ANTISENSE-INDUCED MESSENGER DEPLETION CORRECTS A COL6A2 DOMINANT MUTATION IN ULLRICH MYOPATHY

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Running title: AON modulation of a UCMD dominant COL6A2 mutation

Abstract

Collagen VI genes mutations cause Ullrich and Bethlem muscular dystrophies. Pathogenic mutations frequently have a dominant negative effect, with defect in collagen VI chains secretion and assembling. It is agreed that, conversely, collagen VI haploinsufficiency has no pathological consequences. Thus, RNA targeting approaches aimed at preferentially inactivating the mutated COL6 messenger may represent a promising therapeutic strategy. By in vitro studies we obtained the preferential depletion of the mutated COL6A2 messenger, by targeting a common SNPs, cistronic with a dominant COL6A2 mutation. We used a 2'OMePS antisense oligonucleotide covering the SNP within exon 3, which is out of frame. Exon 3 skipping has the effect of depleting the mutated transcript via RNA nonsense-mediated decay, recovering the correct collagen VI secretion and restoring the ability to form an interconnected microfilament network into the extracellular matrix. This novel RNA modulation approach to correcting dominant mutations may represent a therapeutic strategy potentially applicable to a great variety of mutations and diseases.

Introduction

Ullrich muscular dystrophy (UCMD-MIM#254090) and Bethlem myopathy (BM-MIM#158810) are congenital disorders caused by defects in collagen type VI, a multimer of different α(VI) chains (Bertini et al., 2011). These chains assemble intracellularly as monomers (1:1:1 ratio), which unite to form dimers of antiparallel monomers, and subsequently tetramers; these are stabilized by disulfide bonds, secreted into the extracellular space, and aligned end-to-end as microfibrils, providing structural and mechanical stability by interacting with different connective tissue basement membrane constituents (Allamand et al., 2011). As collagen VI is a multimer, an abnormal mutated chain retaining the ability to be assembled into growing supramolecular structures, will sequester normal chains into non-functional complexes, thus exerting a dominant negative effect (Baker et al., 2007). Indeed, dominant *de novo* COL6A genes mutations occur in more than 50% of UCMD patients (Allamand et al., 2009, 2011), with both missense changes in glycins within the α(VI) chains' triple-helix domains and intron-splicing mutations being common (Allamand et al., 2009). This suggests that a significant proportion of collagen VI-related phenotypes could benefit from therapeutic approaches that selectively reduce/abolish the expression of COL6A genes transcripts carrying dominant negative mutations. This view is reinforced by the evidence that collagen VI haploinsufficiency, which occurs in the parents of recessive UCMD cases, who carry large deletions or premature truncating mutations leading to non-sense mediated decay (NMD), is not associated with any pathological phenotype (Bovolenta et al., 2010; Foley et al., 2011).

We set out to test this hypothesis using a novel molecular approach based on exon-skipping, designing a 2'OMePS antisense oligoribonucleotide (AON) to target a common COL6A2 SNP occurring in *cis* with the *de novo* dominant intron 9 c.954+17_954+22del28 UCMD mutation. Transfecting patient fibroblasts with this AON induced preferential skipping of the mutated exon via SNP recognition, and caused COL6A2 messenger out-of-framing, thereby depleting the mutated transcript via NMD. Skipping quantification confirmed AON efficacy, RNA transcript count evidenced the preferential mutated transcript depletion, protein and morphological studies showed reduced expression of the mutated COL6A2 allele. Both histology and electron microscopy studies revealed promotion of collagen VI integration into the ECM and focal recovery of the microfibrillar network, thus confirming the feasibility of mutated messenger depletion, and thereby dominant mutation correction, via out-of-frame exon-skipping.

Materials and Methods:

UCMD mutation and P221(T)AON design. The UCMD mutation is a *de novo*, heterozygous 28bp deletion within intron 9 of the COL6A2 gene (c.954+17_954+22del28) causing exon 9 skipping and predicting a triple-helix domain internal deletion (Gly310_lys318del). In UCMD patient, the mutation is cistronic with the T-allele of the common COL6A2 c.663C>T SNP (P221P) occurring in the out-of-frame exon 3 (Fig. 1A). A

2'OMePS-modified RNA AON (referred as P221(T)AON) was designed, according to published guidelines (Aartsma-Rus, 2012) to target this T allele (supplementary Figure 1A); synthesis and purification were as previously described (Spitali et al., 2009). Different AON doses and timing were tested (see supplementary table).

