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Defective collagen VI α6 chain expression in the skeletal muscle of patients with collagen VI-related myopathies

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ABSTRACT

Collagen VI is a non-fibrillar collagen present in the extracellular matrix (ECM) as a complex polymer; the mainly expressed form is composed of α1, α2 and α3 chains; mutations in genes encoding these chains cause myopathies known as Ullrich congenital muscular dystrophy (UCMD), Bethlem myopathy (BM) and myosclerosis myopathy (MM). The collagen VI α6 chain is a recently identified component of the ECM of the human skeletal muscle. Here we report that the α6 chain was dramatically reduced in skeletal muscle and muscle cell cultures of genetically characterized UCMD, BM and MM patients, independently of the clinical phenotype, the gene involved and the effect of the mutation on the expression of the "classical" α1α2α3 heterotrimer. By contrast, the collagen VI α6 chain was normally expressed or increased in the muscle of patients affected by other forms of muscular dystrophy, the overexpression matching with areas of increased fibrosis. *In vitro* treatment with TGF-β1, a potent collagen inducer, promoted the collagen VI α6 chain deposition in the ECM of normal muscle cells, whereas, in cultures derived from collagen VI-related myopathy patients, the collagen VI α6 chain failed to develop a network outside the cells and accumulated in the endoplasmic reticulum. The defect of the α6 chain points to a contribution to the pathogenesis of collagen VI-related disorders.

1. Introduction

Collagen VI is an extracellular protein forming a distinct microfibrillar network in most interstitial connective tissues; it has a pivotal role in maintaining skeletal muscle integrity and function [1,2]. In humans, the most widespread collagen VI form is composed of α1, α2 and α3 chains that associate inside the cell in a stoichiometric ratio to form heterotrimeric monomers that assemble into dimers and tetramers [3,4]. After secretion, tetramers associate end-to-end finally forming microfibrils with a beaded filament structure [5] by interactions of their non-collagenous domains [6]. In the extracellular matrix, collagen VI interacts with several other components, including collagen I [7], collagen II and decorin [8], collagen XIV [9], perlecan [10] and the microfibril-associated glycoprotein MAGP1 [11].

Recently, three novel chains, α4, α5 and α6, were identified [12,13]. The new chains share sequence homology and domain structure with the α3 chain; each of them consists of seven von Willebrand factor type A (VWA) domains followed by a short collagenous domain, two or three C-terminal VWA domains and a chain-specific domain [13]. In mouse, the novel chains show a highly differential, often complementary expression [14], pointing to the possibility of tissue-specific roles of the novel chains in collagen VI assembly and function. Due to their similar structure and phylogenetic conservation, the new chains may have the potential to replace the α3 chain forming α1α2α4, α1α2α5 and α1α2α6 heterotrimers, thereby increasing the structural and functional versatility of collagen VI [13]. This hypothesis is supported by results obtained in the *Col6a1* −/− mouse model in which all collagen VI chains are largely absent, demonstrating that the presence of the collagen VI α1 chain is a prerequisite for collagen VI secretion [14,15]. In contrast, an *in vitro* study indicated that the α6 chain does not assemble with α1 and α2 chains, pointing to an alternative collagen VI assembly process [12,16].

In humans, the *COL6A4* gene which encodes the α4 chain, is inactivated because of a pericentric chromosomal inversion [13,17]. The expression of *COL6A5* mRNA is restricted to a few tissues, including the lung, testis, colon [12] and skin [18]. The selective expression of the

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α5 chain at the dermal–epidermal junction [18] and at the myotendinous junction in the skeletal muscle [19], points to a specialized function of α5 at junctional areas, perhaps important for the resistance to tensile stress. *COL6A6* mRNA is expressed in a wide range of fetal and adult tissues [12]; in the skeletal muscle the α6 chain is localized in the endomysium and perimysium, however, unlike the α3 chain, it is not associated with basement membranes [19]. The differential distribution of the novel chains points to specific roles in development, tissue homeostasis or pathogenesis of inherited diseases.

Mutations in the *COL6A1*, *COL6A2* and *COL6A3* genes cause a group of congenital muscular dystrophies: Ullrich congenital muscular dystrophy (UCMD) is a severe disorder characterized by congenital muscle weakness with axial and proximal joint contractures and coexisting distal joint hypermobility [20]; Bethlem myopathy (BM) is an autosomal dominant [21,22,23] or recessive [24] disorder characterized by slowly progressive axial and proximal muscle weakness with finger flexion contractures; myosclerosis myopathy (MM) is a recessive disorder characterized by progressive all-joint contractures [25]. The majority of collagen VI gene mutations impair the secretion and deposition of collagen VI in skeletal muscle and/or fibroblast cultures [26–28]. However, several dominant mutations in BM patients have no detectable effect on collagen VI assembly and secretion, but compromise protein function in the extracellular matrix of muscle [29]. We recently demonstrated that mutations in *COL6A1* and *COL6A2* impair the expression of α5 and α6 chains in the skin of UCMD patients, pointing to a contribution of these chains to the skin phenotype. In contrast, the α5 and α6 chains were apparently unaffected in the skin of UCMD patients with *COL6A3* mutations, suggesting that they may compensate for the deficiency of the α3 chain by forming α1α2α5 or α1α2α6 heterotrimers [18].

To give insights into the pathogenesis of collagen VI-related skeletal muscle disease we explored the expression and distribution of the newly identified α6 chain in muscle biopsies and muscle cell cultures derived from patients affected by collagen VI-related myopathies.

2. Materials and methods

2.1. Patients and muscle biopsies

Skeletal muscle biopsies from three healthy subjects and sixteen genetically characterized patients with collagen VI-related disease (eight BM, seven UCMD and one MM patients) were collected (Table 1). Muscle biopsies were also obtained from patients affected by myopathies not related to collagen VI (one DMD, one MDC1A, one Miyoshi myopathy, one EDMD, one rigid spine syndrome (RSS) caused by FHL1, and one RSS caused by SEPN1). All samples were frozen in melting isopentane and stored in liquid nitrogen. All patients were previously diagnosed by genetic, biochemical and/or immunohistochemical analyses. All participants provided written informed consent, and approval was obtained from the Ethics Committee of the Rizzoli Orthopedic Institute.

2.2. Muscle cell cultures

Primary cell cultures derived from two normal controls, three dominant BM, one recessive BM and two UCMD patients carrying mutations in the *COL6A1*, *COL6A2* or *COL6A3* genes were established as described [30] and maintained in DMEM plus 20% fetal calf serum [31]. Muscle cells were grown until confluence, treated with 0.25 mM L-ascorbic acid (Sigma) and, when needed, supplemented with 10 ng/ml TGF-β1 (Sigma) for 96 h.

