Collagen VI–NG2 axis in human tendon fibroblasts under conditions mimicking injury response

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Abstract

In response to injury, tendon fibroblasts are activated, migrate to the wound, and contribute to tissue repair by producing and organizing the extracellular matrix. Collagen VI is a microfibrillar collagen enriched in the pericellular matrix of tendon fibroblasts with a potential regulatory role in tendon repair mechanism. We investigated the molecular basis of the interaction between collagen VI and the cell membrane both in tissue sections and fibroblast cultures of human tendon, and analyzed the deposition of collagen VI during migration and myofibroblast trans-differentiation, two crucial events for tendon repair. Tendon fibroblast displayed a collagen VI microfibrillar network closely associated with the cell surface. Binding of collagen VI with the cell membrane was mediated by NG2 proteoglycan, as demonstrated by *in vitro* perturbation of collagen VI–NG2 interaction with a NG2-blocking antibody.

Cultures subjected to wound healing scratch assay displayed collagen VI–NG2 complexes at the trailing edge of migrating cells, suggesting a potential role in cell migration. In fact, the addition of a NG2-blocking antibody led to an impairment of cell polarization and delay of wound closure. Similar results were obtained after *in vitro* perturbation of collagen VI extracellular assembly with the 3C4 anti-collagen VI antibody and in collagen VI-deficient tendon cultures of a Ullrich congenital muscular dystrophy patient carrying mutations in *COL6A2* gene.

Moreover, *in vitro* treatment with transforming growth factor β1 (TGFβ1) induced a dramatic reduction of NG2 expression, both at protein and mRNA transcript level, and the impairment of collagen VI association with the cell membrane. Instead, collagen VI was still detectable in the extracellular matrix in association with ED-A fibronectin and collagen I, which were strongly induced by TGFβ1 treatment.

Our findings reveal a critical role of the NG2 proteoglycan for the binding of collagen VI to the surface of tendon fibroblasts. By interacting with NG2 proteoglycan and other extracellular matrix proteins, collagen VI regulates fibroblasts behavior and the assembly of tendon matrix, thereby playing a crucial role in tendon repair.

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Introduction

The healing process of an injured tendon comprises three phases characterized by distinctive cellular and molecular cascades: inflammation, cell proliferation, and tissue remodeling [1]. These phases can overlap and their duration depends on the site and entity of damage. Two cellular mechanisms of tendon healing, known as extrinsic and intrinsic healing, have been suggested. The proposed mechanisms consist of an early migration of fibroblasts and inflammatory cells from the tendon periphery to the damaged area (extrinsic healing), followed by activation of intrinsic cells of the endotenon (tendon fibroblasts) which proliferate and migrate to the injured site, contributing to extracellular matrix (ECM) synthesis and reorganization (intrinsic healing). Tendon healing does not effectively restore the native structure and function of the tendon, and results in fibrotic scar formation with increased risk of re-rupture. Therapeutics have been largely ineffective because fundamental mechanisms that underlie pathogenesis of impaired tendon healing remain unknown [2]. The transforming growth factor-β1 (TGF-β1)-induced myofibroblast trans-differentiation of tendon fibroblasts is one of the most important features of scar fibrosis formation [3–5]. Myofibroblasts are fibroblasts with characteristics of smooth muscle cells, expressing α-smooth muscle actin (α-SMA) and developing a contractile apparatus. Activated myofibroblasts generate traction forces that are required for wound closure and ECM remodeling [6]. However, hyper-activation of myofibroblasts induces tendon contractures [7].

Tendons are composed of relatively rare cells (tendon fibroblasts) scattered within a predominant dense connective tissue arranged in an highly ordered ECM, mainly constituted by collagen fibrils which are hierarchically organized to withstand tensile forces transmitted from muscles to bone axis [8]. Fibrils contain mostly collagen I and other components, which contribute to fibrillogenesis, such as collagen types III, V, VI, XII, and XIV, as well as proteoglycans and glycoproteins [9]. Tendon fibroblasts, the ECM-producing cells, are distributed longitudinally among collagen fascicles and are surrounded by the pericellular matrix (PCM) consisting of specialized ECM proteins [10,11]. Proteomic analysis of the PCM of mouse developing tendon identified regulatory molecules for collagen fibril assembly, such as collagen type V and VI, fibrillin and fibronectin. Integrins and a variety of proteins involved in signal transduction, cell adhesion, and matrix turnover [11] have also been identified, suggesting that the PCM represents a critical site with potential impact on tendon repair.

Collagen VI, a microfibrillar collagen widely expressed in most tissues, has been identified in the PCM of both developing [11] and mature [10] tendon fibroblasts and, more recently, in the PCM of human rotator cuff [12]. Morphologically, collagen VI forms a network of beaded filaments anchored to the cell surface [13,10]. The best characterized and widely expressed form of collagen VI is the [α1(VI)α2(VI)α3(VI)] heterotrimer that further assembles intracellularly into dimers and tetramers. After secretion, tetramers undergo end-to-end association, giving rise to the typical 100 nm-spaced beaded microfibrils [13], which may form, alternatively, fibrils by parallel alignment, or web-like structures by multiple interconnections, depending on the association with cell receptors and ECM-binding proteins [13–17]. In humans, two novel collagen VI subunits, the α5(VI) and α6(VI) chains, were recently identified, which structurally resemble the α3 chain but display a more restricted and often alternative distribution pattern [18–21]. In tendons, the [α1(VI)α2(VI)α3(VI)] heterotrimer is abundantly expressed [12], whereas the α5(VI) chain is selectively detected at the myotendinous junction and the α6(VI) chain is absent [21].

Collagen VI interacts with a variety of cell membrane and ECM proteins. NG2/CSPG4 proteoglycan [22] and integrins [23,24] are potential collagen VI cell membrane receptors. Furthermore, collagen VI interacts with a large number of ECM molecules, reviewed in [25], including collagens I, II, IV and XIV, microfibril-associated glycoprotein-1, perlecan, decorin, biglycan, hyaluronan, heparin and fibronectin.

Mutations in *COL6A1*, *COL6A2*, *and COL6A3* genes cause muscular dystrophies, which comprise two major clinical forms, Bethlem myopathy (BM [MIM 158810]) and Ullrich congenital muscular dystrophy (UCMD [MIM 254090]), and the limb girdle and the myosclerosis myopathy (MM) variants [26]. UCMD is a severe disorder characterized by congenital muscle weakness; BM is a mild form characterized by slowly progressive axial and proximal muscle weakness; MM is characterized by slender muscles with firm 'woody' consistence and restriction of movement at many joints. The majority of collagen VI gene mutations affect the assembly and secretion of collagen VI in the extracellular matrix of both skeletal muscle [27] and fibroblasts cultures [28,29]. Collagen VI myopathies are also characterized by joint hyperlaxity and contractures [26]. *Col6a1*$^{-/-}$ mice, a collagen VI null model [30], and *Col6a3* deficient mice [31] develop a mild myopathy and tendon dysfunction possibly due to altered tendon fibrillogenesis [32,31]. Altogether, these data point to an involvement of collagen VI in the regulation of tendon function.

In this paper, we explored the molecular basis of collagen VI–cell membrane interactions in human tendon fibroblasts, and investigated collagen VI distribution during cell migration and TGFβ1-mediated myofibroblast trans-differentiation, both key events for tendon repair.

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Results

Collagen VI organization in the PCM of human tendon fibroblasts

Immunofluorescence analysis of longitudinal and cross sections of human tendon showed that collagen VI was localized among the longitudinally aligned fibrillar matrix and around the fibroblasts, extending along with their processes (Fig. 1A). Double labeling for collagen VI and NG2 proteoglycan, a major transmembrane collagen VI receptor [22], revealed that tendon fibroblasts expressed NG2 (Fig. 1A).

We explored whether human tendon fibroblasts maintain the ability to organize a collagen VI PCM *in vitro*. Tendon fibroblasts were grown to sub-confluence onto coverslips. Immunofluorescence microscopy showed that collagen VI was closely associated with the cell surface, and filamentous structures anchored at the cell membrane were also frequently detected (Fig. 1B, left panel).

To assess the dynamics of the extracellular assembly of collagen VI with respect to binding partners in the ECM, tendon fibroblasts were grown ten days post-confluence. Immunofluorescence microscopy revealed that collagen VI was abundantly present in the ECM and displayed a filamentous arrangement parallel to the cell axis. Collagen I was also present (Fig. 1B, middle panel) and partly co-localized with collagen VI, as revealed by 3D-reconstruction confocal imaging (Fig. 1B, right panel).

