RESEARCH ARTICLE

Transcriptome Analysis of Ullrich Congenital Muscular Dystrophy Fibroblasts Reveals a Disease Extracellular Matrix Signature and Key Molecular Regulators

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Abstract

Background

Collagen VI related myopathies encompass a range of phenotypes with involvement of skeletal muscle, skin and other connective tissues. They represent a severe and relatively common form of congenital disease for which there is no treatment. Collagen VI in skeletal muscle and skin is produced by fibroblasts.

Aims & Methods

In order to gain insight into the consequences of collagen VI mutations and identify key disease pathways we performed global gene expression analysis of dermal fibroblasts from patients with Ullrich Congenital Muscular Dystrophy with and without vitamin C treatment. The expression data were integrated using a range of systems biology tools. Results were validated by real-time PCR, western blotting and functional assays.

Findings

We found significant changes in the expression levels of almost 600 genes between collagen VI deficient and control fibroblasts. Highly regulated genes included extracellular matrix components and surface receptors, including integrins, indicating a shift in the interaction between the cell and its environment. This was accompanied by a significant increase in fibroblasts adhesion to laminin. The observed changes in gene expression profiling may be

under the control of two miRNAs, miR-30c and miR-181a, which we found elevated in tissue and serum from patients and which could represent novel biomarkers for muscular dystrophy. Finally, the response to vitamin C of collagen VI mutated fibroblasts significantly differed from healthy fibroblasts. Vitamin C treatment was able to revert the expression of some key genes to levels found in control cells raising the possibility of a beneficial effect of vitamin C as a modulator of some of the pathological aspects of collagen VI related diseases.

Introduction

Ullrich congenital muscular dystrophy (UCMD) is caused by mutations in collagen VI genes (*COL6A1, COL6A2 and COL6A3*) and is characterised by congenital hypotonia, proximal muscle weakness and distal joint hyperlaxity. Multiple joint contractures and progressive respiratory insufficiency develop over time [1,2]. In addition to these skeletal features, UCMD patients show a recognisable skin pathology in the form of follicular hyperkeratosis pilaris and abnormal scarring following skin injury mainly in the form of hypertrophic scars and keloids [3,4]. These constitute a fibrotic process due to the excessive deposition of collagen and other extracellular matrix proteins during the wound healing process [5] and can cause pain and pruritus and significant discomfort in patients that require frequent surgical interventions. Expression of collagen VI mRNA has been shown to increase in the early phases of wound healing and in keloids [6] as well as in other fibrotic processes such as systemic sclerosis [7] suggesting a role in matrix reorganisation, fibrosis and scaring in general. Wound healing of tendons and ligaments is generally similar to that of skin [8]. Collagen VI is abundant in the extracellular matrix of many tissues, where it binds to cell-surface receptors, integrins [9] and NG2 [10] and to other extracellular matrix (ECM) components including fibrillar collagens, collagen IV and fibronectin. Via these interactions collagen VI mediates cell adhesion and extracellular matrix organisation [11–13]. Collagen also VI triggers intracellular signaling events regulating for instance cell cycle progression [14] and apoptosis [15].

Fibroblasts, which are the main source of collagen VI, also synthesise and secrete many other ECM proteins, growth factors, matrix metalloproteinases and other soluble molecules such as chemokines which are necessary to maintain correct tissue homeostasis and function [16].

In order to investigate the effect of collagen VI mutations on skin fibroblasts function and how they may contribute to abnormal scarring and other pathological features seen in collagen VI deficiency we performed global gene expression profiling of skin fibroblasts from healthy controls and UCMD patients. Albeit the recognized differences between skin, muscle and other tissue specific fibroblasts we thought that this approach may also help us shed some light into the mechanisms controlling some of the skeletal changes that are observed in patients with collagen VI defects.

Vitamin C is necessary for the hydroxylation of lysyl residues and secretion of different collagen types. In addition, it is a potent antioxidant and has other cellular functions such as promoting cell proliferation, migration, and regulation of gene expression [17]. To find out if collagen VI deficiency has an effect on the fibroblast response to vitamin C we also compared UCMD and healthy fibroblasts that had been previously treated with ascorbic acid.