2.3. Immunofluorescence and confocal microscopy

Frozen sections (7 μm-thick) from muscle of healthy donors and dystrophic patients were fixed with 2% paraformaldehyde in phosphate buffered saline (PBS), permeabilized with 0.15% Triton X100, incubated with the affinity-purified rabbit polyclonal antibody against the α6 chain [18], and detected with anti-rabbit TRITC-conjugated IgG (DAKO). All sections were double labeled with a mouse monoclonal antibody against the laminin γ1 chain (Chemicon) revealed with anti-mouse FITC-conjugated IgG (DAKO). The expression and distribution of classical collagen VI (α1α2α3) was assessed with a polyclonal antibody (Fitzgerald Industries International) and compared with the staining obtained with a rat monoclonal antibody against perlecan (Chemicon), used as a basement membrane marker. Muscle cells were grown on coverslips, fixed with cold methanol, washed with PBS and incubated with rabbit anti-α1α2α3 or anti-α6 chain antibodies. Alpha-smooth muscle actin (α-SMA) expression was assessed in cultured cells subjected to TGF-β1 treatment and compared to untreated cells by labeling with anti-α-SMA antibody (Sigma). All samples were counterstained with DAPI to detect nuclei, mounted with an anti-fading reagent (Molecular Probes) and observed with a Nikon epifluorescence microscope.

Double labelings with anti-Limp II (Santa Cruz Biotechnologies) or anti-Grp78 (StressGene Biotechnologies) antibodies and the anti-α6 antibody were performed after TGF-β1 treatment. The confocal imaging was performed with an A1-R confocal laser scanning microscope (Nikon), equipped with a Nikon 60×, 1.4 NA objective, and with 488 and 561 nm laser lines to excite FITC (green) and TRITC (red) 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 a z-step size of 300 nm).

2.4. Immunoblotting

Cultured cells were washed and scraped into lysis buffer (20 mM Tris–HCl, pH 7.0, 1% Nonidet P-40, 150 mM NaCl, 10% glycerol, 10 mM EDTA, 20 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors). The total protein content was quantified using a Bradford protein assay (Bio-Rad). Samples (20 μg of protein) were reduced with 20% β-mercaptoethanol and separated by SDS-PAGE on 4–20% gradient polyacrylamide gels (Bio-Rad). The fractionated proteins were blotted onto a nitrocellulose membrane at 30 V overnight at 4 °C and the membrane was blocked with 5% dry milk solution for subsequent immunodetection with specific antibodies: anti-α6 chain was used at 1:500; anti-α1 chain (Santa Cruz) at 1:1000; anti-collagen VI (Fitzgerald) at 1:500, and anti-pSMAD 2/3 Ser 423/425 (Santa Cruz) at 1:500. Loading controls were performed with an antibody against GAPDH (Millipore), at 1:8000. Secondary antibodies conjugated with horseradish peroxidase were employed (GE Healthcare) and chemiluminescent detection of proteins was carried out by using the enhanced chemiluminescence method (GE Healthcare) according to the supplier's instructions.

2.5. RNA analysis

Total RNA was isolated from myoblasts of patients by using the RNeasy Kit (QIAGEN, Chatsworth, CA) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). In order to quantify the steady state level of *COL6A6* transcripts, commercially available TaqMan expression assays (Applied Biosystems) were used for the target gene (*COL6A6*: Hs01029204_m1 exons 12–13) and for β-actin as a housekeeping reference gene (ACTB Endogenous Control). Real-time PCR was performed in triplicate on the Applied Biosystems Prism 7900HT system, using 10 ng of cDNA and default parameters. Evaluation of *COL6A6* transcript level was performed by the comparative CT method (ΔΔCT method; Applied Biosystems User Bulletin #2). cDNAs from control myoblasts were utilized as calibrators.

Table 1
Description of the clinical phenotypes, mutations, and immunofluorescence analyses of collagen VI α1α2α3 and α6 chains' expression in muscle biopsies of collagen VI-related patients. Abbreviations: DL, distal laxity; FC, finger's contractures; SC, skin changes (keloid formation, follicular hypercheratosis); MV, nocturnal mechanical ventilation; W, walker; NAW, never able to walk; RI, respiratory insufficiency; CHD, congenital hip dislocation. R, reduced; MR, marked reduction.

Patient	Phenotype	Clinical features	Mutation	a1 a2 a3	a6
UCMD-1	UCMD	Floppy at birth, CHD, NAW, DL, FC, SC, RI and death age 11 years	COL6A1 exon 22 c.1465delG horn; (p.Ala489Profs*16) [32]	Absent	Absent
UCMD-2	UCMD	Floppy at birth, NAW, DL, FC, SC, RI age 10 years	COL6A1 exon 9 c.819-833del het.; (p.Pro274_Gly278del) [33]	R at the basement membrane of muscle fibers	Traces
UCMD-3	UCMD	Floppy at birth, CHD, DL, FC, SC, walked 20 months–6 years, MV age 11 years	COL6A1 exon8–intron 8 c.798 804 + 8del 15 het.; (p.Pro254_Glu268 del) [34]	R at the basement membrane of muscle fibers	Traces
UCMD-4	UCMD	Floppy at birth, walker 3–7 years, DL, FC, SC, MV age 10 years	COL6A2 intron 17 c.1459-2 A>G het; (p.Gly487-Ala495delAspfs*48); COL6A2 intron 23 c.1771-1 G>A; (p.Glu591-Cys605delThrfs*148) [35]	Traces	Traces
UCMD-5	UCMD	Floppy at birth, CHD, FC, DL, SC, aided walker age 5 years	COL6A2 exon 28 c.2572 C>T horn; (p.Gln858*)	R at the basement membrane of muscle fibers	Traces
UCMD-6	UCMD	Floppy at birth, never able to run, DL, FC, SC, indoor walker age 9 years, RI age 12 years	COL6A2 exon 12 c.1096 C>T het (p.Arg366*); COL6A2 intron 8 c.927 + 5 G>A het; (p.Lys318fs*6) [34]	R at the basement membrane of muscle fibers	Traces
UCMD-7	UCMD	Floppy at birth, CHD, DL, FC, slow walker at age 7 years	COL6A3 intron 16 c.6210 + 1 G>A het; (p.Gly2053_Pro2070del)	R at the basement membrane of muscle fibers	Traces
BM-1	BM-AD	Moderate weakness age 30 years, FC, W age 60 years, mild RI	COL6A1 intron 3 c.428 + 1 het; (p.Tyr122-Gly143del) [38]	Normal	Traces
BM-2	BM-AD	Unable to run age 6, DL, FC, MV age 49 years, W age 50 years	COL6A2 exon 6 c.802 G>A het; (p.Gly268Ser)	Normal	Traces
BM-3	BM-AD	Unable to run age 18 years, FC, W age 36 years, mild RI.	COL6A2 intron 25 c.1970-3 C>A het; (p.Thr656_Ala698del)	Normal	MR
BM-4	BM-AD	CHD, first steps age 2.5 years, FC, DL, W age 24, moderate RI.	COL6A2 exon 7 c.883 G>A het; (p.Gly295Arg)	Normal	MR
BM-5	BM-AD	Clubfoot, mild distal limb weakness age 6 years, FC, W age 29, normal respiratory function.	COL6A3 exon 11 c.4928 T>G het; (p.Leu1643Arg)	Normal	Traces
BM-6	BM-AD	First steps at 2 years, never able to run, DL, FC, W age 42, moderate RI.	COL6A3 exon 17 c.6230 G>A het; (p.Gly2077Asp)	Normal	Traces
BM-7	BM-AD	Floppy at birth, CHD, first steps age 2.5 years, never able to run, FC, DL, W at age 29, moderate RI.	COL6A3 exon 16 c.6158 G>T het; (p.Gly2053Val)	Normal	Traces
BM-8	BM-AR	Floppy at birth, first steps age 13 months, unable to run age 6 years, FC, DL, slow W age 30, moderate RI.	COL6A3, exon 5 c.1393 C>T horn; (p.Arg465*) [30]	R at the basement membrane of several fibers	MR
MM	MM	Early childhood weakness, severe diffuse contractures, slow W age 21, severe RI.	COL6A2 exon 27 c.2455 C>T horn; (p.Gln819*) [25]	R at the basement membrane of vessels and several muscle fibers	Traces