Replicas obtained by rotary shadowing were analyzed by transmission electron microscopy to better define *in vitro* collagen VI organization. Colloidal gold-labeled collagen VI microfilaments were detected both along the cytoplasmic processes and in the extracellular space (Fig. 1C). Parallel aligned microfilaments were associated with the cell surface (Fig. 1C, left panel), while web-like structures were detected in the extracellular space (Fig. 1C, right panel), in agreement with the reported functional interactions of collagen VI with membrane binding partners and ECM components, reviewed in [25], which appear to be maintained in our *in vitro* model.

NG2 proteoglycan is required for collagen VI localization in the PCM of cultured tendon fibroblasts

The *in vivo* expression of NG2 proteoglycan by tendon fibroblasts enabled us to further study its role in collagen VI PCM organization. Immunofluorescence microscopy for the NG2/CSPG4 proteoglycan, showed that NG2 was present at the cell membrane of cultured tendon fibroblasts with a punctuate pattern. Moreover, double labeling with collagen VI and NG2 antibodies showed a clear co-localization at discrete areas of the cell membrane (Fig. 2A). Strikingly, *in vitro* treatment with a monoclonal antibody against the NG2 core protein, which prevents collagen VI–NG2 binding [33], led to a strongly reduced association of collagen VI with the cell membrane (Fig. 2B), and the protein was mainly found in the ECM. In agreement with this, western blot analysis showed a reduced amount of the α3(VI) chain in the lysate of cells treated with NG2-blocking antibody (Fig. 2C). Interestingly, the amount of NG2 was also reduced by the blocking antibody treatment (Fig. 2C), suggesting a possible protein shedding and proteolytic effect due to the inhibition of the interaction with collagen VI [33].

Collagen VI–NG2 axis is involved in cell polarization and migration

Given the critical role of NG2 proteoglycan for the association of collagen VI with the cell surface, we further explored the functional consequences of collagen VI–NG2 interaction under conditions mimicking injury response. Motility is important for tendon fibroblasts during wound repair [1], therefore we performed scratch wound assays, a widely used method to study cell motility and migration in response to *in vitro* injury [34,35]. Confluent tendon fibroblast cultures were scratched and images were captured at regular intervals. Tendon fibroblasts facing the wound promptly started migrating toward the empty space created by the scratch. Cell migration was monitored by time-laps and, at the end of the experiment, samples were fixed and processed for collagen VI and NG2 immunofluorescence microscopy (Fig. 3A, B; Movie S1). Collagen VI was clearly associated with the trailing edge of the moving cells, as determined by comparing direction of cell movement and immunohistochemistry. In contrast, collagen VI was never detected at the leading edge (Fig. 3B). Strikingly, collagen VI was found associated with the tail of polarized cells (Fig. 3C), pointing to a role of collagen VI in stabilizing the direction of the cell migration/polarization [36].

To explore the functional consequences of the loss of the collagen VI–NG2 binding during migration, scratch wound assays were performed in the presence or absence of NG2-blocking antibody. As a marker of cell polarization we used golgin-97, a protein of the Golgi apparatus, which is oriented toward the leading edge of migrating cells [37]. As expected, in untreated migrating cells the Golgi apparatus was located between the cell's leading edge and the nucleus (Fig. 4A, upper row; Supplementary Fig. 1A). Strikingly, in the presence of NG2-blocking antibody a significant number of cells

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facing the scratch displayed a random distribution of the Golgi apparatus (Fig. 4A, lower row; Supplementary Fig. 1), suggesting an impairment of cell polarization. In addition, application of the NG2 blocking antibody delayed the migration of tendon fibroblasts in the wound area, resulting in a partial closure of the scratched area as compared to untreated samples (Fig. 4B, C).

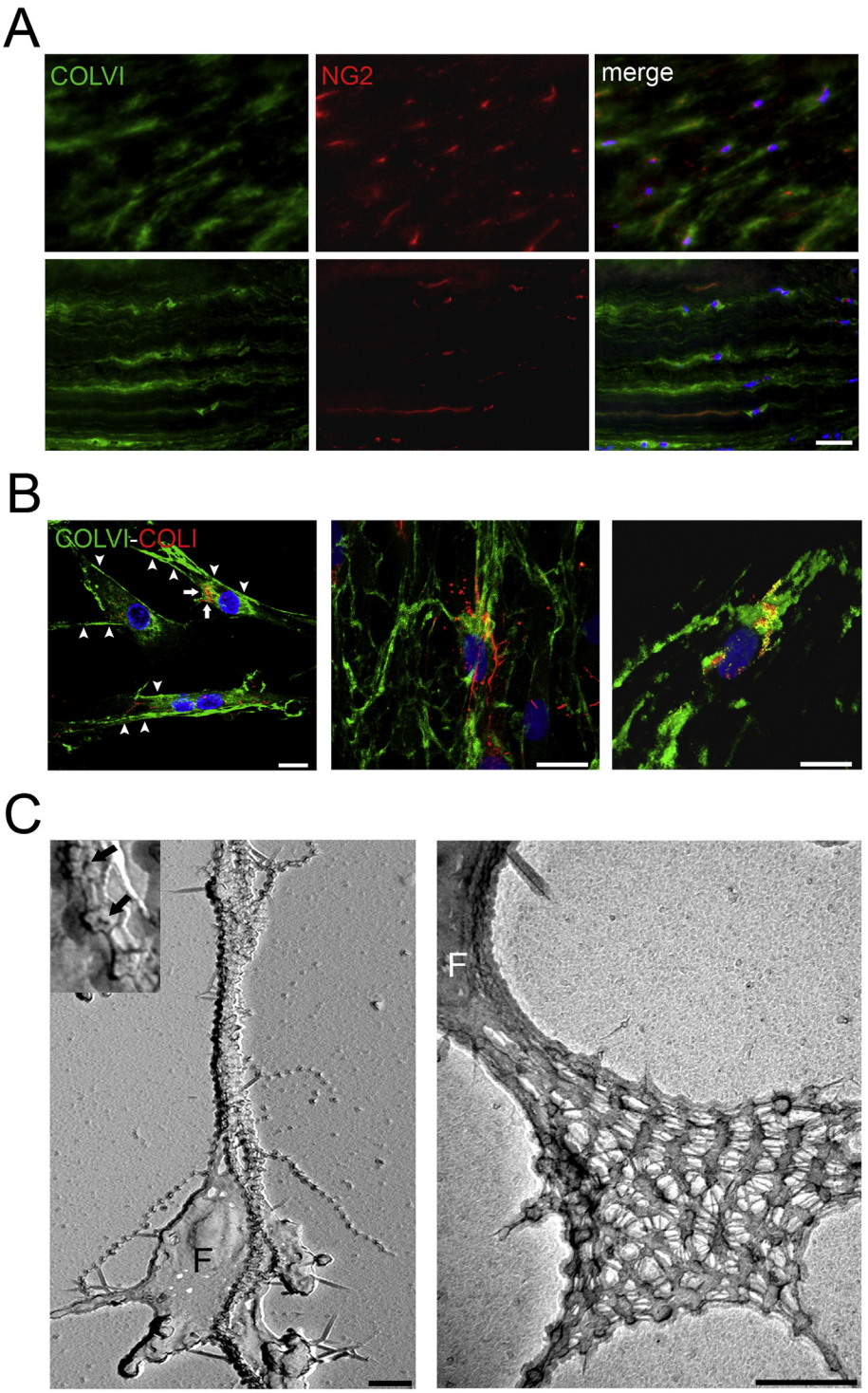

Fig. 1 (*legend on next page*)

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To address further the role of collagen VI in cell polarization we analyzed tendon fibroblasts after *in vitro* perturbation of collagen VI extracellular assembly with the 3C4 antibody, a monoclonal antibody raised against the globular domain of collagen VI which blocks sites of collagen VI polymerization [13,38], and in collagen VI-deficient tendon fibroblasts of a UCMD patient carrying mutations in the *COL6A2* gene [39]. Immunofluorescence analysis of collagen VI in 3C4-treated cultures displayed a dose dependent effect on the protein expression in the extracellular matrix (Fig. 5A), consistent with perturbation of collagen VI fibril assembly. In addition, collagen VI was dramatically reduced in UCMD patient's culture (Fig. 5B), in accordance with the low protein level reported in skeletal muscle [27] and skin fibroblast cultures of this patient [39]. Double labeling of collagen VI and NG2 proteoglycan revealed a moderate reduction of NG2 expression and decreased association of collagen VI with the cell surface in 3C4-treated samples. In UCMD tendon fibroblasts, NG2 staining was barely detectable while collagen VI was apparently not associated with the cell surface (Fig. 5C). A consistent reduction of both collagen VI and NG2 was also demonstrated by western blot analysis (Fig. 5D). Scratch wound assay of tendon fibroblasts in the presence of 3C4 antibody, and of UCMD fibroblasts showed a dramatic effect on wound closure when compared to control cells (Fig. 5E, F). Furthermore, we found a significant increase of incorrectly polarized cells in both 3C4-treated and UCMD fibroblasts, as detected by golgin-97 immunolabeling (Supplementary Fig. 1B). Altogether, these data indicate that collagen VI microfibrils, through interaction with the cell membrane may play a dual role by protecting NG2 proteoglycan from proteolytic degradation, and regulating cell behavior during *in vitro* wound healing.