Materials and Methods

Ethics Statement

This work has been approved by the Ethical Committee of "Fundació Sant Joan de Déu". Written informed consent for research was obtained from all patients and controls (or their parents/guardians) according to the Hospital Sant Joan de Déu forms and regulations.

Patients and samples

UCMD patients with confirmed mutations in *COL6A* genes have been described previously [18]. All patients showed a deficiency in collagen VI secretion by dermal fibroblasts that ranged from a mild to a severe reduction in extracellular collagen VI.

Skin biopsies from the forearm were obtained from UCMD and Bethlem myopathy (BM) patients and from children not affected by a neuromuscular condition. In the microarray analysis we included 6 UCMD and 6 aged-matched control fibroblast cell lines Primary skin fibroblasts cultures were established as previously described [19]. Confluent fibroblasts (passages 2 or 3) were treated or not with 50 μg/mL of L-ascorbic acid phosphate magnesium (Wako Chemicals GmbH, Neuss, Germany) for 5 days before RNA extraction. Open quadriceps muscle biopsies were snap-frozen in liquid nitrogen before RNA extraction. Serum samples were collected, separated by centrifugation and stored at -80°C until the moment of the analysis.

RNA isolation

Total RNA was extracted with RNeasy Fibrous Tissue mini kit (Qiagen, Hilden, Germany) and its quantity and quality assessed as previously described [18]. For miRNA isolation from serum Trizol extraction was applied.

Microarray and Systems Biology Analysis

Expression data have been submitted to NCBI's Gene Expression Omnibus database (GSE56741, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56741).

Biotin-labelled and fragmented target RNA samples were loaded into Affymetrix Gene-Chip® (Human Genome U219) Array Plate (Affymetrix, Santa Clara, CA, USA). The current format of these arrays interrogates more than 36,000 transcripts and variants, which represent more than 20,000 annotated genes in the human genome. Washes and scanning of the arrays were performed according to manufacturer instructions. Raw data was normalized using Robust Multichip Analysis (RMA) method to assure comparability across samples [20]. Statistical differential gene expression analysis between groups was made by the non-parametric approach Rank Prod [21] which detects genes that are consistently highly ranked in a number of replicate experiments. Those Affymetrix probesets having changes between groups with false discovery rate (FDR) lower than 0.05 were considered significant. David tool [22] was used for the functional enrichment analysis using significant gene lists, and Gene Ontology Biological Process (www.geneontology.org) and KEGG pathways (Kyoto Encyclopedia of Genes and Genomes, www.genome.jp/kegg) databases were considered.

Physical interaction networks have been constructed using the Ingenuity Pathways tool (IPA) (www.ingenuity.com), based on extensive records maintained in the Ingenuity Pathways Knowledge Base (IPKB) database.

Pearson correlations were computed considering expression values of interesting genes to construct and display networks using Cytoscape tool (|R|>0.8, p-value<0.005) [23].

Real-Time Quantitative RT-PCR

High-throughput real-time qPCR was performed on the BioMark 48.48 Dynamic Array (Fluidigm®, South San Francisco, CA, USA) with Taqman Gene Expression Assays as previously described [24]. *HPRT1* was used as the housekeeping gene.

Adhesion assay

Fibroblasts were seeded into 96-well culture 8 well-strips coated with vitronectin, fibronectin, laminin, collagen type I and collagen type IV (Millicoat™ Screening Kit, Millipore, Billerica, MA, USA). Cells were incubated for 1 hour at 37°C/ 5% CO_2. The adherent cells were washed with HBSS and stained with 0.2% crystal violet solution in 10% ethanol for 5 min. Excess dye was removed by washing with PBS, the violet stain was solubilized with a mixture of 0.1M NaH_2PO_4 pH 4.5 and 50% ethanol and absorbance was measured at 540 nm on a microplate reader (Emax, Molecular Devices).

