiar combination of signs was considered to be of diagnostic value. Interestingly, the unusual combination of basement membrane thickening and duplication was also detected in blood vessels of muscles of a myosclerosis patient carrying a mutation in the collagen VI α2 chain [3]. This indicates that although Col6a1 null mice do not display an overt...
wound healing defect, some features seen in skin of collagen VI related myopathy patients are also present in Col6a1 null mice. These mice are therefore the most relevant animal model available to study mechanistic aspects of the skin pathology in collagen VI related disease.

**Materials and Methods**

**Ethics statement**

This study was carried out in strict accordance with the German federal law on Animal Welfare, and the protocols were approved by the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (permit No. 8.87–51.04.20.09.338 for wound healing experiments; permit No. 8.87–50.10.31.08.197 for bleomycin induced fibrosis experiments).

**Histology and morphometric analysis**

Frozen sections (7 μm) were fixed with 2% paraformaldehyde and stained with haematoxylin and eosin (H/E) to determine the granulation tissue area and the distance between the ends of the panniculus carnosus as described [38] using the ImageJ software. Significance of differences was analyzed using the two-tailed t-test. All data were presented as the mean ± SD, a p value of <0.05 was considered significant. Staining with picrosirius red was used to examine collagen distribution and characteristics.

**Immunofluorescence microscopy**

Immunofluorescence microscopy was performed on frozen sections (7 μm) of healthy or fibrotic skin and of wounds from wild type and Col6a1 null mice [32]. Sections were fixed with 2% paraformaldehyde and treated with bovine testicular hyaluronidase. Primary antibodies were applied overnight at 4°C followed by incubation with secondary AlexaFluor 546-conjugated goat anti-rabbit IgG (Molecular Probes), AlexaFluor 488-conjugated goat anti-rabbit IgG (Molecular Probes), AlexaFluor 546-conjugated goat anti-guinea pig IgG, or AlexaFluor 546-conjugated rat anti-mouse pig IgG (Molecular Probes). The antibodies raised against recombinant N-terminal fragments of the collagen VI α3, α5 and α6 chains have been described [15] and those against the collagen VI α2 and α3 chains were raised by immunization with recombinant C-terminal fragments of these chains. The ER marker protein disulphide isomerase (PDI) was detected with a purified anti-rabbit PDI antibody (Stressgene), the endothelial marker CD31 with a purified rat anti-mouse CD31 antibody (MEC13.3, BD-Pharmingen) and nerves with a purified rat anti-mouse neurofilament antibody (Dako). Nuclei were stained with DAPI (Sigma Aldrich).

**Protein extraction from skin and wounds**

Skin and wound tissues were frozen in liquid nitrogen and pulverized by pestle and mortar, lysed in 50 mM Tris, pH 7.4, containing 150 mM NaCl, 10 mM MgCl2, 0.5 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 2% SDS and 1% Triton-X100 together with protease inhibitors (complete, Roche), incubated at 70°C for 10 min and homogenized by a Rotor stator homogenizer. The samples were clarified by centrifugation at 4°C.

**Gel electrophoresis and immunoblot**

For SDS-PAGE samples were reduced with 5% β-mercaptoethanol and subjected to electrophoresis on 4–12% (w/v) gradient polyacrylamid gels. Samples for electrophoresis on 0.5% agarose-2.4% polyacrylamide composite gels were pretreated with 2 M urea [39]. In both cases proteins were electrophoretically transferred to Immobilon-P membranes (Millipore). The collagen VI α3, α5 and α6 chains were detected with affinity purified antibodies raised by immunization with N-terminal recombinant fragments [15], the α2 and α3 chains with antibodies against C-terminal recombinant fragments. Secondary antibodies were conjugated with horseradish peroxidase and bands were visualized by chemiluminescence (SuperSignal West Pico, Pierce). The experiments were performed with extracts from three different animals per genotype. The individual animals gave similar results.