TGFβ1 mediates myofibroblast trans-differentiation, affects collagen VI–NG2 proteoglycan interaction and determines collagen VI relocalization into the ECM of cultured tendon fibroblasts

TGFβ1 is a potent regulator of ECM production during tendon repair [40], where it induces trans-differentiation of fibroblasts into myofibroblasts [41]. Therefore, we treated tendon fibroblasts with 10 ng/ml TGFβ1 for either four or ten days and assessed the cellular phenotype by western blot analysis. Consistent with myofibroblast trans-differentiation, increased levels of ED-A fibronectin splicing isoform and α-SMA were detected; in contrast, tenomodulin, a tenocyte marker, was reduced. A time-dependent effect of TGFβ1 was evident, as cells treated for ten days showed an increase of myofibroblast markers when compared with cells treated for four days (Supplementary Fig. 2A). The trans-differentiation into myofibroblasts was supported by the presence of α-SMA-positive cells (Supplementary Fig. 2), which also displayed typical thick stress fibers, as revealed by phalloidin staining (Supplementary Fig. 2C) and transmission electron microscopy (Supplementary Fig. 2D).

Strikingly, treatment of tendon fibroblasts with TGFβ1 induced a strong reduction of NG2 and an almost complete loss of collagen VI in the PCM (Fig. 6A). Instead, collagen VI was still detectable in association with ED-A fibronectin (Fig. 6B) and collagen I (Fig. 6C), which were strongly induced by TGFβ1 treatment. Marked changes in the pericellular pattern of collagen VI were also confirmed by rotary shadowing transmission electron microscopy, which showed the absence of a collagen VI network associated with the cell surface (Fig. 7A). Single microfilaments focally connected to the cell membrane were occasionally detected (Fig. 7A, middle panel). In addition, colloidal gold-positive collagen VI microfilaments were found in the

Fig. 1. Collagen VI forms a microfibrillar network in the PCM of tendon fibroblasts. A. Immunofluorescence analysis of collagen VI (COLVI, green) and NG2 proteoglycan (NG2, red) on cross (upper lane) and longitudinal (lower panel) sections of a quadriceps tendon biopsy. Collagen VI is localized among the longitudinally aligned fibrillar matrix and around fibroblasts. Tendon fibroblasts, identified by DAPI nuclear staining express NG2 proteoglycan. Scale bar, 20 μm. B. Immunofluorescence microscopy with collagen VI (COLVI, green) and collagen I (COLI, red) primary antibodies in sub-confluent (left panel) and ten days post-confluent (middle and right panels) tendon fibroblast cultures. Collagen VI networks are detected at the cell surface, and collagen VI structures anchoring the cells to the substrate are also detected (left panel, arrowheads). In sub-confluent cultures, due to the short term condition, collagen I is mainly detectable inside the cells (left panel, arrows). In long-term cultures (middle and right panels), collagen VI is abundantly secreted and appears aligned with the cell axis, with collagen I partly co-localized with the collagen VI network, as also demonstrated in merged 3D-reconstruction (right panel). Nuclei were stained with DAPI (blue). Scale bar, 10 μm. C. Electron microscopy of rotary shadowed replicas of proliferating cultured tendon fibroblasts immunolabeled with an α3(VI) chain specific primary antibody and a 5-nm colloidal gold-conjugated secondary antibody. Colloidal gold particles (box in the left panel, arrows) are associated with the globular domains of collagen VI tetramers. Aligned collagen VI parallel microfilaments appear associated with the cell surface (left panel), with web-like structures detected in the extracellular space, anchoring cell processes (F) to the substrate (right panel). Scale bar, 300 nm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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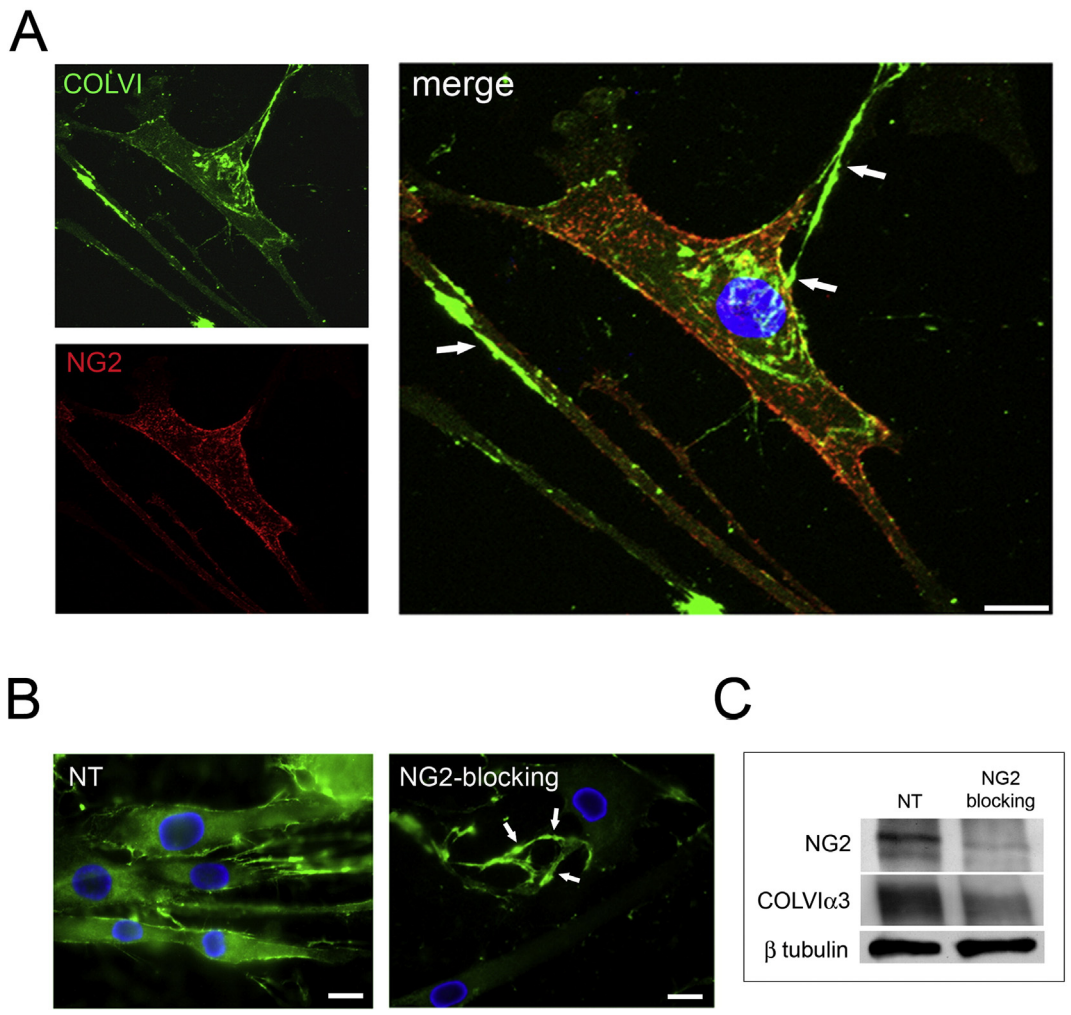

Fig. 2. NG2 proteoglycan contributes to collagen VI pericellular organization. A. Confocal fluorescence microscopy of cultured tendon fibroblasts incubated with primary antibodies against collagen VI (COLVI, green) and NG2 proteoglycan (NG2, red). Collagen VI co-localizes with NG2 at the cell surface. Collagen VI filamentous structures connected to the cell surface are also detectable (arrows). Nuclei were stained with DAPI (blue). Scale bar, 10 μm. B. Representative immunofluorescence microscopy images of collagen VI in tendon fibroblasts, showing reduced association of collagen VI to the cell surface in the presence of 40 μg/ml of NG2-blocking antibody (right panel, NG2-blocking) compared to cells cultured under basal conditions (left panel, NT). Treatment with NG2-blocking antibody does not affect the deposition of collagen VI in the extracellular space (arrows). Nuclei were stained with DAPI (blue). Scale bar, 10 μm. C. Western blot analysis of tendon fibroblast lysates harvested under basal conditions (NT) and after treatment with NG2-blocking antibody (NG2 blocking), showing a marked reduction of α3(VI) chain (COLVIα3) and NG2 in treated cells. β-tubulin was used as loading control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

ECM (Fig. 7A, right panel), confirming the recruitment of collagen VI in extracellular filamentous complexes observed by immunofluorescence analysis.