Immunofluorescence and Western blot

Fibroblasts were fixed in 2% paraformaldehyde on ice for 10 minutes. Blocking was performed using 2% BSA in PBS-Tween 0.05%. Mouse monoclonal anti-integrin- α3 (Millipore MAB1952, 1:50) was used and detected with a secondary anti-mouse IgG conjugated with Alexa-488. Images were captured with a Leica epifluorecent microscope and LAS software. For Western blot analysis total protein extracts were separated on 10% Mini-PROTEAN ® TGX (Bio-Rad, Hercules, CA, USA) and transferred onto a nitrocellulose membrane using Trans-Blot® TurboTM Mini Nitrocellulose Transfer Pack and Tans-Blot® Turbo TM Transfer System (BioRad, Richmond, USA). After blocking, membranes were incubated with primary antibodies over night at 4°C. A HRP-conjugated donkey anti -mouse secondary antibody (Jackson Immuno Research) was used. Proteins were detected using chemiluminescence (ECL, Pierce, Rockford, USA). The protein content was expressed in arbitrary units relative to α-Tubulin (Sigma, Missouri, USA) as the protein loading control. The intensity of protein bands was determined by densitometry with ImageJ Software.

miRNA analysis

miRNA analysis was performed as described previously [25]. Real-time PCR was performed in duplicates using Taqman Gene Expression Master Mix and Taqman Assays for selected miRNAs (U6 snRNA, hsa-miR-181a and hsa-miR-30c) in 7500 real-time PCR System using Applied Biosystems software v.2.0.4 (Foster City, CA, USA). U6 snRNA was used as a reference to normalize transcription levels among patients. Fold changes were calculated as mean values of 2-ΔΔCT or 1/2-ΔΔCT relative to control. A fold change above or below 1.5 was considered significant.

Results

Overview

Statistical analysis with Rank Prod revealed important changes in gene expression in all four comparisons analysed (Table 1). The complete list of unique under-expressed and over-expressed genes in each comparison is provided in S1 Table. The Top 10 down- and up-regulated genes are summarised in Table 2 for P-C comparison (untreated patient versus untreated control cells) and in S2 Table for the other three comparisons. These changes were validated for a selection of genes of interest by real-time PCR (S3 Table). There were 314 genes in common (approximately 50% for both comparisons) between P-C and PAA-CAA comparisons,

Table 1. Differentially expressed genes in each contrast (FDR<0.05).

Unique genes/Contrast	P-C	P_{AA}-P	P_{AA}-C_{AA}	C_{AA}-C
UP	360	298	442	540
DOWN	233	276	189	544
TOTAL	593	575	631	1084

P: patients; C: controls; P_{AA}: patients treated with ascorbic acid (AA); C_{AA}: controls treated with AA. FDR: false discovery rate.

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mainly enriching TGF-beta signaling and ECM-receptor interaction pathways (see below). We recently described the transcriptomic profile of UCMD muscle [24]. We compared those data with the results from the fibroblasts cultures and found 52 differentially expressed genes in common. KEGG pathway analysis of those common genes revealed that they were involved either in immunity or cell adhesion (Table 3). In the first group we found several genes for class I and class II antigens as well as components of the complement cascade indicating that collagen VI deficiency leads to an inflammatory signal both in skin and muscle. Regarding cell adhesion we found similar fold changes in fibroblasts and muscle in common genes including those for laminin-α4, tenascin X, caveolin-1, thrombospondin-4 and WNT1 inducible signaling pathway protein (WISP2). Thus, these proteins may play an important role in disease pathogenesis.

Functional Enrichment analysis:

Patient versus control fibroblasts. Gene Ontology (GO) and KEGG pathway databases were used for functional enrichment analysis as previously described [24]. The most over-

Table 2. Top-ten down- and up-regulated genes in the comparison P-C.

Gene symbol	Gene name	FC	FDR
DACT1	dishevelled-binding antagonist of beta-catenin 1	-6.31	0
SCRG1	stimulator of chondrogenesis 1	-5.88	0
ID4	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	-4.31	0
COL11A1	collagen, type XI, alpha 1	-4.01	0
GPC4	glypican 4	-3.94	0
RDH10	retinol dehydrogenase 10 (all-trans)	-3.58	0
TPD52L1	tumor protein D52-like 1	-3.57	0
GDF6	growth differentiation factor 6	-3.47	0
INHBE	inhibin, beta E	-3.30	0
SLC38A4	solute carrier family 38, member 4	-3.15	0
APCDD1	adenomatosis polyposis coli down-regulated 1	10.34	0
C10orf116	adipogenesis regulatory factor (ADIRF)	9.06	0
EPDR1	ependymin related 1	5.09	0
CLEC3B	C-type lectin domain family 3, member B	5.01	0
INHBB	inhibin, beta B	4.91	0
LY6K	lymphocyte antigen 6 complex, locus K	4.90	0
OLFML2A	olfactomedin-like 2A	4.73	0
NTN4	netrin 4	4.67	0
SLC7A14	solute carrier family 7, member 14	4.41	0
PSPH	phosphoserine phosphatase	4.23	0

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Table 3. KEGG pathway analysis of genes commonly regulated in collagen VI deficient skeletalmuscle (Paco et al., 2103) and skin fibroblasts (FDR<30).