Cell culture and transfection. Primary human skin fibroblasts (obtained for diagnostic procedures with Ethics Committee approval n° 7/2009) were cultured at 37°C and 5% CO2 as described (Martoni et al., 2009). Cells were transfected for 4 hours with either AON (200nM) plus 3.3 ul/ug AON ExGen500 polyethylenimine (PEI) (MBI Fermentas) or with PEI alone, as described (Evers et al., 2011).

RNA studies. Cells were harvested 24hrs post-transfection, and total RNA isolated and retro-transcribed as reported (Martoni et al., 2009). RT-PCR was performed using COL6A primers within exons 2–10 and 4–10 (sequence available upon request) to quantify exon 3 skipping, and to assess the proportions of wild-type (exon 9-plus) vs. mutated (exon 9-minus) COL6A2 transcripts, calculated as the ratio between each type of transcript and the sum of all transcripts, on an Agilent High Sensitivity DNA chip (Agilent Technologies, Santa Clara, CA) (Supplementary Figure 1B). TaqMan expression assays (Applied Biosystems) were used to quantify COL6A2 and COL6A1 transcripts (Hs00242484_m1 Ex27/28; Hs00242448_m1 Ex20/21) with respect to beta-actin, the endogenous control. Real-time PCR was performed as described (Martoni et al., 2009). cDNAs from untreated fibroblasts served for calibration.

Western Blot. 16 hrs post-transfection, fibroblasts were supplemented with 0.25mM sodium ascorbate for 24 hours. Medium was collected, and cell layer was scraped and solubilized in RIPA buffer. Protein content was quantified by BCA Protein Assay kit (Pierce). Western blotting was performed as previously reported (Martoni et al., 2009). Loading check was performed using anti-beta-catenin antibody (1:1000, BD Transduction Laboratories) for cell-layer extracts and Coomassie staining for culture medium.

Immunostaining. 16 hrs post-transfection and after 24 hours in sodium ascorbate, cells were fixed with cold methanol and double-stained with anti-collagen VI (Fitzgerald) and perlecan (Chemicon) antibodies, as previously reported (Sabatelli et al., 2001). After nuclei staining with DAPI, samples were mounted with anti-fade reagent (Molecular Probe) and analysed under Nikon Eclipse 80i fluorescence microscope.

Immunoelectron Microscopy. Cultures were incubated with 3C4 monoclonal antibody (Chemicon), diluted 1:50 in culture medium at 37°C for 3 hours, and revealed with anti-mouse IgG 5-nm gold-conjugated antibody (Amersham Biosciences), diluted 1:50 for 1 h at 37°C. Rotary-shadowing electron microscopy was performed as described (Zhang et al., 2002). Samples were examined under Philips EM 400 or Jeol JEM 1011 electron microscope at 100 kV. For quantitative analysis of microfibrillar tetramer composition, 20 fields at 17000x magnification were evaluated for each condition.

Results:

P221(T)AON induces exon-3 skipping, transcript out-of-framing and COL6A2 transcript degradation:

Patient-derived fibroblasts were transfected (in three independent experiments) with 200nM P221(T)AON, and total RNA was isolated after 24 hours. RT-PCR and DNA-chip analysis showed average exon 3 skipping of 30.3% (range 21.3%–39.3%), while none was observed in cells treated with control AON (against dystrophin exon 51) (Goemans et al., 2011). Following P221(T)AON treatment, real-time PCR showed COL6A2 mRNA depletion to about 60% of baseline (0.62±0.10); COL6A1 expression was not significantly affected (1.07±0.20) (Fig. 1B).

P221(T)AON reduces collagen VI at cell layer and in the medium:

In parallel with transcript depletion, P221(T)AON treatment caused a decrease in the global amount of collagen VI in UCMD patient cell layer (Fig. 1D, left). P221(T)AON-treated samples showed even more pronounced reduction of collagen VI in the medium fraction (Fig 1D, right) with respect to basal conditions. As medium extract is composed of proteins neither retained in cells nor deposited on ECM, the difference

in collagen-VI depletion between the two fractions seems to indicate more efficient post-treatment ECM deposition of the protein.

P221(T)AON affects mutated/wild-type COL6A2 allele ratio:

To assess the sequence-specificity and efficacy of P221(T)AON, the proportions of mutated vs. wild-type COL6A2 transcripts were compared in treated vs. untreated cells. In untreated cells, the mutated COL6A2 allele (missing exon 9) comprised 82.72% ± 1.86 of the wild-type. After P221(T)AON transfection, this pathological messenger was reduced by 10% to 72.87±1.18. Control AON did not significantly affect the allele ratio (80.70±1.00) (Fig. 1C).