3. Results

3.1. The collagen VI α6 chain distribution is severely affected in the skeletal muscle of genetically characterized UCMD, BM and MM patients

We previously showed that the α6 chain is expressed in the endomysium and perimysium of the normal human skeletal muscle, with a more restricted pattern than the α3 chain [19]. To define the impact of *COL6A1*, *COL6A2* and *COL6A3* gene mutations on α6 chain distribution, we investigated muscle biopsies of genetically characterized UCMD, BM and MM patients (Table 1) by immunofluorescence analysis. Patients are described and grouped on the basis of their clinical phenotype and the gene involved.

The UCMD-1 patient carried a homozygous deletion in *COL6A1* exon 22 that resulted in a frameshift mutation at amino acids 504–505 in the triple helical domain, causing a complete absence of collagen VI in skin fibroblast culture extracellular matrix [32]. The immunofluorescence analysis on muscle sections confirmed the expected absence of α1α2α3 in the endomysium and perimysium, while perlecan, a marker of basement membranes, was normally expressed. This mutation also compromised the α6 chain expression, which could also not be detected (Fig. 1).

The UCMD-2 [33] (Fig. 1) and UCMD-3 [34] (Supplementary Fig. 1A) patients, carrying heterozygous mutations in the *COL6A1* gene (Table 1), showed a partial deficiency in α1α2α3 expression, characterized by focal loss of protein in the basement membrane of muscle fibers and a marked reduction of the α6 chain (Fig. 1, supplementary Fig. 1A), which appeared to be intracellular in some interstitial fibroblasts in UCMD-2 (Fig. 1). These data suggest that the human α1 chain, in accordance with the reported *Col6a1* −/− mouse model [13], is a prerequisite for α6 chain assembly and localization in the muscle.

We also examined muscle biopsies of UCMD patients carrying mutations in *COL6A2*. The UCMD-4 patient with heterocompound mutations in the *COL6A2* gene [35] showed an almost complete absence of α1α2α3 expression; by immunolabeling, only a few perivascular deposits of the α6 chain were detectable in the endomysium (Fig. 1). α6 chain deposition was also dramatically reduced in muscle biopsies of UCMD-5, carrying a *COL6A2* homozygous mutation in exon 28, and UCMD-6, with a heterozygous mutation in *COL6A2* exon 12 [34], although both mutations did not completely prevent the ECM deposition of α1α2α3 chains (Supplementary Fig. 1A). A severe deficiency of the α6 chain was detected in the muscle biopsy of the MM patient [25] with a homozygous mutation in *COL6A2* (Fig. 1). In this MM patient, the mutation causes instability of α1α2α3 tetramers which do not localize correctly at the basement membranes of muscle fibers (Fig. 1). These data suggest that the α2 chain, similarly to α1, is essential for α6 chain localization in the muscle.

We previously reported that collagen VI deficient UCMD and BM patients with mutations in the *COL6A3* gene display a broad α6 chain expression pattern in the skin [18], pointing to a compensation by the α6 chain when the α3 chain is altered. To evaluate whether a similar compensatory mechanism occurs in the muscle, we analyzed the

muscle biopsy of a UCMD patient carrying a *de novo* heterozygous mutation in the *COL6A3* gene (UCMD-7) and in a BM patient (BM-8) [36] with recessive mutations in the *COL6A3* gene, whose skin results were mentioned above [18]. UCMD-7 displayed a selective reduction of α1α2α3 chains at the basement membrane of muscle fibers, whereas the α6 chain was almost completely absent (Fig. 2). The BM-8 patient almost exclusively expresses a shorter, alternatively spliced N6–C5 isoform of the α3 chain [37], which caused a focal loss of α1α2α3 chains and a marked deficiency of α6-containing microfibrils (Fig. 2). These data indicate that the α6 chain does not compensate for the absence of the α3 chain in the skeletal muscle.

We investigated the distribution of the α6 chain in muscle biopsies of seven BM patients with dominant mutations in the *COL6A1*, *COL6A2* and *COL6A3* genes (BM-1 mutation described in [38], the other mutations are reported in the Leiden Muscular Dystrophy pages: http://www.dmd.nl COL6A1 homepage, http://www.dmd.nl COL6A2 homepage, and http://www.dmd.nl/ COL6A3 homepage), without evident alterations in the expression of the α1α2α3 chains. As indicated by immunofluorescence analysis, the α1α2α3 chains appeared unaffected in all BM patients (Fig. 3 and Supplementary Fig. 1B). Surprisingly, the expression of the α6 chain was markedly reduced in the interstitium of all patients (Fig. 3 and Supplementary Fig. 1B), or weakly detectable only in the perimysium of BM-2 and BM-6 patients (Fig. 3).