Western blot analysis of cell lysates in the absence of ascorbic acid did not reveal significant quantitative changes of the α3(VI) chain in cells treated with TGFβ1 (Fig. 7B). However, analysis of the culture medium after induction of collagen VI secretion with ascorbic acid revealed an increased amount of α3(VI) chain in the medium of both four and ten days TGFβ1 treated cells (Fig. 7C). Interestingly, NG2 expression was dramatically reduced, both at protein (Fig. 6B) and transcript (Fig. 7D) levels. These data suggest that TGFβ1 treatment did not affect the synthesis of collagen VI chains, but inhibited collagen VI retention on the cell surface by means of its interaction with NG2.

Discussion

In this study we investigated the molecular basis of the interaction of collagen VI with the plasma

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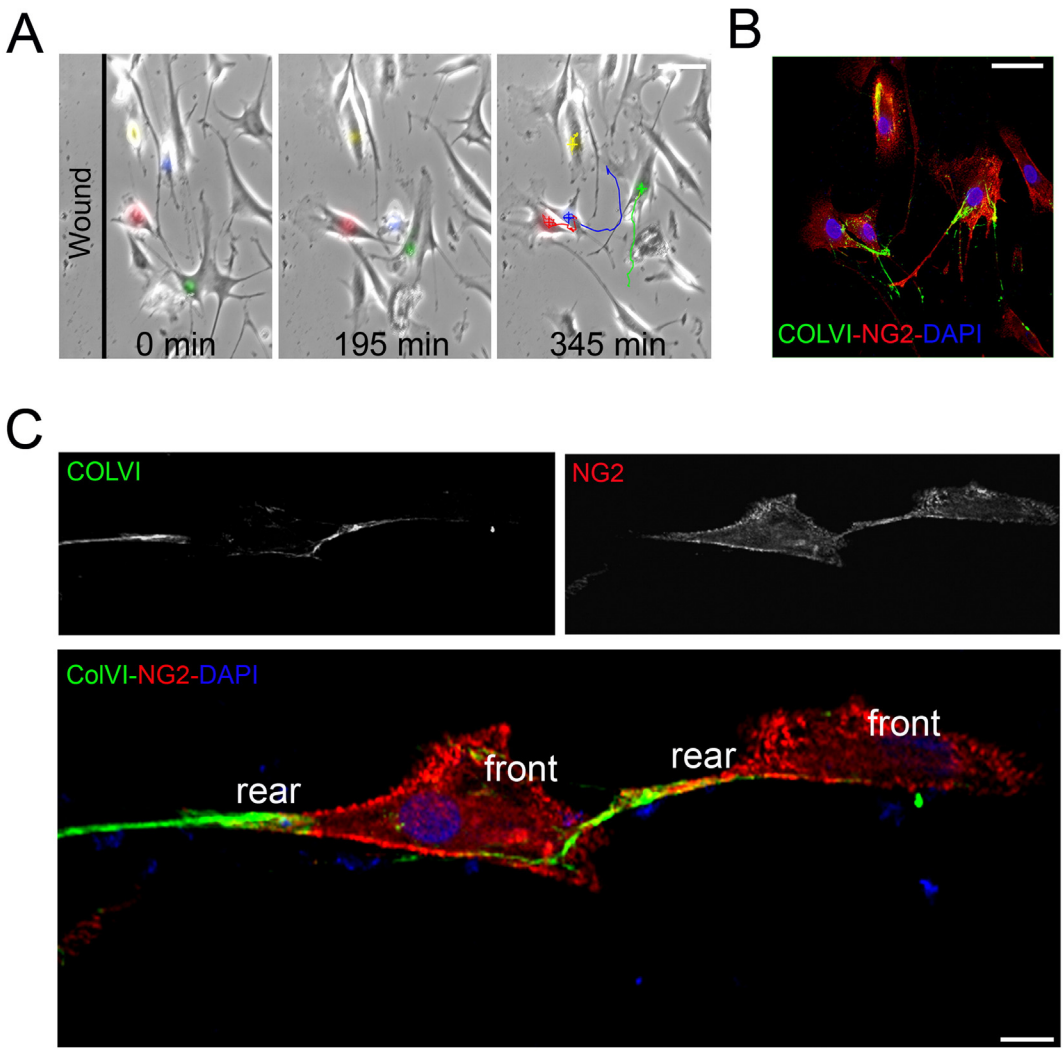

Fig. 3. Collagen VI localizes at the trailing edge of migrating cells. A. Phase contrast microscopy images of cultured tendon fibroblasts during *in vitro* scratch wound assay. Tracking of the individual cell movement is visualized with different artificial colors. Time is indicated in minutes. Scale bar, 100 μm B. Double immunofluorescence microscopy for collagen VI (COLVI, green) and NG2 of the tracked cells shown in the phase contrast microscopy images of panel A. Collagen VI localizes at the rear of moving cells (red, green and blue cells in A), while it associates with the cell body of a stationary cell (yellow cell in phase contrast images in A). Nuclei were stained with DAPI. Scale bar, 100 μm C. Confocal imaging of two polarized cells labeled with antibodies against collagen VI and NG2 showing elongated collagen VI structures connected to NG2-positive rear ends of the cells. The maximum intensity projections of the single channels (white) are shown on the upper row, while the merged image of confocal projections is shown in the lower panel. Scale bar, 10 μm. See also Movie S1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

membrane of human tendon fibroblasts, and analyzed the expression of collagen VI during tendon fibroblast migration and in response to TGFβ1, two events of tendon repair. We found that *i*) NG2 proteoglycan is expressed both *in vivo* and *in vitro* by tendon fibroblasts and is required for the attachment of collagen VI to the cell surface; *ii*) binding of collagen VI to NG2 influences cell behavior, *e.g.* cell motility and polarization; *iii*) in response to TGFβ1 treatment, NG2 proteoglycan is down-regulated and, as a consequence, binding of collagen VI to the surface of tendon fibroblasts is impaired, while the association of collagen VI with ECM partners is maintained, consistent with its role in ECM organization. These data point to a differential spatial localization and three-dimensional organization of collagen VI microfilaments, in order to optimize their regulatory roles on the cell-surface or ECM-linked events required for tendon repair.

The cell surface-associated collagen VI microfibrillar network we found in tendon fibroblast cultures is analogous to that previously described in the whole