P221(T)AON partially recovers collagen-VI based ECM:

In untreated UCMD patient fibroblasts, collagen VI secretion appeared severely affected, with little protein deposited in the ECM in a spot-like arrangement, indicating a decreased ability of secreted mutant tetramers to associate into microfibrils. P221(T)AON treatment appeared to increase the amount of collagen VI associated to the ECM (Fig. 2A); the secreted protein was organized in a microfibrillar network and co-localized with perlecan labelling, as per control (Fig. 2B). EM analysis showed improved tetramer-tetramer association and enhanced ability to form a microfilament network (Fig. 3A). In untreated UCMD culture, 83% of microfibrils were constituted by 2 to 10 tetramers, whereas microfibrils with more than twenty tetramers were absent. After treatment, about 80% of fibrils contained more than 20 tetramers (Fig. 3B). No effects were seen in fibroblasts treated using AON on dystrophin exon 51 (data not shown).

DISCUSSION

Correcting dominant mutations by splicing modulation is appealing, since the only way to intervene in these cases is by specifically silencing the mutated allele, a feat which has been achieved using various genetic methodologies (Wood et al., 2007; Le Roy et al., 2009). In particular, siRNAs' exquisite sequence specificity has been used with encouraging results for allele-specific knockdown in several non-muscular diseases *in vitro* and in animal models (Wood et al., 2007), and a phase 1b trial based on mutation-targeted siRNA, locally injected within skin lesions of pachyonychia congenita, is underway (Leachman et al., 2010). Nevertheless, concerns regarding delivery, interference with endogenous RNAi processing and non-specific off-target effects have thus far hampered a wider clinical exploitation of siRNA-based therapies (Wood et al., 2007; Wang and Wientjes, 2010). In contrast, AON-based treatment has reached phase 1-2a clinical trials in neuromuscular diseases. In DMD patients, 2'OMePS-AONs and PMO against dystrophin exon 51 have been systemically utilized to induce in-frame exon-skipping, which can restore dystrophin production, apparently without overt toxic effects, paving the way to extended treatment phases (Cirak et al., 2011; Goemans et al., 2011) .

We adopted exon-skipping, exploring the efficacy of an SNP-specific 2'OMePS-AON to induce out-of-framing skipping and consequent allele degradation, as a corrective approach for a COL6A2 gene dominant negative mutation. This strategy was based on the following evidence: i) 2'OMePS-AONs sequence specificity and efficacy in inducing exon removal has been largely demonstrated in humans (Spitali et al., 2009); ii) COL6 genes transcripts with nonsense mutations almost invariably undergo RNA nonsense-mediated degradation, and quantification of transcripts has been proposed as a tool for pinpointing the mutated gene (Allamand et al., 2011); iii) COL6 genes are highly polymorphic and several common SNPs are known which, if cistronic with pathogenic mutations, can be specifically targeted by AONs to induce transcript depletion in large patient populations; iv) dominant *de novo* mutations represent the predominant type, being the 67% of the COL6 genes missense mutations, 57% of small deletions and 44% of splice site mutations leading to in-frame exon exclusion (Allamand et al., 2011). By AON-targeting a common SNP, we were able to target the causative mutation and deplete the mutated transcript, partially correcting the associated severe cellular phenotype. Treatment was not completely specific and caused a

global reduction of COL6A2 mRNA level, preferentially involving the mutated allele. We observed that a relatively low efficiency of mutated-allele depletion (10% variation in mutated/wild type allele ratio) was nevertheless able to significantly improve extracellular collagen VI assembly, and restore its ability to develop a microfilament network, in single-dose and short-term treatment. The collagen VI complex assembly process, which explains the dominant negative behaviour of mutations leading to aberrant chains, also accounts for this "magnification effect" of protein correction (Fig. 3C).

Progress in unravelling the pathogenic mechanisms of collagen VI-related myopathies (Grumati et al., 2010) has opened intriguing therapeutic perspectives; nevertheless, RNA-splicing modulation is very appealing, since it is based on safe and highly reliable drugs in terms of systemic administration and tissue targeting (Cirak et al., 2011; Goemans et al., 2011). Furthermore, exon-skipping can be personalized, treating specific mutations using dedicated AONs.

We provide proof of principle that out-of-frame exon-skipping by AONs induces allele targeting and corrects COL6 gene dominant mutations, highlighting molecular splicing as a potential treatment for collagen VI-related myopathies. In addition, since our AON design targeted a very common SNP linked to the mutation, we envisage a novel AON therapy that may facilitate personalized treatments, targeting "common variations embracing rare mutations".