3.2. The collagen VI α6 chain expression is normal or increased in the skeletal muscle of patients affected by muscular dystrophies unrelated to collagen VI

We investigated the α6 chain expression pattern in muscle biopsies of other forms of inherited muscle pathologies with primary defects affecting different cellular compartments; in particular we analyzed Duchenne muscular dystrophy (DMD), laminin alpha2 chain deficiency (MDC1A), dysferlin deficiency (Miyoshi myopathy), Emery–Dreifuss muscular dystrophy carrying a mutation in the *LMNA* gene (EDMD2), Rigid Spine Syndrome (RSS) caused by mutation in SEPN1, and RSS with mutation in FHL1. We found that the α6 chain was expressed at normal levels in the skeletal muscles of Miyoshi myopathy, EDMD2, SEPN1 and FHL1 patients, while it was markedly increased in the muscles of DMD and MDC1A patients (Fig. 4). Double labeling with anti-collagen III, used as fibrosis marker, showed a strong increase of the α6 chain in collagen III-positive areas of MDC1A muscle biopsy (supplementary Fig. 2A), in agreement with the ECM augmentation in fibrosis [19].

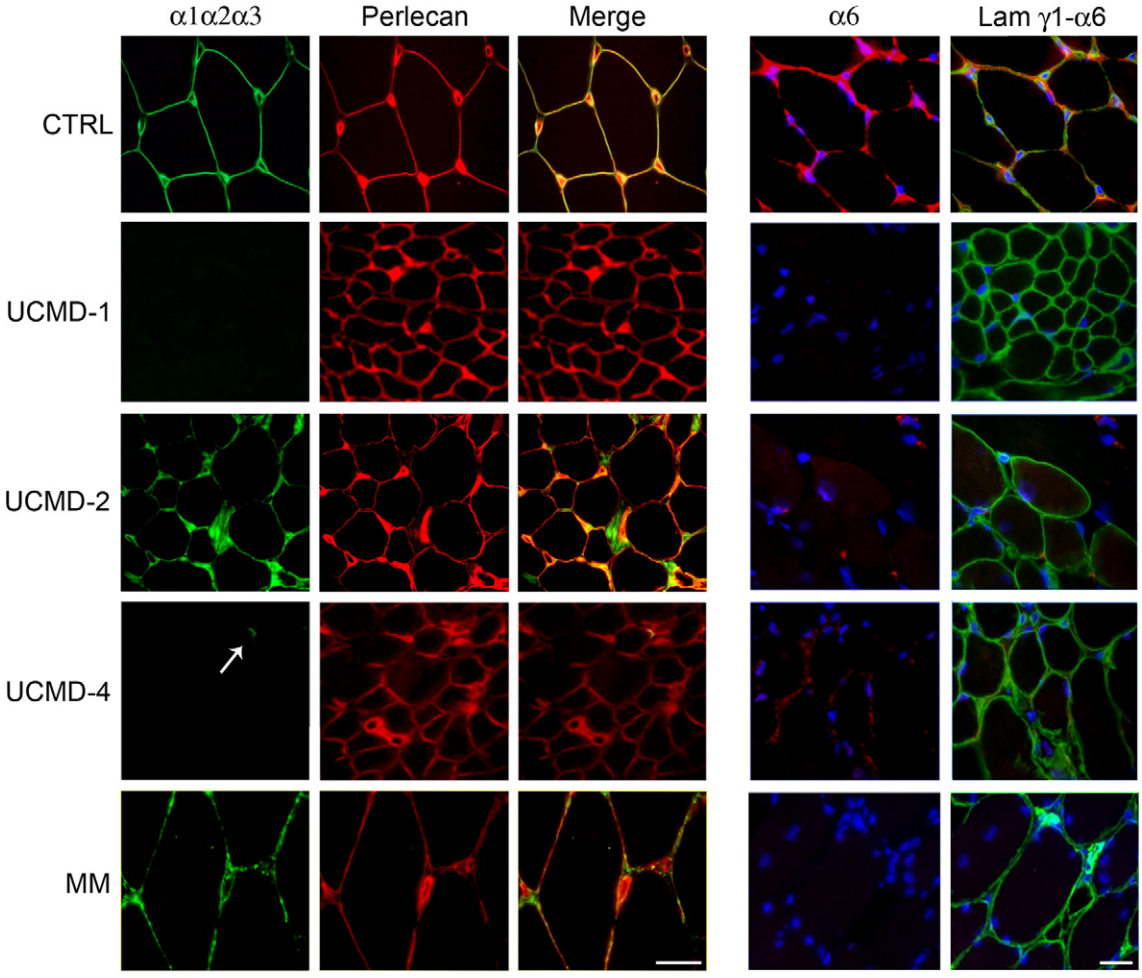

Fig. 1. Immunofluorescence analysis of transversal sections of muscle biopsies from one healthy control (CTRL), and UCMD-1, UCMD-2, UCMD-4 and MM patients. In the left panel, α1α2α3 heterotrimers (green) were labeled with a specific polyclonal antibody. UCMD-1 showed a complete absence of α1α2α3 heterotrimers while these were strongly reduced at the basement membranes of UCMD-2 muscle fibers, and present as a few aggregates in UCMD-4 muscle interstitium (arrow). α1α2α3 heterotrimers were also reduced at the basement membrane of muscle fibers of MM patients. By contrast, perlecan (red), a marker of the basement membrane was normally expressed. Scale bar, 50 μm. In the right panel, the α6 chain (red) was absent in UCMD-1 and markedly reduced in all patients as compared to control. The laminin γ1 chain (green) was used as a basement membrane marker. Nuclear staining, DAPI. Scale bar, 50 μm.

3.3. The collagen VI α6 chain deposition is impaired in the extracellular matrix of BM and UCMD muscle cell cultures

To further characterize the consequences of α6 chain deficiency observed in muscle sections, skeletal muscle cell cultures of BM and UCMD patients (BM-1, BM-2, BM-6, UCMD-2, UCMD-6 and BM-8 patients) were established and analyzed by RT-PCR, immunofluorescence and western blot.

By RT-PCR analysis a *COL6A6* transcript up-regulation was detected in all samples (fold change ranging from 3.297 up to 171.254). Transcript upregulation appeared to be very strong in cultures derived from BM-2 and BM-6 patients (Fig. 5A), without any apparent correlation to the mutated chain or to the clinical phenotype.

By immunofluorescence analysis, the α1α2α3 chain showed a normal amount and organization in BM-2 and BM-6 cultures, a moderate deficiency in BM-1 and BM-8 and a marked reduction in UCMD-2 and UCMD-6 cultures (Fig. 5B). Intracellular retention of α1α2α3 chains was detected in UCMD-2 patient muscle cells (Fig. 5B, inset). These data were confirmed by biochemical analysis. Ascorbic acid-treated cell layers analyzed by western blot revealed a normal pattern in BM-2 and BM-6 cultures, while a reduced amount of α1–α2 and α3 chains was detected in BM-1, BM-8 and UCMD-6 cultures (Fig. 5C). UCMD-2 showed an almost normal amount of α1α2α3 chains (Fig. 5C), possibly due to a secretion defect, as also indicated by the intracellular retention detected by immunofluorescence analysis (Fig. 5B). The α6 chain content was evaluated in the same muscle cell lysates. A severe reduction of the α6 chain was observed in cell lysates of all patients compared to controls (Fig. 5C). Immunofluorescence analysis of the α6 chain was not performed, because under basal conditions the α6 chain is poorly detected in the extracellular matrix [19].