**Electron microscopy**

Tissue was fixed in 2% paraformaldehyde, 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, at 4°C for 24–48 h. Post-fixation was in 2% osmium tetroxide buffered at pH 7.3 with sodium cacodylate for 2 h at 4°C. Biopsies were washed, stained in 1% uranyl acetate, dehydrated through a series of graded ethanols and embedded in epon resin. Semithin sections (300 nm) were cut with a glass knife on an ultramicrotome (Reichert) and stained with methylene blue. Ultrathin sections (70 nm) for electron microscopic evaluation were processed on the same microtome with a diamond knife and placed on copper grids. The ultrathin sections were analyzed with a Zeiss 902A Transmission Electron Microscope (Zeiss) and the TEM Imaging Platform iTEM Software (Soft Imaging Systems).

**Wound healing**

Wounding and harvesting of wound tissue was performed as previously described [19,20]. Briefly, mice were anesthetized by intraperitoneal injection with ketamine/xylazine and full-thickness wounds comprising the epidermis, dermis, subcutaneous fat and the panniculus carnosus muscle were created by a biopsy punch on the shaved backs. Animals were housed under a 12/12 hours light/dark cycle with free access to food and fresh water ad libitum. Mice were euthanized by carbon dioxide. For histological analysis, wounds were excised at different times after injury (4–14 days), bisected in the caudocranial direction, and the tissue was either fixed overnight in 4% formaldehyde or embedded in optimal cutting temperature compound (Tissue-Tek, Sakura).

**Bleomycin induced fibrosis**

Experimental skin fibrosis was induced by repeated intradermal injection of bleomycin sulphate (100 μl, 1 mg/ml in 0.9% NaCl; Medac) into the back skin of six anaesthetized (isoflurane) and shaved 6 week old female C57Bl6/N mice on 5 days/week for 4 weeks as described [40]. Five control mice received intradermal injections of NaCl (100 μl, 0.9%). Animals were housed under specific pathogen-free conditions in a 12/12 hours light/dark cycle with free access to food and fresh water ad libitum. Mice were euthanized by carbon dioxide.

**Mechanical testing**

The tensile strength of skin of 8-week-old wild type (n = 9) and Col6a1 null (n = 12) mice was analysed using a material testing machine (Z2.5/TN1S; Zwick). Two stripes of back skin were dissected in hourglass-shaped form (width: 5 mm in middle and 10 mm at the ends, length: 25 mm) using a punch. These samples were fixed between two ruffled clamps. After preloading (0.05 N, 0.1 mm/s) the skin was stretched until failure with a crosshead speed of 15 mm/s. To calculate the stress, skin thickness was assessed in sections prepared for histological analysis. Significance of differences was analyzed using the two-tailed t-test.
Supporting Information

Figure S1  Histological analysis of unwounded skin and wounds (a, b) and immunofluorescence analysis of collagen VI α1 and α2 chains (c). (a) H/E staining of skin from 10 week-old wild type and Col6a1 null mice. (b) H/E staining of wounds from wild type and Col6a1 null mice at 4, 7, 10 and 14 days after wounding. (c) Sections from day 10 wounds were incubated with affinity purified antibodies against the collagen VI α1 and α2 chains, followed by Alexa 546 (red) labeled secondary antibody. Nuclei were stained with DAPI (blue). d = dermis, e = epidermis, g = granulation tissue, h = hair follicle, st = scar tissue, pe = panniculus carnosus, arrow heads = ends of the panniculus carnosus. Bar, 100 μm. (TIF)

Figure S2  Quantification of the granulation tissue area (a) and of the distance between the ends of the panniculus carnosus (b). The granulation tissue area and the distance between the ends of the panniculus carnosus from wild type and Col6a1 null mice at 4, 6, 7, 8, 9, 10 and 14 days after wounding was determined using the ImageJ software. The standard deviation is indicated. The significance was determined using a two-tailed t-test. There were no significant differences. N (wild type/Col6a1 null) = 6/6 (d4), = 7/8(d6), = 14/11 (d7), = 4/6 (d8), = 4/6(d9), = 6/8(d10) and 7/9 (d14). (TIF)

Figure S3  Immunofluorescence analysis of the collagen VI α5 chain during wound healing. Frozen sections of wounds from wild type and Col6a1 null mice at days 4, 7, 10 and 14 after wounding were incubated with an affinity purified antibody against the collagen VI α5 chain followed by Alexa 546 labeled secondary antibody (red). Nuclei were stained with DAPI (blue). d = dermis, e = epidermis, g = granulation tissue, st = scar tissue. Bar, 200 μm. (TIF)

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