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Legend to Figure 1:

A) Schematic representation of the UCMD mutation within the COL6A2 gene. cDNA sequencing showed the intronic c.954+17_954+22del28 mutation (which causes exon9 skipping) to be in cis with the T allele of the c.663C>T (P221P) SNP within COL6A2 exon 3; B) Real-Time PCR analysis on RNA from UCMD patient fibroblast after treatment with P221(T)AON and control dys51 AON. P221(T)AON transfection had no effect on COL6A1 transcript level, whereas COL6A2 mRNA was reduced to 60% of pre-treatment level (beta actin as reference). Control AON had no significant effect on either COL6A1 and COL6A2 transcript levels. The graphs are representative of three independent experiments. Data are given as means ± SD and the statistical significance has been calculated with the Student's t-test for unpaired data (*p< 0.005); C) Relative proportion of mutated vs. wild-type COL6A2 transcript before and after P221(T)AON treatment. RT-PCR was performed using COL6A primers within exons 4-10, and products were run on an Agilent High Sensitivity DNA chip for relative quantification the COL6A2 mutated allele (lacking exon 9) versus the wild type allele (including exon 9). Pre-treatment, the mutated allele represents the 82.72% ± 1.86 of the wild-

type allele. After P221(T)AON transfection, this ratio was lowered to 72.87±1.18. Control AON had no effect on the allele ratio. Statistical significance has been calculated with the Student's t-test for unpaired data (*p< 0.005). D) *Western Blot analysis after treatment with P221(T)AON.* Samples corresponding to cell layers (20 μg, left) and culture media (right), from skin fibroblasts of the UCMD patient (before and after P221(T)AON treatment) and healthy donor (CTRL), were separated by SDS-PAGE onto 8% polyacrylamide gels under reducing conditions. Collagen VI was detected with an antibody recognizing all three alpha-chains. Protein bands were quantified using ImageJ software. Collagen VI quantity was calculated as an absolute value and normalized using beta-catenin for cell layer extract and with coomassie staining for culture medium. Densitometric analysis (histograms) shows a decrease in the amount of collagen VI, after p221(T)AON treatment, in UCMD patient samples, more pronounced in the medium. As medium extract is composed of proteins neither retained in cells nor deposited on ECM, the increase in protein in the medium of untreated samples is probably related to the inefficient deposition of mutated collagen VI, significantly ameliorated by treatment. The blots are representative of three independent experiments. Data are given as means ± SD and the statistical significance has been calculated with the Student's t-test for unpaired data (*p< 0.005).

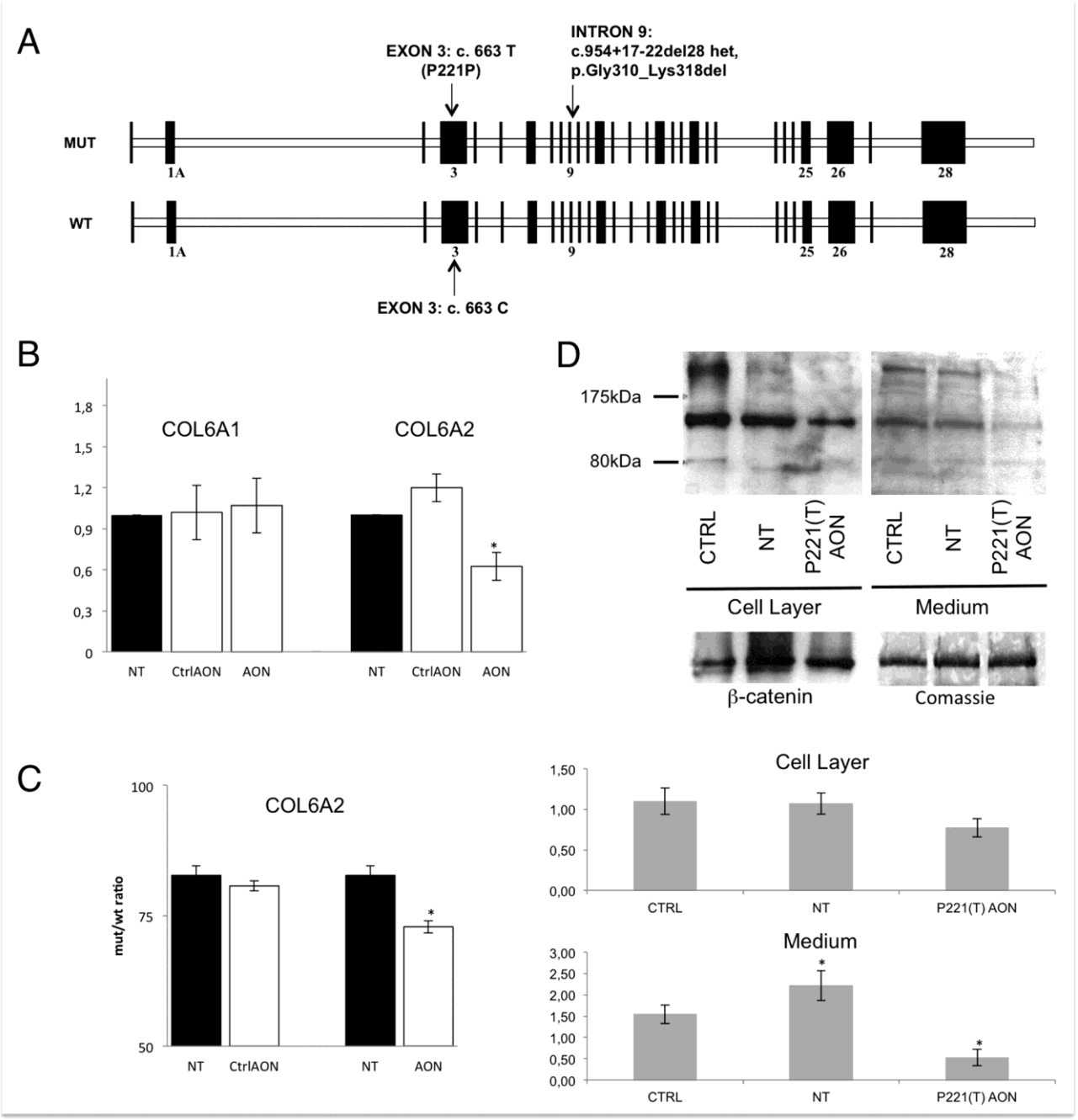
Legend to Figure 2:

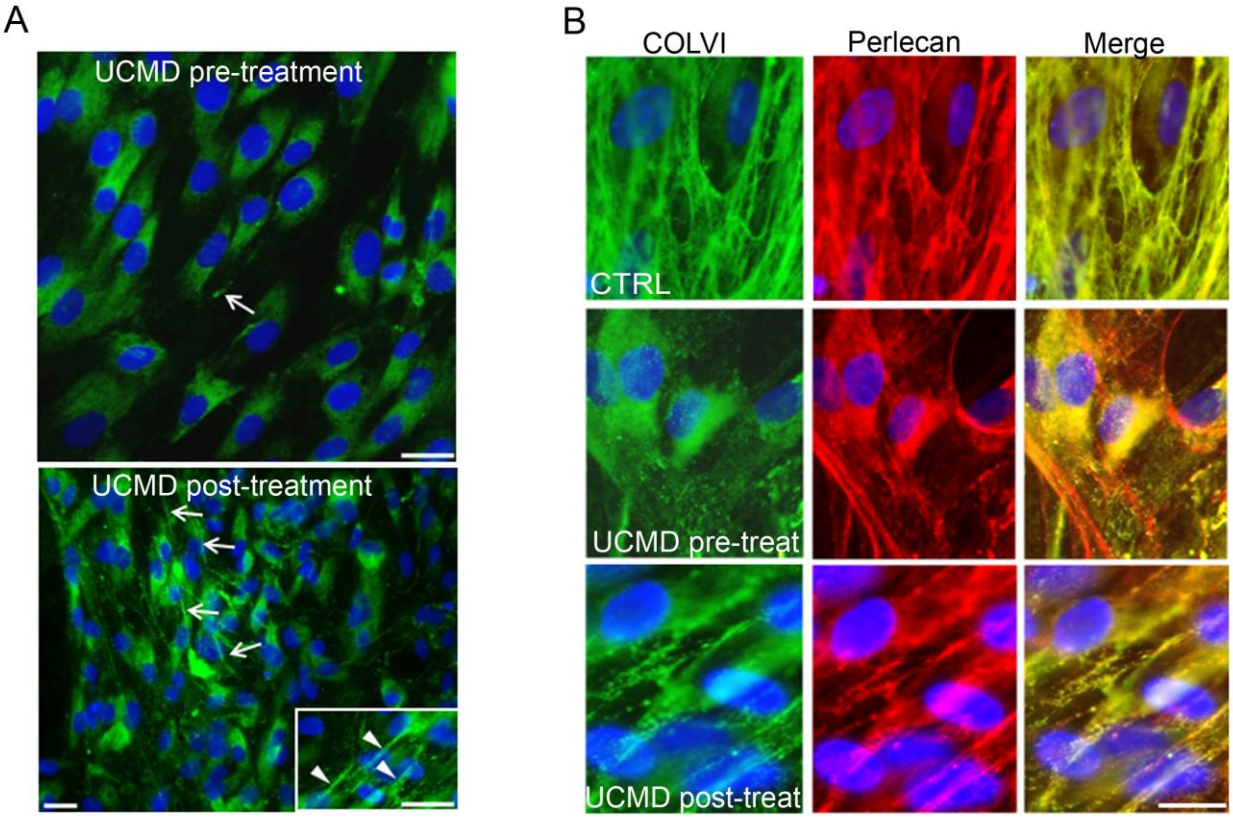
A) Immunofluorescence analysis of collagen VI in fibroblast cultures of the UCMD patient before (UCMD pre-treat) and after treatment with P221(T) AON (UCMD post-treat). Before treatment, collagen VI shows an intracellular localization and only rare deposits can be detected in the ECM (arrow). After treatment, collagen VI secreted in the ECM appears to be increased (arrows) and displays a fibrillar arrangement (inset, arrowheads). Nuclei are stained with DAPI. Bar, 20 μm. B) Immunofluorescence analysis of collagen VI (green) and perlecan (red) in fibroblast cultures of a control (CTRL), and the UCMD patient before (UCMD pre-treat) and after treatment with P221(T)AON (UCMD post-treat). In normal cells, collagen VI develops a dense microfibrillar network which overlaps with perlecan labelling, as showed by yellow staining in merge. In the culture of the UCMD patient under basal condition (UCMD pre-treat), collagen VI appears mainly accumulated in the cell cytoplasm; some spot-like collagen VI-positive structures can be detected in the extracellular matrix. After treatment with P221(T)AON, the amount of protein secreted and associated to the ECM is increased and shows a fibrillar arrangement. The co-localization of collagen VI with perlecan is also recovered, as showed in the merge image. Nuclei are stained with DAPI. Bar, 10 μm.