KEGG Pathway	Genes	FDR
Type I diabetes mellitus	*IGF2, HLA-DPA1, HLA-DPB1, HLA-F*	6.28E-04
Viral myocarditis	*CAV1, HLA-DPA1, HLA-DPB1, HLA-F*	0.003
Focal adhesion	*CAV1, LAMA4, IGF1, COL1A1, THBS4*	0.008
Allograft rejection	*HLA-DPA1, HLA-DPB1, HLA-F*	0.009
Graft-versus-host disease	*HLA-DPA1, HLA-DPB1, HLA-F*	0.01
Cell adhesion molecules (CAMs)	*HLA-DPA1, HLA-DPB1, JAM2, HLA-F*	0.016
Autoimmune thyroid disease	*HLA-DPA1, HLA-DPB1, HLA-F*	0.018
Complement and coagulation cascades	*F10, C3, CFD*	0.032

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represented GO_BP terms amongst up-regulated genes were related to cell adhesion, immunity, blood vessel development and wound healing (S4 Table). Hypertrophic scars and keloids, which are common in collagen VI deficiency, are a manifestation of abnormal wound healing. Wound healing is a highly orchestrated process which consists of a series of stages: hemostasis and coagulation, inflammation, proliferation and remodeling [8], [5]. Within the GO terms related to scaring and blood vessel development we found genes involved in one or more stages of wound healing as summarized in Table 4.

On the other hand, KEGG database enrichment analysis identified 9 significantly enriched pathways for the total 593 differentiated genes in untreated patient cells relative to untreated control cells. The top 3 pathways were ECM-receptor interaction, nitrogen metabolism and TGF-β signaling (Table 5). Nitrogen metabolism genes included cysteine-protein-sulfhydrase (*CTH* -2.5), which is involved in the synthesis of cysteine and asparagine synthetase (*ASNS* -2.11) which is induced by starvation. Glutamate-ammonia ligase (*GLUL* +1.7) is responsible for the synthesis of glutamine from glutamate and ammonia whereas the enzyme that catalyses the reverse reaction in mitochondria was under-expressed (*GLS* -2.21). Both enzymes regulate ammonia levels and therefore acid-base balance. Also, in this category we found changes in cytosolic and extracellular isoforms of carbonic anhydrase (*CA13*–1.65 and *CA12* +2.5) which are necessary for maintaining tissue PH. These data suggest that collagen VI deficiency in fibroblasts may result in imbalances of PH and an adaptative metabolic response involving amino acids.

Table 4. Genes involved in wound healing that are either up-regulated or down-regulated in P-C fibroblasts comparison.

Coagulation	Inflammation	Angiogenesis	Adhesion	ECM remodelling
F10 (+2.6) coagulation factor X	*CD59* (+1.8) inhibitor of complement membrane attack	*COL18A1* (+.2.2) precursor of endostatin	*CD44* (+1.8), *ITGA7* (+2.8)	*TIMP3* (+1.7) inhibitor of matrix metalloproteinases
SERPINB2 (+2.4) *TFPi* (+2.2) Protease inhibitors	*CD40* (+1.7) IFN-γ and CD40L receptor	*MEOX2* (+2.2), *ITGA7* (+2.8), vascular smooth muscle development	*CD9* (+3.8)	*PLAUR* (+1.8) receptor for urokinase plasminogen activator
PLAUR (+1.8) receptor for urokinase plasminogen activator	*C3* (-2.2), *CFD* (-1.6) complement cascade	*TBX1* (+2.7), *ANPEP* (+1.9), *ARHGAP22* (+1.9), *CD44* (+1.8) Endothelial cell formation and proliferation		
	HLA-DPA1 (+4), *HLA-DPB1* (+2.2), *HLA-F* (+1.8), major histocompatibility complex II and I	*LAMA4* (+ 1.7), *ENG* (+1.8)) endothelial cell membrane and basal lamina		
	SERPING1 (+1.7), complement 1 inhibitor			

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Table 5. KEGG pathway analysis of all regulated genes in P-C comparison with an FDR $\leq$ 0.3.