We previously reported that TGF-β1 promotes the deposition of the α6 chain in the extracellular matrix of normal muscle cells by inducing muscle cell trans-differentiation into a myofibroblast phenotype [19]. Healthy and UCMD/BM muscle cell cultures were treated with TGF-β1 for 96 h in the presence of ascorbic acid to induce the secretion of collagen VI. The efficacy of the treatment was assessed by evaluating the phosphorylation of its downstream effector Smad2/3 in each culture. As expected, Smad2/3 phosphorylation on Ser 423/425 was increased after TGF-β1 treatment in control as well as in patient cells (Supplementary Fig. 2B). Moreover, TGF-β1 treatment strongly induced transdifferentiation of muscle cells into myofibroblast-like cells as indicated by the increased expression of α-SMA (data for the UCMD-2 patient are shown in Supplementary Fig. 2C and are representative for all patients).

The TGF-β1 treatment also induced the expression of the α6 chain in cell cultures from all patients as well as in healthy cells; however, a lower expression level of the protein in patients compared to controls could still be observed by western blot analysis (Fig. 5D). The immunofluorescence analysis showed a less abundant and organized α6-composed extracellular matrix in UCMD/BM cultures than in controls, confirming the biochemical results obtained under the same experimental conditions. In addition, α6(VI) chain aggregates were detected in all BM/UCMD muscle cell cultures, and in particular in BM-1 and BM-6 derived cells (Fig. 5E). Double labeling of the α6 chain with an antibody against-Limp II, a lysosome marker, excluded a targeting of the α6 chain into the degradative pathway (Fig. 6A). Instead, α6 chain aggregates displayed an intense labeling with anti-glucose-related protein 78 (Grp78), an endoplasmic reticulum (ER) marker, suggesting a protein accumulation in the ER (Fig. 6B).

4. Discussion

Collagen VI has long been thought to be composed exclusively of three α chains, α1, α2 and α3. The discovery of three additional collagen VI chains in mouse (α4, α5, α6) of which two are also present in humans (α5, α6), suggested the existence of additional assembly forms of collagen VI [12,13,14]. Based on their structural high homology with the α3 chain, it is conceivable that in humans the new chains may form α1α2α5 and α1α2α6 heterotrimers.

Our study aimed to analyze the expression pattern of the α6 chain in the skeletal muscle in UCMD, BM and MM patients to define its involvement in the pathogenesis of collagen VI-related myopathies. The expression of the α6 chain was reduced in skeletal muscle biopsies of such patients, independently of their clinical phenotype, of the collagen VI chain mutated, and of the effect of the mutations on the assembly and secretion of classical collagen VI heterotrimers. The strongly reduced expression of the α6 chain in the muscle of UCMD patients

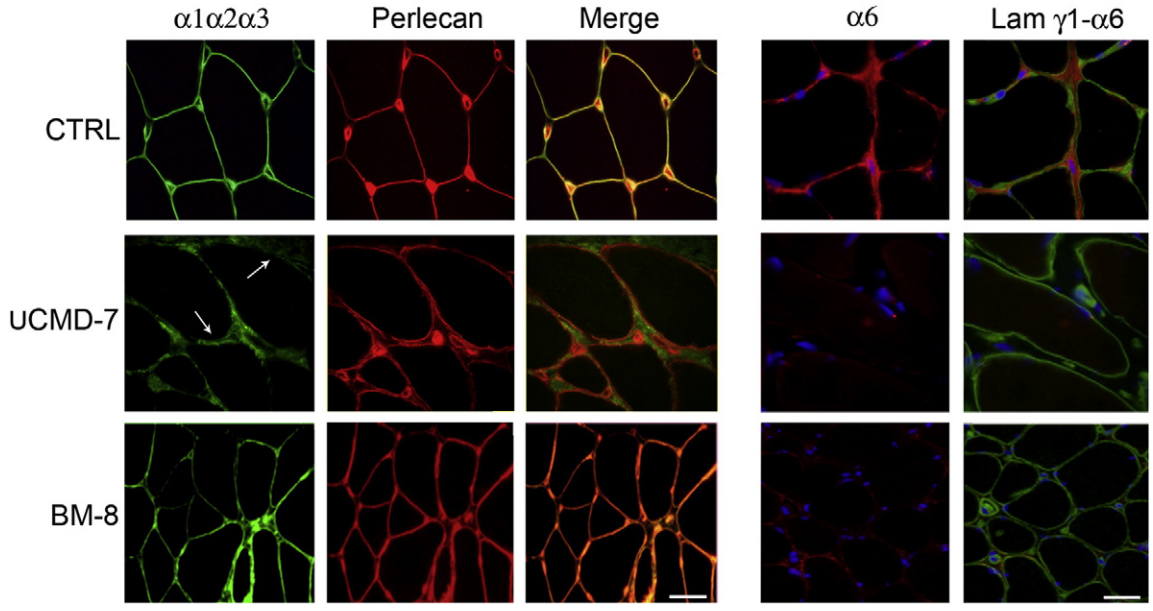

Fig. 2. Immunofluorescence analysis of transversal sections of muscle biopsies from one healthy control (CTRL), UCMD-7 and BM-8 patients, both carrying COL6A3 mutations. In the left panel, α1α2α3 chains (green) appeared reduced at the basement membrane of muscle fibers as indicated by lack of co-localization with perlecan labeling in discrete areas (arrows) of the UCMD-7, and to a lesser extent, of the BM-8 patient. Scale bar, 50 μm. In the right panel, the α6 chain (red) is poorly detectable; laminin γ1 chain (green) was used as basement membrane marker. Nuclear staining, DAPI. Scale bar, 30 μm.