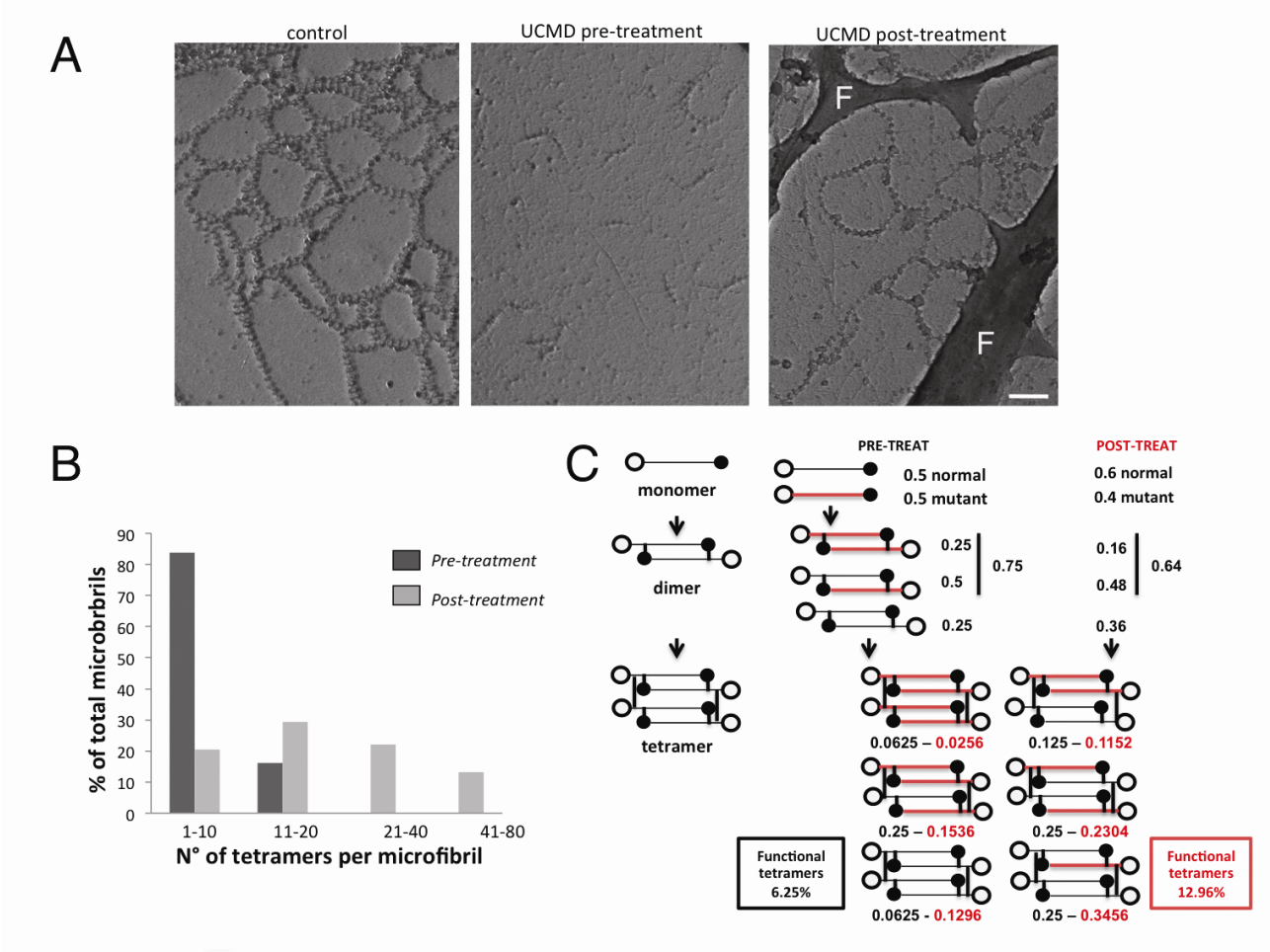
Legend to Figure 3:

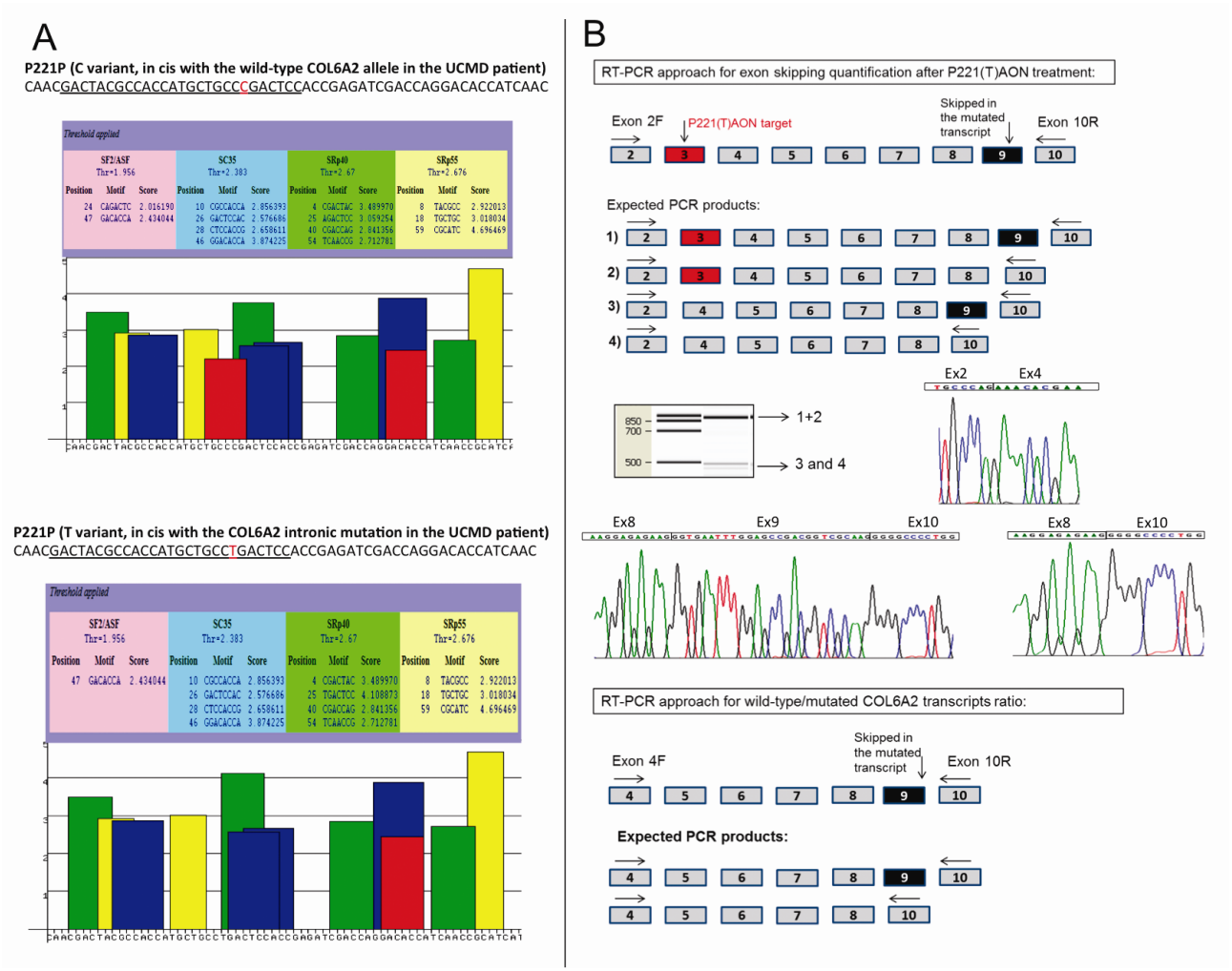
A) Electron microscopy analysis (rotary-shadowed replicas) of cultured fibroblasts. Fibroblasts from control and the UCMD patient under basal conditions (UCMD pre-treat) and after P221(T)AON treatment (UMD post-treat), were immunolabelled with anti-collagen VI and 5-nm colloidal gold conjugates and rotary shadowed. TEM analysis of replicas obtained from control cells reveals the presence of long interconnected microfilaments forming a complex network. In the UCMD patient culture, under basal conditions single short microfilaments can be detected outside the cells. After treatment with P221(T)AON, the length of the isolated microfilaments was increased, as well their ability to develop an interconnected network. Bar, 500 nm. F, fibroblast cytoplasmic process. B) Quantitative analysis of collagen VI tetramer composition. Collagen VI deposited in the extracellular matrix of the UCMD patient fibroblasts before and after