KEGG Pathway	Genes	FDR
ECM-receptor interaction	COL4A1, TNXB, TNXA, TNC, ITGA3, LAMA4, ITGA6, CD44, COMP, ITGA7, COL1A1, COL11A1, THBS4	5.30E-04
Nitrogen metabolism	CTH, GLUL, CA13, CA12, GLS, ASNS	0.002
TGF-beta signaling pathway	INHBB, ACVR2A, INHBA, INHBE, COMP, GDF6, GDF5, ID4, DCN, ID3, THBS4	0.002
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	JUP, TCF7, DSG2, SGCG, ITGA6, ITGA7, LMNA, SGCD, ITGA3, TCF7L2	0.003
Complement and coagulation cascades	F10, MASP1, C3, F3, CD59, TFPI, SERPING1, CFD, PLAUR	0.006
Hypertrophic cardiomyopathy (HCM)	ACTC1, IL6, SGCG, ITGA6, ITGA7, LMNA, IGF1, SGCD, ITGA3, TPM1	0.007
Viral myocarditis	CAV1, SGCG, MYH2, SGCD, HLA-DPA1, HLA-DPB1, CD40, HLA-DRA, HLA-F	0.008
Hematopoietic cell lineage	CD9, IL6, CD44, ITGA6, CD59, ANPEP, ITGA3, CD14, HLA-DRA	0.023
Renin-angiotensin system	AGTR1, AGT, ANPEP, ENPEP	0.03

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It has been previously described that TGF-β signaling contributes to muscular dystrophies and myopathies by promoting fibrosis formation [26]. Expression of different components of this pathway including members of TGF-β superfamily of cytokines such as inhibins (*INHBA -1.96*, *INHBE -3.3* and *INHBB*, *+4.90*), growth differentiation factors (*GDF5 +2.16* and *GDF6– 3.47*), ECM components that modulate the ligand access to TGF-β receptors (*COMP +1.68*, *DCN +1.82* and *THBS4 +1.85*), type II transmembrane serine/threonine kinase receptor (*ACVR2A -1.76*) and effectors of down-stream Smad signaling pathway (*ID3–1.74* and *ID4– 4.31*) were significantly either up-regulated or down-regulated in our dataset. The TGF-beta signaling pathway is complex and while some of those components may be exerting a stimulatory effect on fibrosis others may be acting on the opposite direction and therefore it is difficult to foresee from changes at gene expression level whether this pathway is activated or not. We studied phosphorylation of Smad2 after treatment with TGF-β of UCMD and control cells and although we detected phosphorylation of Smad2 we did not find significant differences between patients and control cells (data not shown). In addition, IPA tool could not find statistical evidence of this pathway being either activated or inhibited.

ECM-receptor interaction and cell adhesion. Extracellular Matrix (ECM)—receptor was the most highly over-represented KEGG canonical pathway with 12 genes (Table 5). Given the position of collagen VI as a key component of the ECM we decided to focus on this pathway for a more detailed analysis.