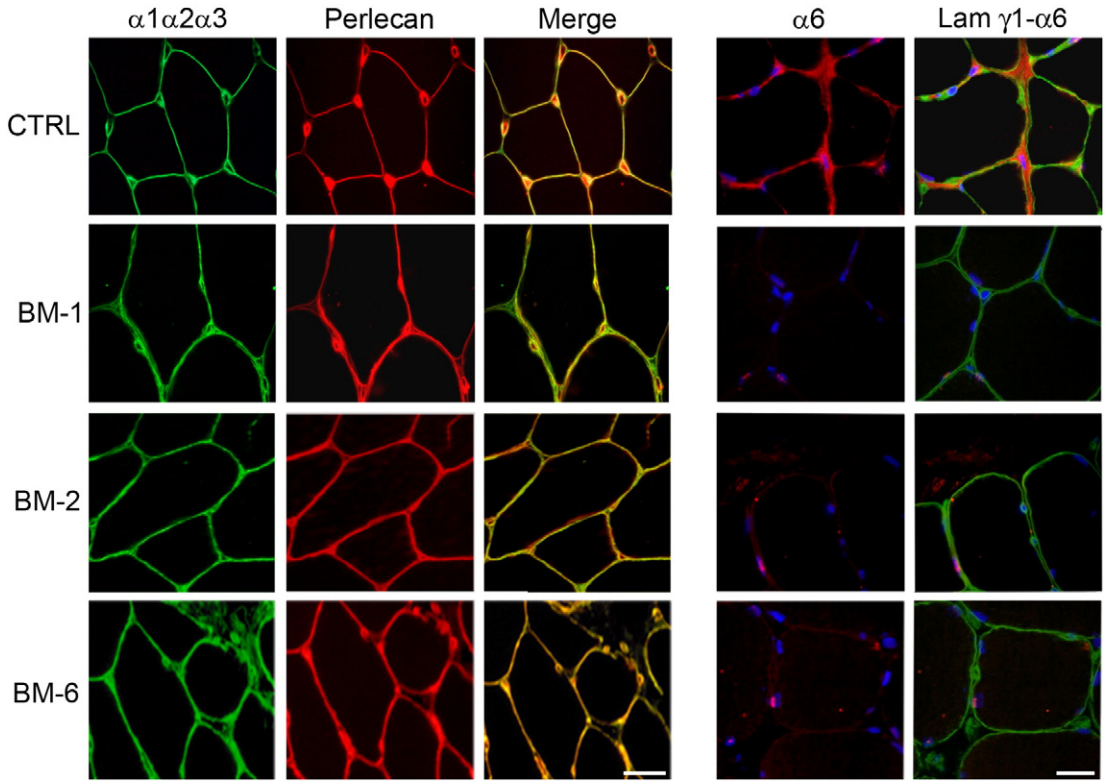

Fig. 3. Immunofluorescence analysis on transversal sections of muscle biopsies from one healthy control (CTRL), and BM-1, BM-2 and BM-6 patients. In the left panel, α1α2α3 chains (green) appeared normally expressed at the basement membrane and co-localized with perlecan (red fluorescence). Scale bar, 50 μm. In the right panel, the α6 chain expression (red) was strongly reduced; the laminin γ1 chain (green) indicates the basement membrane. Nuclear staining, DAPI. Scale bar, 30 μm.

carrying mutations in *COL6A1* or *COL6A2* matches the results obtained for the skin [18], and reflects the complex assembly mechanism of collagen VI microfibrils. Given the high homology of the α6 and α3 chains, we expected a compensative role of the α6 chain in UCMD patients with mutations in the *COL6A3* gene, as we already detected in the skin of the same patients [18]. On the contrary, we found a severe deficiency in the muscle, indicating that the α3 chain, as well the α1 and α2 are necessary for the integrity and/or normal secretion of collagen VI α6 chain. The observation that in normal cultured muscle cells the α6 chain does not form an independent microfilamentous network in the extracellular matrix [19] strongly supports this hypothesis.

Furthermore, we found that the α6 chain did not substitute and compensate for α3 deficiency in the skeletal muscle, in agreement with a recent study on Col6a3(+/d16) mice [39]. The absence of compensatory mechanisms in the muscle of patients with mutations in *COL6A3* could depend on the different distribution pattern of α6 chain with respect to the α3. In the skin of *COL6A3* patients, the α6 chain compensated for the absence of the α3 in the papillary dermis, where α3 and α6 chains are both expressed. In the skeletal muscle, while α3 is clearly part of the basement membrane of muscle fibers, the α6 chain is only interstitial and absent in the basement membrane. Considering that *COL6A3* patients studied in this paper showed a

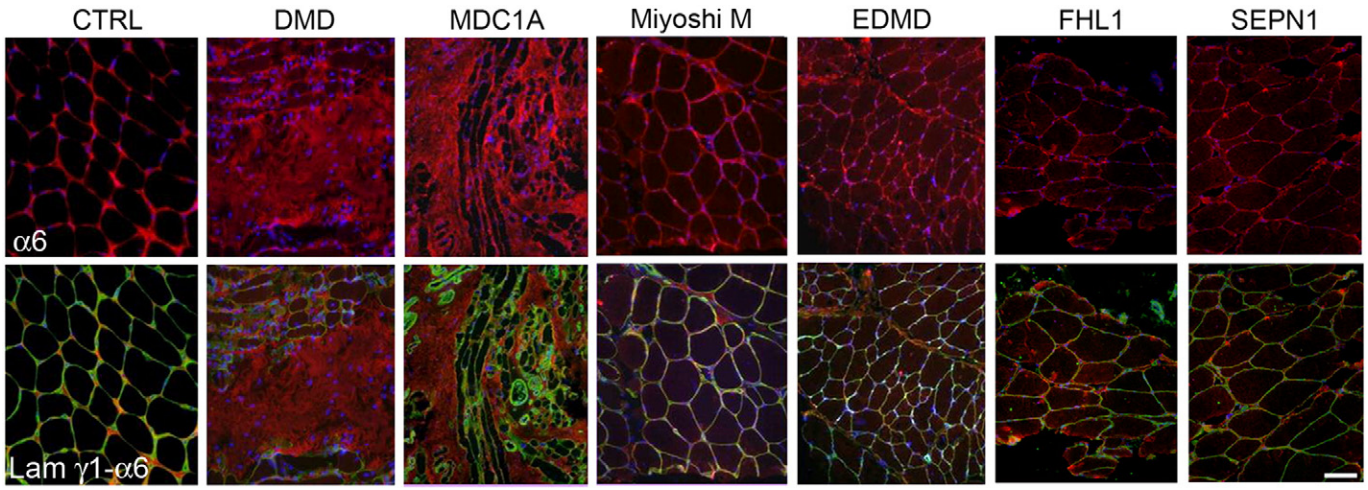

Fig. 4. Immunofluorescence analysis of α6 chain expression on transversal sections of muscle biopsies from one healthy control (CTRL), DMD, MDC1A, one Miyoshi myopathy (Miyoshi M), one EDMD2 (EDMD), FHL1 and SEPN1 patients. The α6 chain (red) appeared normally expressed in EDMD, and Miyoshi myopathy FHL1 and SEPN1 patients and upregulated in DMD and MDC1A patients. The laminin γ1 chain (green) was used as a basement membrane marker. Nuclear staining, DAPI. Scale bar, 20 μm.