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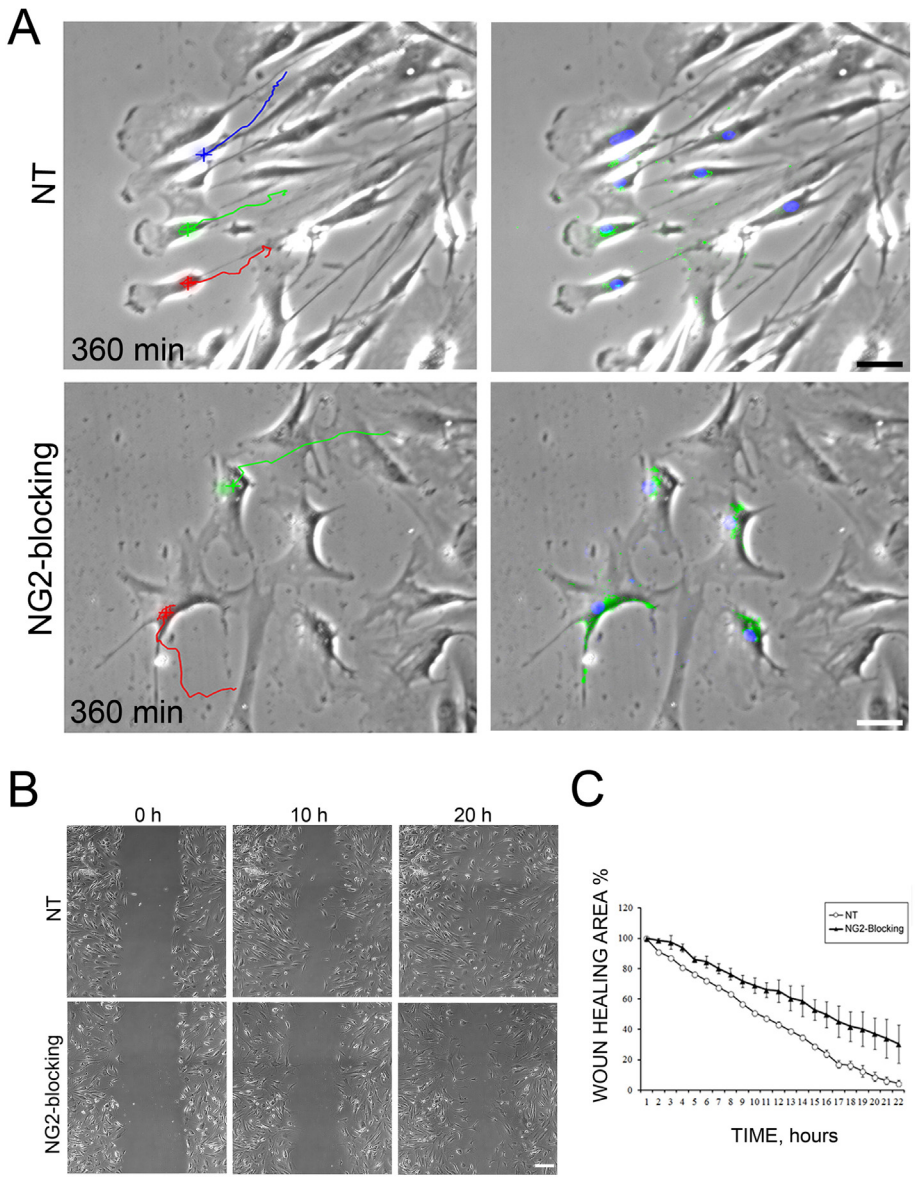

Fig. 4. Effect of NG2-blocking antibody on cell polarization. A. Scratch wound migration assay performed on tendon fibroblasts under basal condition (upper row, NT) and in presence of NG2- blocking antibody (lower row, NG2-blocking). Tracking of the individual cell movement is visualized by different colors in the phase contrast microscopy images of the left panels. Time is indicated in minutes. The positions of the Golgi apparatus, stained with a golgin 97 antibody, and the positions of the nuclei, stained with DAPI (blue), are superimposed in the right panels. Scale bar, 50 μm. B. Representative phase contrast microscopy images of scratch wound assays of tendon fibroblast cultures under basal conditions (NT) and in the presence of a NG2-blocking antibody (NG2-blocking) at the indicated times. Scale bar, 100 μm. C. Graphical representation of the percentage of the wound area at the indicated times in scratch assays carried out in tendon fibroblasts cultured under basal condition (NT) or treated with NG2-blocking antibody (NG2-blocking). Data represent mean ± SE of three independent experiments. $p < 0.01$ *vs* untreated samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tendon [10]. Furthermore, in long-term cultures, we found that collagen VI co-localizes with collagen I fibrils at discrete sites of the cell surface. This result reflects the close association of collagen VI with collagen I fibrils *in vivo* [13], and supports the hypothesis of a role of this ECM component in cell-directed early collagen fibrillogenesis [42]. In addition, our data indicate that collagen VI expressed by cultured tendon fibroblasts maintains similar interactions with cellular and ECM binding partners as in *in vivo* condition.

It is well-known that collagen VI interacts with a variety of cell membrane receptors [23,24,22]. We

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found that tendon fibroblasts express the NG2/CSPG4 proteoglycan and that NG2 and collagen VI co-localized at the cell surface. Notably, *in vitro* treatment with a NG2 blocking antibody affected the association of collagen VI with the cell membrane, suggesting that NG2 is involved in mediating the association of collagen VI with the cell membrane. Previous evidence for this aspect was raised by Nishiyama and Stallcup in studies where cell lines unable to express NG2 were transfected with a full length NG2 transcript, leading to enhanced collagen VI retention at the cell surface [43]. Remarkably, our data indicate a role of NG2-collagen VI interaction under physiological conditions, showing that the endogenous production of NG2 by tendon fibroblasts influences collagen VI deposition, thus potentially acting in the organization of collagen VI-based PCM. The presence of collagen VI in the PCM has

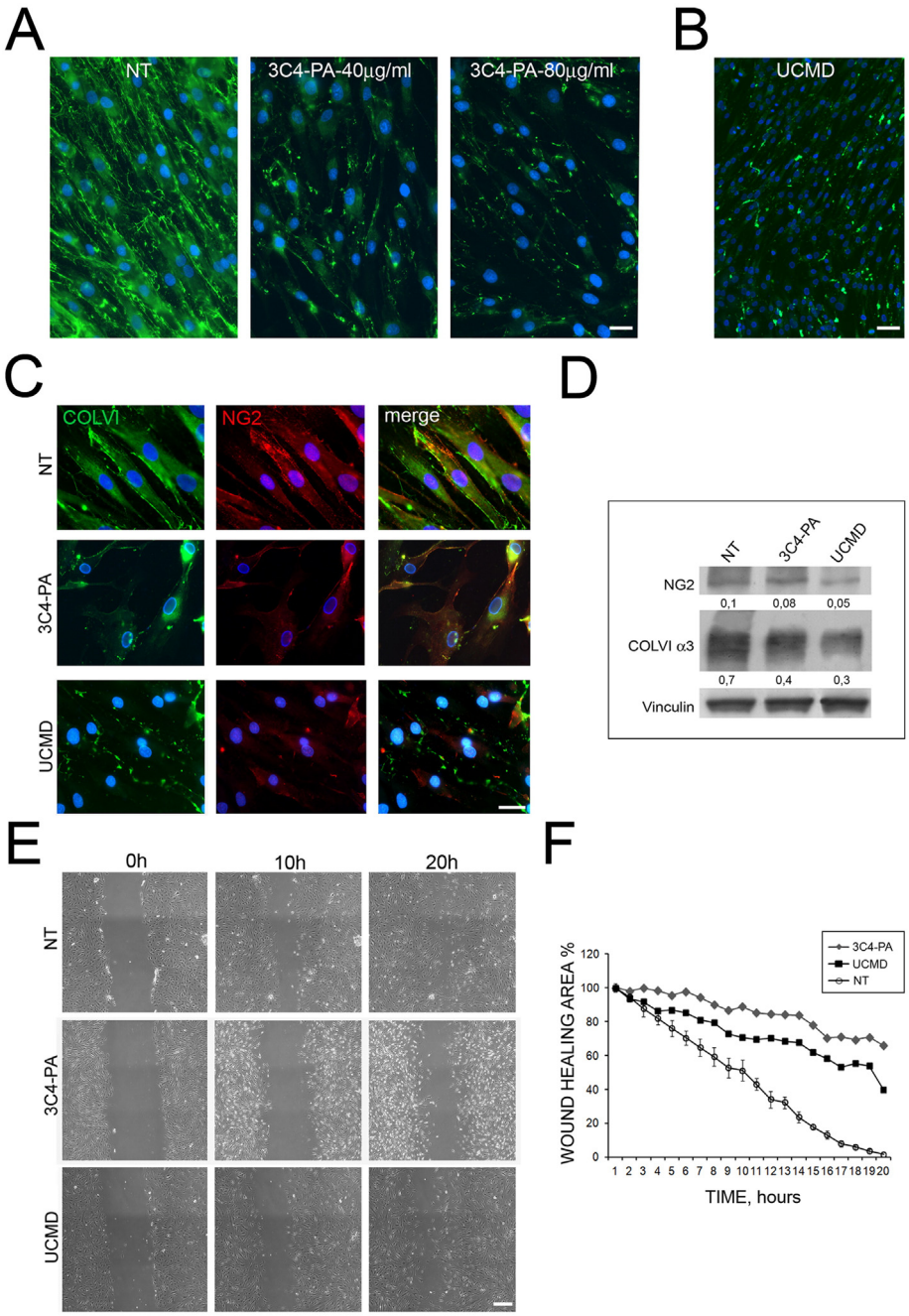

Fig. 5 (*legend on next page*)

been reported in several tissues, as cartilage [44], skeletal muscle [45,46], tendon [10] and ligaments [33]. Notably, chondrocytes [47], myoblasts [48], and ligament fibroblasts [33] express NG2 both *in vivo* and *in vitro*, pointing to a pivotal role of this transmembrane proteoglycan in collagen VI PCM regulation. Notably, we found that NG2 proteoglycan was also expressed by tendon fibroblasts *in vivo*, further supporting the role of collagen VI in tendons.