In order to find out more about the possible functional consequences of the observed gene signatures from a systems biology point of view, we decided to analyse and visualize correlations between individual genes and to build networks using Cytoscape tool. This is represented in "Fig 1". The depicted network includes only those correlations that were significant (R >0.8, p-value<0.005) between selected genes in the KEGG Pathway "ECM-receptor interaction (hsa04512)", mainly those encoding for integrins and other cell surface receptors, laminins, collagens, tenascins and thrombospondin, and collagen VI genes that were not previously described on that pathway. Using this tool we were able to see that the connectivity between several ECM components and transmembrane receptors was altered in UCMD fibroblasts relative to control fibroblasts. For example, three of the collagen binding integrins, *ITGA2*, *ITGA10* and *ITGA11* were largely correlated with genes for collagen I, III, IV, V, VI and XI chains in
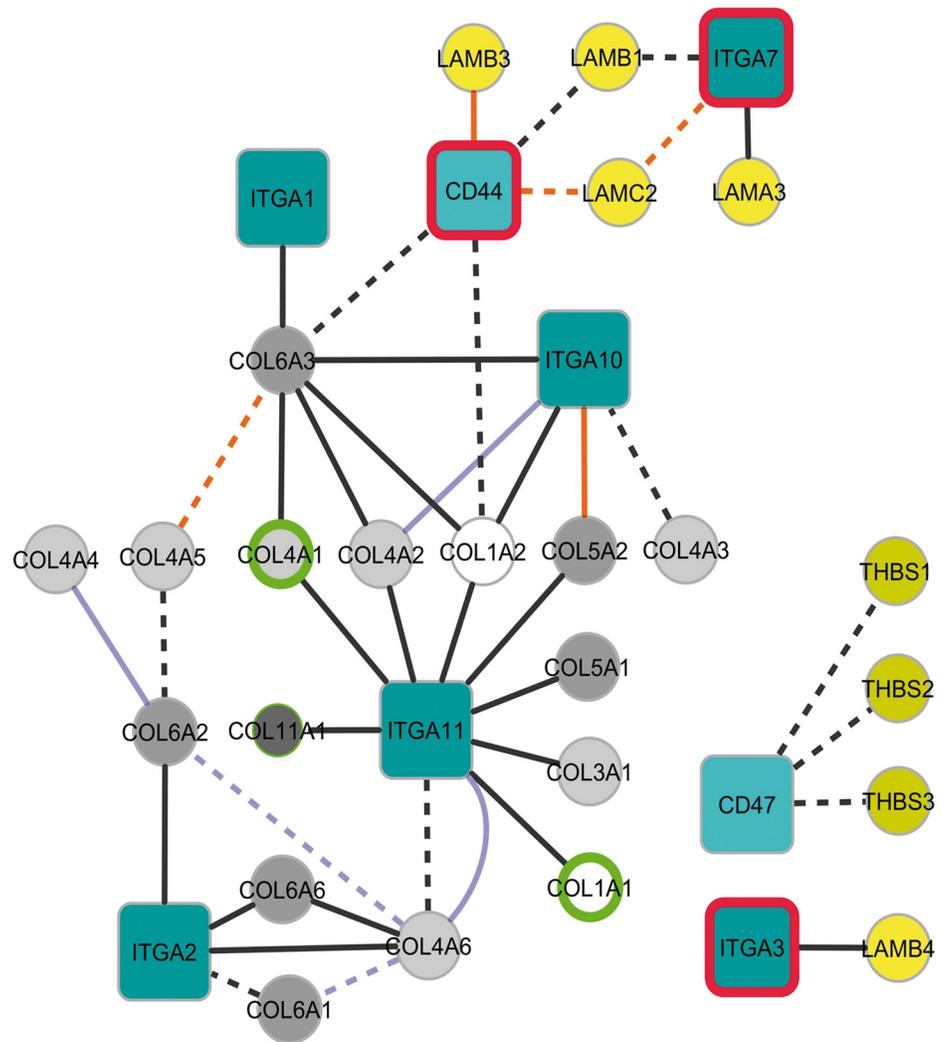
Fig 1. Extracellular matrix gene correlation network. This network represents Pearson correlations (R >0.8, p-value<0.005) computed considering expression values of genes of interest according to the microarray data and using Cytoscape tool. We selected those genes on the ECM-receptor interaction KEGG pathway and COL6A genes. Continuous lines represent positive correlations and discontinuous lines negative ones. Those correlations that are significant in patients´cells only are represented in black lines. Orange lines represent those correlations that are present in both patients and control cells but are of different sign (positive or negative) whereas those that have the same sign are represented by lilac lines. Red lines around gene symbols represent significantly over-expressed genes and green lines those that were under-expressed in patients´ fibroblasts relative to control fibroblasts.