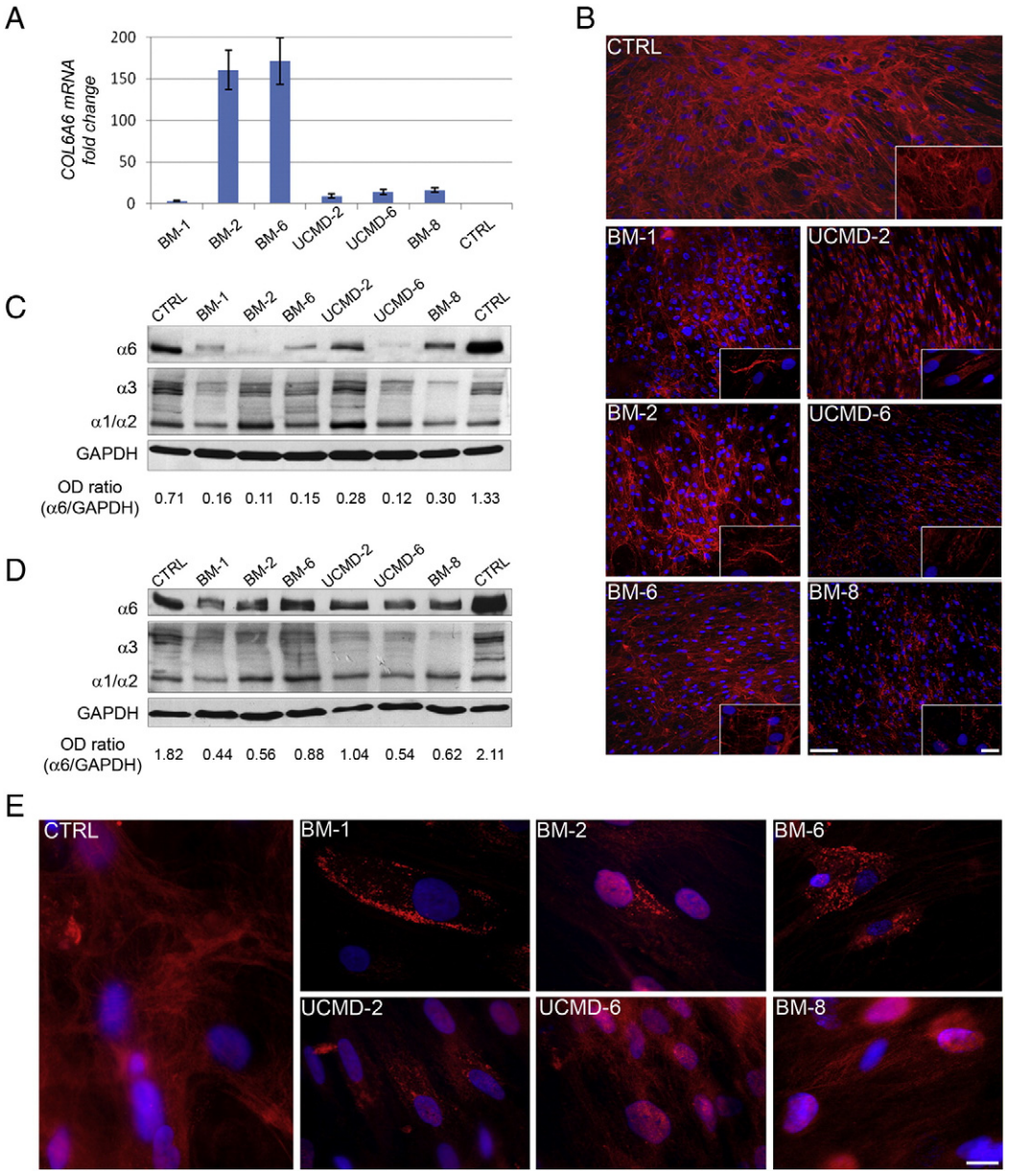

Fig. 5. (A) Relative quantification of the endogenous *COL6A6* mRNA in two UCMD and four BM patients. The *COL6A6* gene is up-regulated in all patients (fold change ranging from 3.297 up to 171.254). The *COL6A6* mRNA level in control myoblasts (CTRL) is set to 1. The values reported in the graph are the average between two independent experiments. (B) Immunofluorescence analysis of the α1α2α3 heterotrimer in control, four BM and two UCMD-confluent muscular fibroblasts after 4 days of L-ascorbic acid treatment. The α1α2α3 chains appeared normally assembled and secreted in BM-2 and BM-6 cultures, while they were reduced in BM-1 and in all UCMD patient cultures. A particularly strong intracellular retention was detected in the UCMD-2 patient (inset). Nuclear staining, DAPI. Scale bar: 20 μm. (C) Western blot analysis of cell layer from muscle cell cultures derived from two unaffected donors (CTRL), and BM-1, BM-2, BM-6, UCMD-2, UCMD-6 and BM-8 patients. Cells were treated for 4 days with L-ascorbic acid post-confluence, scraped and samples were separated on a 4–20% polyacrylamide gel under reducing conditions. The α1α2α3 heterotrimer and the α6 chain were evaluated by specific antibodies. The loading control was performed using a GAPDH antibody. Lower numbers indicate α6 chain/GAPDH ratio by densitometry. (D) Western blot analysis of cell layers from two unaffected donors (CTRL), and BM-1, BM-2, BM-6, UCMD-2, UCMD-6 and BM-8 patients treated for 4 days with L-ascorbic acid and 10 ng/ml TGF-β1. Cells were scraped and samples were separated on a 4–20% polyacrylamide gel under reducing conditions. The α1α2α3 heterotrimer and the α6 chain were evaluated by specific antibodies. GAPDH was used as a loading marker. Lower numbers indicate the α6 chain/GAPDH ratio by densitometry. (E) Immunofluorescence analysis of the α6 chain in control (CTRL), BM-1, BM-2, BM-6, UCMD-2, UCMD-6 and BM-8 patient muscle cell cultures treated for 4 days with TGF-β1 and L-ascorbic acid. All patients' cells displayed a strongly reduced amount of the α6 chain, with a significant intracellular retention in BM-1 and BM-6 muscle cells. Nuclear staining, DAPI. Scale bar, 10 μm.

selective deficiency in the basement membrane of muscle fibers, the absence of compensatory mechanisms could be attributed to the fact that the α6 chain is not part of this specific structure. The study of patients with the complete absence of collagen VI caused by homozygous mutations in *COL6A3* or models lacking α3 chains could be helpful for a better understanding of the role of the α3 chain in the α6 assembly. On the other hand, we cannot exclude that the α6 chain can be part of tissue specific alternative collagen VI assemblies [16]. However, skin fibroblasts produce the α3 but not the α6 chain (unpublished personal data), thus today, a cell model for biochemical investigation is lacking.