The impact of the binding of collagen VI to NG2 on tendon fibroblast behavior was not studied so far. Our data reveal that the binding of collagen VI to NG2 is essential for the direction of tendon fibroblasts migration *in vitro.* We found that collagen VI localized at the trailing edge of migrating cells anchors the cell tail to the substrate. It has been proposed that in migrating cells the tail acts as a drag mechanism essential for the direction of migration [36]. Collagen VI, by interacting with NG2, may provide a support for the anchorage of the cell to the ECM, and thus contribute to the stabilization of the trailing edge and the polarization of the cell during migration. Our data are consistent with a previous report that demonstrated the involvement of NG2-mediated binding to collagen VI influenced cell migration and polarization during tumor engraftment [49]. In addition, the delayed *in vitro* wound closure we observed in scratch assay experiments in the presence of NG2-blocking antibodies further strengthens the role of NG2-collagen VI binding for regulating tendon fibroblast migration. On the other hand, it is possible that inhibition of the collagen VI binding to NG2 may affect the regulatory role of NG2 in the formation of retraction fibers and in cell migration [50].

Consistent with a role of collagen VI in regulating cell behavior during migration, *in vitro* we found a delay of wound closure in cultures treated with an anti-collagen VI antibody that impairs the extracellular microfibril assembly [38], and in collagen VI deficient cells of a UCMD patient carrying *COL6A2* mutation [39]. It is interesting to note that the expression of NG2 proteoglycan was affected both in 3C4-treated samples and UCMD tendon fibroblasts, indicating that changes in collagen VI expression also cause a parallel change in NG2 expression. It is possible that membrane-associated collagen VI might protect NG2 from proteolytic degradation [22]. Consistent with this hypothesis, NG2 alterations have been previously reported in collagen VI deficient UCMD skeletal muscle [51,52] and in *Col6a1* null mice [52].

It has been established that after migration to the damaged area, tendon fibroblasts may further contribute to the repair of tissue by producing ECM proteins and directing tissue remodeling. These events are modulated by a variety of cytokines released by cells at the injured site [40]. We explored the fate of collagen VI and NG2 in tendon cultures in response to TGFβ1, a potent regulator of tendon repair. We found that the TGFβ1-induced trans-differentiation of tendon fibroblasts into myofibroblasts resulted in dramatic changes of collagen VI localization and NG2 expression. Notably, TGFβ1 induced a marked reduction of NG2, both at protein and transcript levels, with dramatic consequences on collagen VI attachment to the cell membrane, as indicated by the absence of typical collagen VI networks at the cell surface of TGFβ1-treated cells. In addition, western blot revealed that the amount of collagen VI chains synthesized by TGFβ1-treated cells was still comparable to that of untreated cells, thus the decreased association of collagen VI with the cell surface elicited by TGFβ1 treatment most likely relies upon changes in NG2 expression. Consistent with persistent collagen VI synthesis and secretion, collagen VI was still detected

Fig. 5. Collagen VI deficiency affects NG2 expression and cell migration in 3C4-treated and UCMD tendon fibroblasts. A. Immunofluorescence microscopy of collagen VI in peroneal tendon fibroblasts, showing reduced expression in cells subjected to perturbing assay with anti-collagen VI 3C4 antibody (3C4-PA) at different concentrations (40 and 80 μg/ml, middle and right panels, respectively) compared to cells cultured under basal conditions (left panel, NT). Nuclei were stained with DAPI (blue). Scale bar, 20 μm. B. Immunofluorescence microscopy of collagen VI in peroneal tendon culture of the UCMD patient showing a marked reduction of protein secretion associated with altered organization pattern, consistent with the *COL6A2* mutation. Nuclei were stained with DAPI (blue). Scale bar, 40 μm. C. Double immunofluorescence microscopy of collagen VI (COLVI, green) and NG2 proteoglycan (NG2, red) in normal (NT, upper row), 3C4-treated (3C4-PA, middle row) and UCMD patient (UCMD, lower row) tendon cultures, showing that the reduced expression of collagen VI impairs the expression of NG2 in both 3C4-treated and UCMD cells. Nuclei were stained with DAPI (blue). Scale bar, 20 μm. D. Western blot analysis of normal tendon fibroblast lysates harvested under basal conditions (NT) or after treatment with 3C4 antibody (3C4-PA), and of UCMD patient (UCMD). A reduction of the α3chain of collage VI (COLVIα3) and NG2 proteoglycan (NG2) is observed in both 3C4-treated and UCMD patient, although the defect is more evident in UCMD cells. Blots were probed for vinculin to verify equal loading. The numbers below each band show densitometric values that are corrected for loading. E. Representative phase contrast microscopy images of scratch wound assays of tendon fibroblast cultures under basal conditions (NT, upper row) or after treatment with 3C4-antibody (3C4-PA, middle row), and of tendon fibroblasts from UCMD patient (UCMD, lower row) at the indicated times. Scale bar, 100 μm. F. Graphical representation of the percentage of the wound area at the indicated times in scratch assays carried out in tendon fibroblasts cultured under basal condition (NT) or treated with 3C4 antibody (3C4-PA), and in UCMD patient (UCMD). Data represent mean ± SE of three independent experiments. $p < 0.01$ *vs* untreated samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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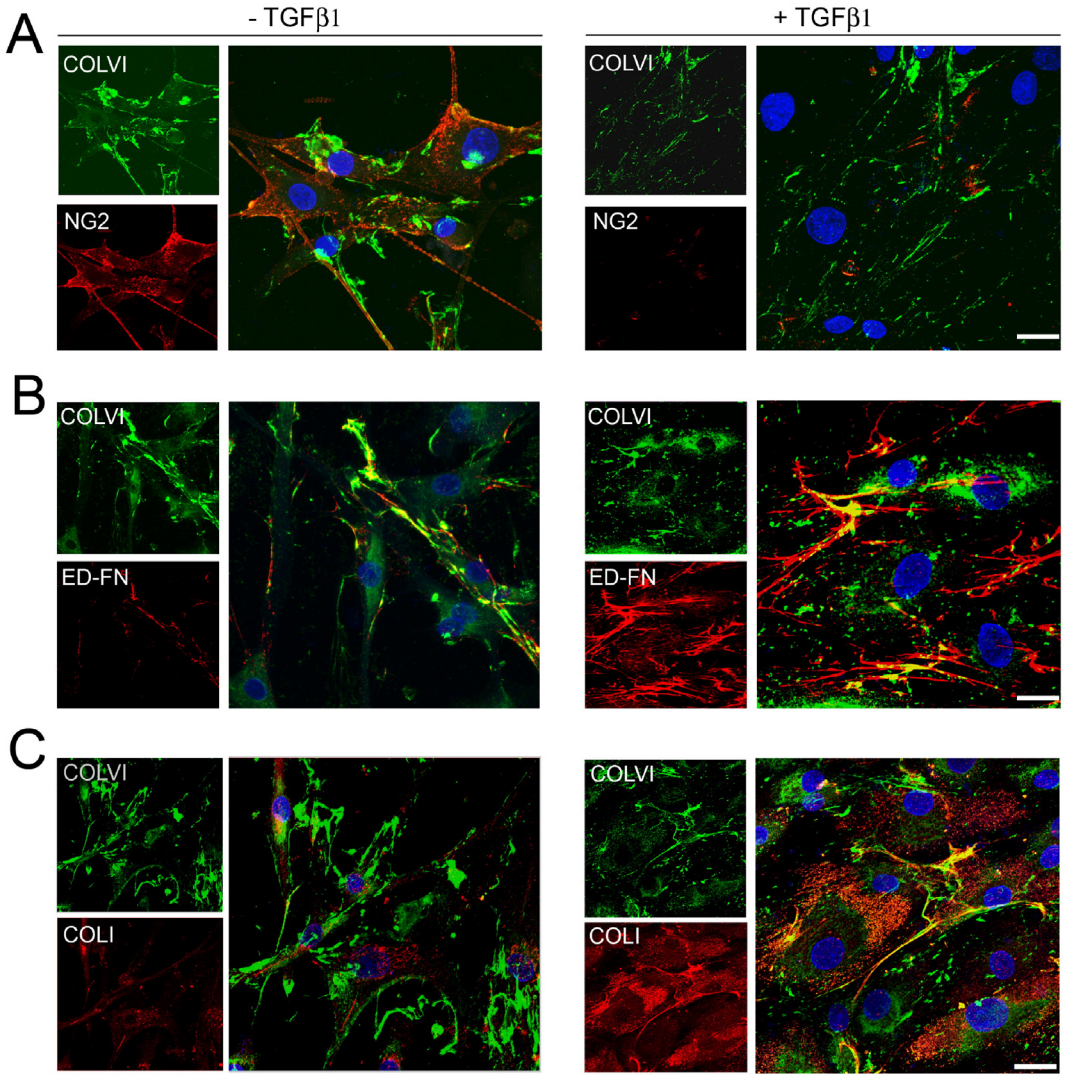