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the case of disease samples only (black lines, "Fig 1"). In particular, new positive correlations appeared between collagen VI chains and cell receptors (*ITGA2* and *ITGA10*) as well as with other collagens (type I and IV). Some connections between *ITGA3, ITGA7, CD44 and CD47* genes were also modified. Moreover, some correlations although found in both patients and controls changed their sign (orange lines). For example in control cells *ITGA7* positively correlated with *LAMC2* (encoding for laminin-γ2 chain) whereas in patient cells this correlation was reversed. These findings suggest that collagen VI defects result in a major reorganization of the ECM and its interaction with cells.
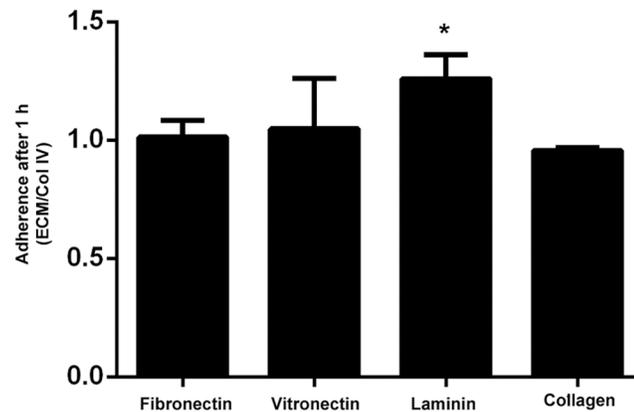
Fig 2. Cell adhesion assay. Adherence of UCMD fibroblasts (n = 7) relative to control fibroblasts (n = 7) for fibronectin, vitronectin, laminin and collagen type I (experiments were performed in triplicate). To normalize adhesion values between experiments, we expressed the results as a ratio between the absorbance values for collagen type IV (which was the substrate that showed the smallest variability between individual cultures and experiments, data not shown) and each ECM protein, (student t-test * p < 0.05).

To investigate this further we studied the capacity of UCMD fibroblasts (n = 7 which included the 6 fibroblasts cell lines analysed in the microarrays and an additional UCMD fibroblast sample with collagen VI deficiency) to adhere to various ECM substrates. We found that the adherence profile of UCMD cells was similar to controls for vitronectin, fibronectin and collagen type I. However, adherence to laminin was significantly increased ("Fig 2"). Any of the three integrin alpha chains that we found up-regulated in the microarray (ITGA3, ITGA6 and ITGA7) could be involved in the observed enhanced adhesion to laminin. Given that integrin-α3 has been shown to mediate fibrosis in lung and skin [27] we decided to look in more detail at the expression of this integrin in patients and control fibroblast. Here we included two additional samples from patients with mutations in collagen VI genes and the milder Bethlem myopathy (BM).

In control fibroblasts we observed a uniform punctuate labeling all over the cell surface. In patients cells the labeling appeared more intense than in control cells and in addition we observed sites of increased staining in areas of contact between two cells ("Fig 3A"). Western blot analysis was carried out from protein extracts from control (n = 2), UCMD (n = 10) and BM (n = 2) fibroblasts. A band of the expected molecular weight (119kDa) was seen in all cases. The intensity of this band was quantified by densitometry relative to α-tubulin as a loading control. On average Integrin-α3 was moderately increased in UCMD (1.3 fold) and BM extracts (1.7 fold) although it only reached statistical significance in BM patients (student t-test, p = 0.006) ("Fig 3B").

Regulation of gene expression: miRNAs

In order to identify key molecules potentially responsible for the observed changes in gene expression we performed Ingenuity Pathways (IPA) interaction network analysis.