The absence of the α6 chain in the muscle of autosomal dominant BM patients with mutations which do not affect the assembly and deposition of the α1α2α3 chains is surprising. Under a basal condition, the protein was scarcely detectable by western blot despite that the transcript was upregulated in all patients compared to controls. The expression of collagen VI chains is highly regulated at different levels such as gene transcription, processing of encoding RNAs,

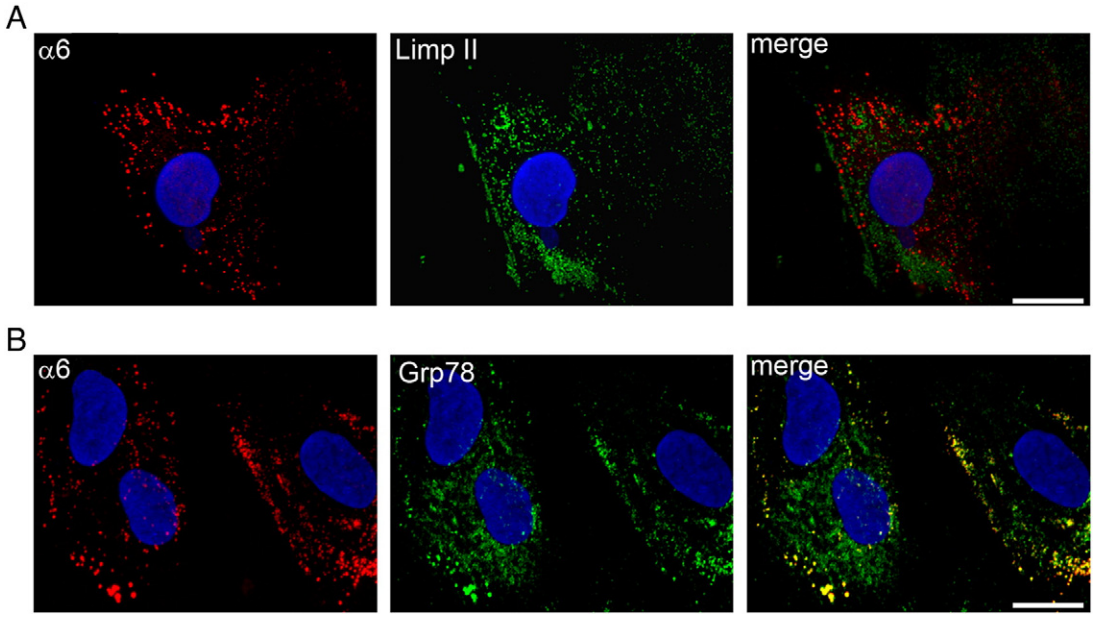

Fig. 6. Confocal analysis of a muscle cell culture derived from patient after treatment with TGF-β1. Double labeling of the α6 chain (red) with Limp II (lysosome marker, in A) and Grp78 (ER marker, in B) excluded lysosomal localization but demonstrated accumulation of protein aggregates in the endoplasmic reticulum. Nuclear staining, DAPI. Scale bar, 20 μm.

efficiency of translation and post-translational modifications [22], and the impairment of the efficiency of each step may affect protein assembly and secretion. Interestingly, TGF-β1 treatment induced the α6 chain expression in patients as well as in control cells; however, in patients with collagen VI-related myopathies, the α6 chain failed to develop a network-like matrix outside the cell and accumulated as aggregates in the ER. This result suggests that the protein retention maybe due to a defect in the post-translational machinery or in the supramolecular protein assembly. Retention of collagen VI in the secretory pathway was recently detected in mouse bronchiolar epithelial cells, where the absence of the phospholipid-binding protein annexin A2 leads to collagen VI retention within the Golgi compartment [40]. However, we cannot exclude that altered mechanisms downstream of the collagen VI deficiency may interfere with the correct α6 chain production selectively in the skeletal muscle, the most affected tissue in collagen VI-related patients. In a paper in collaboration with other groups we demonstrated that *Col6a1* knockout mice and BM–UCMD patient muscles display an impairment of the autophagic mechanism, which leads to inadequate removal of defective mitochondria and spontaneous apoptosis [41]. It has been reported that autophagy is involved in collagen turnover [42], and that the impairment of autophagy induces the accumulation of procollagen aggregates in the endoplasmic reticulum [43].

Diagnosis of collagen VI related myopathies is complex and a definite assignment of collagen VI genes requires molecular genetic testing currently available in only a few medical centers.

The immunohistochemical and western blot analysis of collagen VI in muscle biopsies and skin fibroblasts may be helpful for the diagnosis of UCMD [44], BM with recessive mutations [24] and MM [25], since it reveals a reduced and/or altered expression pattern. However, in several BM patients with dominant mutations, collagen VI immunolabeling is indistinguishable from the normal pattern both in skeletal muscle biopsies and cultured skin fibroblasts [29]. Moreover, the report of collagen VI deficiency in muscle biopsies of patients without mutations in collagen VI genes [45] and with UCMD/BM overlapping clinical features weakens the diagnostic significance of collagen VI protein analysis. So far, specific changes in muscle biopsies of dominant BM patients have never been reported. BM [46] and MM [25] patients may display a secondary reduction of laminin β1 labeling, however this finding is not specific to collagen VI related myopathies since it has also been reported in Emery–Dreifuss muscular dystrophy [47]. The normal level, or even the upregulation, of the α6 chain in muscular dystrophies not related to collagen VI, and in forms with BM overlapping phenotypes [48] indicates that the altered expression of the α6 chain it is not a common mechanism, related to the dystrophic process *per se*. This further supports the assertion that in the principal contractured phenotypes mimicking collagen VI disorders the α6 chain is not affected.

Fibrosis is the most conspicuous pathological alteration in dystrophic muscle. It consists in a non-reversible remodeling of the ECM, which significantly contribute to muscle dysfunction. We previously reported that the α6 chain expression is induced by the profibrotic factor TGF-β1, and that it is strongly up-regulated in DMD muscle fibrosis [19]. Here we show that the α6 chain is up-regulated in MDC1A, another form of muscular dystrophy with prominent fibrosis and involvement of TGF-β1 signaling [49].

5. Conclusions

The discovery of additional collagen VI chains in the human skeletal muscle added new complexity to collagen VI biology, as their roles in development, tissue homeostasis and pathogenesis of inherited diseases are completely unknown. The defect of α6 chain we detected in collagen VI-related myopathies points to a contribution to the pathogenesis of collagen VI-related disorders.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.05.033.

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