Fig. 6. TGFβ1 treatment affects NG2 expression and collagen VI pericellular localization in tendon fibroblast cultures. A. Immunofluorescence microscopy of collagen VI (COLVI, green) and NG2 proteoglycan (NG2, red) on tendon fibroblasts grown for ten days under basal conditions (−TGFβ1) and after treatment with 10 ng/ml TGFβ1 (+TGFβ1). Nuclei were stained with DAPI (blue). Scale bar, 10 μm. B. Immunofluorescence microscopy of collagen VI (COLVI, green) and ED-A fibronectin (ED-FN, red) of cultured tendon fibroblasts grown for ten days under basal condition (−TGFβ1) and after treatment with 10 ng/ml TGFβ1 (+TGFβ1). Nuclear staining, DAPI (blue). Scale bar, 10 μm. C. Immunofluorescence microscopy for collagen VI (COLVI, green) and collagen I (COLI, red) in cultured tendon fibroblasts grown for ten days under basal condition (−TGFβ1) and after treatment with 10 ng/ml TGFβ1 (+TGFβ1). Nuclei were stained with DAPI (blue). Scale bar, 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

in the ECM within multi-protein complexes also containing collagen I and ED-A fibronectin. The recruitment of collagen VI in areas of ECM assembly reflects its suggested role in early collagen fibrillogenesis [42]. The molecular mechanism by which collagen VI regulates collagen fibril morphology remains unclear. Collagen VI interacts with collagen I [53] and with crucial regulators of collagen fibrillogenesis including small leucine rich proteoglycans [54] and collagen XIV [55]. Moreover, it associates with collagen fibrils in developing tendon [56]. It is noteworthy that collagen VI deficient mice display a dysfunctional regulation of collagen fibrillogenesis in tendon, as indicated by the reduced cross-sectional areas of collagen fibrils and decreased tendon stiffness [32,31], and in skin, as indicated by an altered collagen I fibril architecture and decreased tensile strength [57].

Myofibroblasts are mechanically active cells, which promote tissue contraction by establishing cell–cell and cell–ECM specific interactions. Due to

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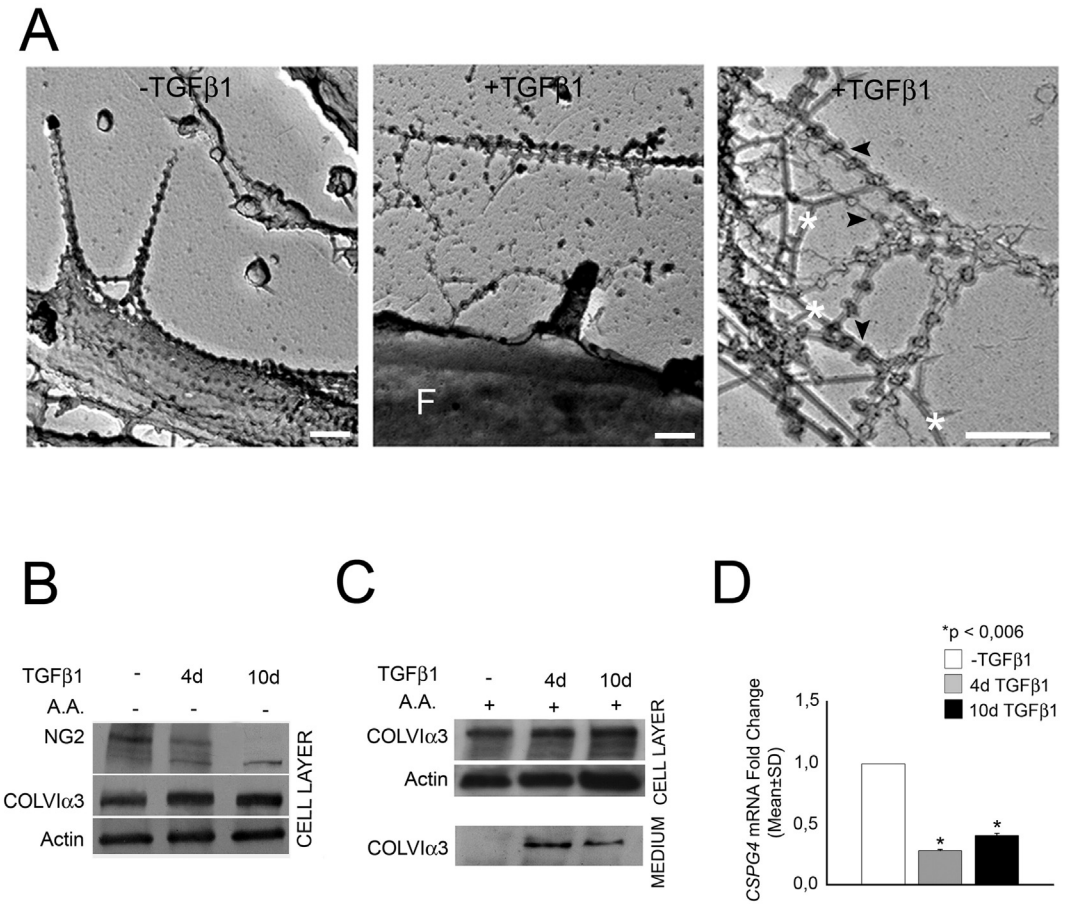

Fig. 7. TGFβ1-related changes of cell surface association of the collagen VI microfibrillar network. A. Electron microscopy of rotary shadowed replicas of tendon fibroblasts cultured under basal condition (–TGFβ1, left panel) and after treatment with 10 ng/ml TGFβ1 (+TGFβ1, middle and right panels), showing the loss of collagen VI network association with the fibroblast surface (F, middle panel) in TGFβ1-treated cells. Collagen VI microfilaments (arrowheads) associated with non-beaded thin fibrils (asterisks) are detected in the extracellular space (right panel). Scale bar, 300 nm. B. Western blot analysis of cell layer derived from tendon fibroblasts cultured under basal condition (–) and after treatment with 10 ng/ml TGFβ1 for four and ten days (4 d, 10 d) post-confluence in absence of L-ascorbic acid (A.A.). Cells were scraped, and the extracts were separated on a 4–20% SDS polyacrylamide gel under reducing conditions. The levels of the α3(VI) chain and NG2 were evaluated with specific antibodies. Actin was used as loading control. C. Western blot analysis of the α3(VI) chain in the cell layers and conditioned media of tendon fibroblasts, grown for four and ten days in the presence of L-ascorbic acid (A.A.) and/or TGFβ1. D. Real time-PCR analysis of *CSPG4* transcription in tendon fibroblasts grown four and ten days post-confluence under basal condition (–TGFβ1) or in the presence of 10 ng/ml TGFβ1 (4 d TGFβ1, 10 d TGFβ1). Reported values are the mean of three independent experiments. *GAPDH* was used as reference transcript, and the relative ratio of *CSPG4*/*GAPDH* mRNAs in untreated cells was normalized to 1. $*p < 0.006$ *vs* basal condition.

this function, myofibroblasts are key players in the classic connective tissue wound healing paradigm but are also associated with scar formation. The mechanism of scar formation in tendon remains elusive. Imbalance between collagen I and collagen III [58] and tissue hyper-contraction related to the presence of myofibroblasts [5] are thought the major cause of scar-related tissue dysfunction. The data presented here suggest that the loss of the collagen VI-based PCM may represent the basis for altered tendon functionality. As a speculation, restoring a collagen VI-based PCM may represent a first step to implement therapeutic strategies aiming at restoring a normal tendon structure at injury sites.