P221(T)AON treatment was visualized by immunogold and rotary shadowing techniques, and the ability of the tetramers to associate end-to-end was quantified. The occurrence of microfibrils containing 1–10, 11¬–20, 21–40 and 41–80 tetramers is shown as a percentage of the total number of microfibrils. Tetramer-tetramer association appears significantly improved after treatment ($P<0.05$). C) Schematic representation of the improvement in tetramer composition after P221(T)AON treatment. Under pre-treatment conditions the actual ratio between normal and mutant COL6A2 transcript was 1:0.8 (the ratio between normal and mutant alpha2(VI) chains was approximated to 1:1); after P221(T)AON treatment, this ratio become 1:0.7, with a 10% relative depletion of the mutated transcript and, theoretically, an equal reduction of the mutated chain. The tetramer assembly process accounts for the “magnification effect”: after treatment, the percentage of functional tetramers is doubled despite of the relatively low efficiency of the mutated allele depletion.









AON dosage	**AON toxicity**	**Time at analysis (hours post-transfection)**					
		16 hours		*24 hours*		*24+24 hours*	
		Exon 3 skipping (%)	ICC analysis (+24hrs AA)	Exon 3 skipping (%)	ICC analysis (+ 24hrs AA)	Exon 3 skipping (%)	ICC analysis (+ 24 hrs AA)
25 nM	No toxicity	Not detectable	nd	Not detectable	nd	Not detectable	Not done
50 nM	No toxicity	12.5%	No variation	11.5%	No variation	Not done	Not done
100 nM	No toxicity	13.5%	No variation	13%	No variation	12%	No variation
200 nM	Low toxicity	21.3%	Focal recovery of collagen VI based ECM (see text)	30.3% (see text)	Only slight focal changes	21%	No variation
250nM	Partial cell detachment	Not done	No variation (cell toxicity?)	Not done	Not done	Not done	Not done
300 nM	High toxicity; Massive cell detachment	Not done	No variation (cell toxicity?)	Not done	Not done	Not done	Not done

Legend to Supplementary Figure:

A) UCMD allele-specific AON was designed against the T variant of the c.663 C/T SNP within COL6A2 exon 3 occurring in cis with the UCMD mutation and also covering internal exon 3 sequences, predicted as putative ESEs by the online open-source programs ESE finder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) and ESE Rescue (http://genes.mit.edu/burgelab/rescue-ese/). AON targeted sequence within exon 3 is underlined. The P221P(T) variant cause the loss of a SF2/ASF recognition site that is conserved in the C allele. A BLAST search of the human EST-databases for sequences homologous to the P221(T)AON did not reveal any perfect hits, suggesting a low probability of unintentional exon-skipping through non-specific interactions. The P221(T)AON is a 2'-O-methyl RNA with a full length phosphorothioate backbone. B) Schematic representation of the RT-PCR strategy utilised to quantify exon 3 skipping after P221(T(AON treatment (upper panel) and to assess the wild-type/mutated COL6A2 transcripts ratio (lower panel).

Legend to Supplementary table:

Summary of different P221(T)AON dosages tested for transfection in UCMD patient fibroblasts. Different timing for RNA and protein analysis were also verified for exon skipping efficiency and protein recovery. Due to the requirement of Ascorbic Acid (AA) supplementation for collagen VI secretion, timing for immunohistochemical analysis is 16+24 and 24+24. In 24+24 hours condition, a second transfection was performed 24 hours after the first, with similar AON concentration.