Our findings establish a critical role for NG2 proteoglycan in mediating collagen VI attachment to the surface of tendon fibroblasts. By interacting with NG2 and ECM partners, collagen VI may regulate cell behavior and influence tendon matrix assembly, two processes that play a pivotal role in tendon repair. Tendon healing represents a clinical challenge to orthopedic surgery, mainly because damaged tissue responds poorly to treatment and requires prolonged rehabilitation. Therapeutic

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options used to repair ruptured tendons consist of suture, autografts, allografts, and synthetic prostheses [40]. None of these alternatives provide a successful long-term solution, and often the restored tendons do not recover their complete strength and functionality [59]. On the other hand, the current knowledge of human tendon biology is limited, therefore the elucidation of critical mechanisms for tendon repair is crucial to develop new treatment options. The identification of collagen VI as a key regulator of critical events of tendon repair has relevant implications for the development of therapeutic strategies, which may accelerate and improve the response to injury.

Experimental procedures

Tendon biopsies and cell cultures

Human quadriceps tendon fragments were harvested from four patients (17, 21, 51, and 60 years old) during standard orthopedic surgery (total knee arthroplasty and anterior cruciate ligament replacement). Peroneal tendon biopsies were harvested from two healthy subjects (17 and 21 years old) during foot surgery and during a Grice procedure from a previously genetically characterized UCMD patient carrying compound heterozygous mutation for a G>A variation at position +5 of COL6A2 intron 8 and a nonsense mutation R366X in *COL6A2* exon 12 [39]. All patients provided informed consent. Quadriceps tendon fragments were frozen in isopentane pre-chilled in liquid nitrogen (LN2) and processed for immunofluorescence microscopy as previously reported [33]. For tendon fibroblast cultures, quadriceps and peroneal tendon fragments were subjected to mechanical dissociation, and maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 1% antibiotics and 10% fetal bovine serum (FBS) [35]. For immunofluorescence analysis, tendon fibroblasts were grown onto coverslips and, when indicated, treated with 10 ng/ml TGFβ1 (Sigma–Aldrich) for 4 and 10 days. L-ascorbic acid (0.25 mM) was added to the medium to allow collagen secretion [60].

Inhibition of collagen VI cell surface binding by NG2-blocking antibody

NG2-blocking antibody (clone 9.2.27, Santa Cruz Biotechnology) was added to the culture medium (40 μg/ml). After 24 h, the medium was replaced with DMEM supplemented with 40 μg/ml NG2-blocking antibody and 0.25 mM L-ascorbic acid. After 24 h, cells were fixed with cold methanol for the immunofluorescence study or collected for western blot analysis.

Antibody perturbation of collagen VI polymerization

Peroneal tendon fibroblasts of control and UCMD patient were grown to confluence and treated with 3C4 anti-collagen VI monoclonal antibody (Millipore) (40 or 80 μg/ml) for 48 h in the presence of L-ascorbic acid (0.25 mM). This antibody specifically binds to the globular domains of collagen VI molecules [13], which are needed for tetramer polymerization and secretion. Monolayer cultures were fixed with cold methanol and processed for immunofluorescence labeling or collected for western blot analysis.

Scratch wound healing assay

Normal and UCMD tendon fibroblasts were seeded onto poly-D-lysine-coated tissue culture dishes and cultured to confluence in 10% FBS-containing medium for 24 h. Normal tendon fibroblasts were incubated with or without NG2-blocking antibody (40 μg/ml) or 3C4 antibody (80 μg/ml) in a serum-containing medium for 24 h. The medium was then replaced with medium containing 0.25 mM L-ascorbic acid and NG2-blocking or 3C4 antibodies and grown for an additional 24 h. A straight scratch simulating a wound was made across the center of the cell monolayer in each dish by using a sterile 200-μL pipette tip. For live imaging, tissue culture dishes were mounted on a dedicated stage incubator (OkoLab) and an Eclipse Ti-E inverted microscope (Nikon). Phase contrast imaging was performed with a 10× (0.25 NA) Nikon objective lens. The experiment was stopped when control samples reached confluence in the scratched area, and the wound areas were determined as percentage of the initial free-cells area at different times. To evaluate the localization of collagen VI and NG2 in migrating cells, confluent cultures were scratched and phase contrast images were acquired at regular intervals of 15 min for 6 h. Cells were then fixed with cold methanol and processed for collagen VI and NG2 or golgin-97 immunofluorescence analysis.

Immunofluorescence and confocal analysis

Cells grown onto coverslips were incubated with an affinity-purified rabbit polyclonal antibody against the collagen VI α3 chain [20], or with an antibody against the collagen VI globular domain (clone 3C4, Millipore), NG2 proteoglycan (Chemicon), ED-A-fibronectin (Enzo), TRITC-conjugated phalloidin (Sigma), golgin 97 (Molecular Probe), or collagen I (Sigma-Aldrich). Double labeling of collagen VI and ED-fibronectin or collagen I were performed with the rabbit anti-collagen VI α3 chain antibody, while the double labeling of collagen VI and NG2 was performed with the mouse monoclonal 3C4 antibody. FITC or TRITC-conjugated anti-mouse or anti-rabbit secondary antibodies

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(DAKO) were used. Cell nuclei were stained with 1 mg/ml DAPI (Sigma-Aldrich). Samples were mounted with an anti-fading reagent (Molecular Probes Life Technologies) and observed with a Nikon epifluorescence microscope. The confocal imaging was performed with a Nikon A1-R confocal laser scanning microscope, equipped with a 60×, 1.4 NA objective and with 405, 488 and 561 nm laser lines to excite DAPI (blu), FITC and TRITC fluorescence signals. Each final confocal image, of 1024 × 1024 pixels and 4096 gray levels, was obtained by maximum intensity projection of ten optical sections passed through the central region of the cells (recorded at z-step size of 300 nm). Volume view with 3D rendering was carried out using the NIS Elements Advanced Research software (Nikon).

Western blot analysis

Cultured tendon fibroblasts were harvested by scraping. The media recovered from the different culture conditions were concentrated with Vivaspin sample concentrators (Vivaspin 2 MWCO10000, GE Healthcare) according to the manufacturer's operating procedures. Cell lysates and concentrated culture media were resolved by standard SDS–PAGE, electro-blotted onto a nitrocellulose membrane [61] and incubated with antibodies against ED-A fibronectin (Enzo), collagen VI α3 [20], tenomodulin, β-tubulin, actin, αSMA, vinculin (Sigma-Aldrich), and NG2 (Chemicon, MAB5320), followed by incubation with anti-mouse or anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies. Chemiluminescent detection of proteins was carried out with the ECL detection reagent Kit (GE Healthcare Amersham, Pittsburgh, PA) according to the supplier's instructions.

Rotary shadowing

Tendon fibroblasts were grown onto coverslips and, after confluence, treated for 24 h with 0.25 mM L-ascorbic acid. *In vitro* immunolabeling was performed with a polyclonal antibody against collagen VI α3 chain [21]. Rotary shadowing of immuno-gold labeled samples was performed following reported procedures [29]. Replicas were washed with distilled water, collected on copper grids, and examined with a Philips EM 400 electron microscope at 100 kV.

RNA isolation and real-time qRT-PCR

Total RNA from cells was isolated using Trizol reagent (Life Technologies) following the manufacturer's instructions. cDNA products were generated from 600 ng of total RNA with SuperScript III reverse transcriptase (Invitrogen) and analyzed by qRT-PCR with the Rotor Gene SYBR Green PCR kit (Qiagen). Data were normalized to *GAPDH* expression. The following primer pairs were used for the PCR amplification: *CSPG4* (Forward: 5′-GGCCCCCA CTTCCAGCC-3′, Reverse: 5′-TCTCACCGAAGAA GGAAGCCGC-3′); GAPDH (Forward: 5′-GTC AAGGCTGAGAACGGGAA-3′, Reverse: 5′-AAAT-GAGCCCCAGCCTTCTC-3′).

Statistical analysis

Statistical analysis was performed by Student's t-test with the Statistical Package for the Social Sciences software (SPSS, Chicago, IL). The results were considered statistically significant for P values <0.05.

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Abbreviations used:
TGFβ1, transforming growth factor β1; PCM, pericellular matrix.

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