A well scored network of genes involved in cell cycle, skeletal and muscular system development ("Fig 4") was particularly interesting because 12 miRNAs were added by the tool, interacting with the significantly altered genes of the P-C comparison. The striking central "hub" (highly connected) of the network was miR-30c. This mRNA has been previously detected in fibroblasts where it is implicated in fibrosis since it is a powerful negative regulator of CTGF (Connective tissue growth factor) expression [28] which was down-regulated in our array (FC
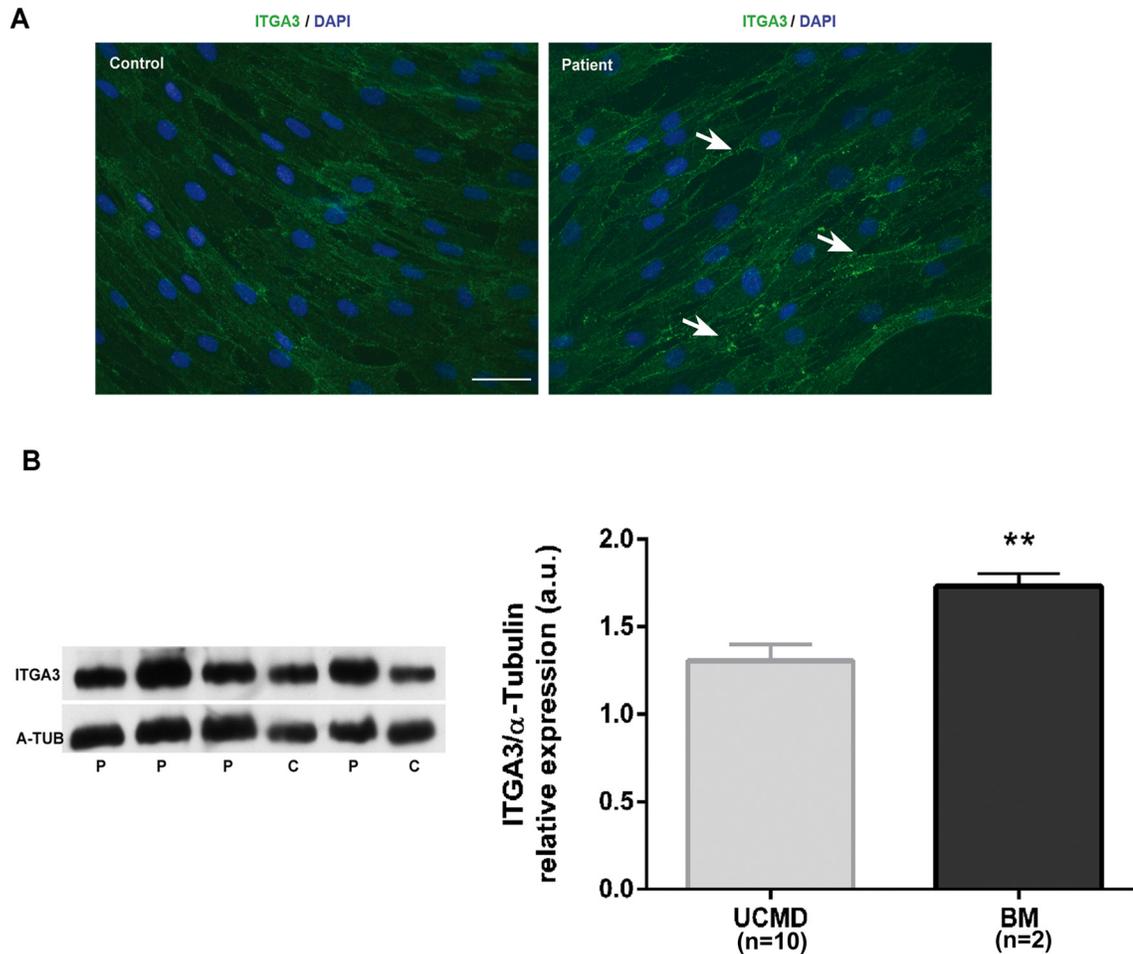
Fig 3. Integrin-α3 expression in collagen VI deficient fibroblasts. A. Immunofluorescence for integrin-α3 in confluent fibroblast cultures. B. Representative western blot analysis. The intensity of the bands corresponding to integrin-α3 (ITGA3) was quantified by densitometry using α-tubulin (A-TUB) as a loading control and expressed as arbitrary units relative to the control samples.

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-2.29). Another well scored network of genes involved in protein synthesis and fibrosis was also identified (data not shown) incorporated 4 "hubs" miRNAs including miR-181a which is expressed in skeletal muscle [29].

Besides their role as regulators of gene expression, miRNAs represent useful clinical bio-markers of different diseases including muscular dystrophies. By means of qRT-PCR we quantified miR-181a and miR-30c in UCMD muscle (n = 5) and fibroblasts (n = 9). We found that miR-181a was over-expressed in UCMD muscle relative to healthy muscle whereas miR-30c was under-expressed ("Fig 5A"). Levels of both miRNAs were decreased in RNA from UCMD skin fibroblasts ("Fig 5B") although they did not reach the -1.5 threshold.

We also measured miR-181a and miR-30c in serum samples from UCMD (n = 6), BM (n = 7) and DMD (n = 9) patients relative to healthy controls (either children or adult). Both miRNAs were significantly elevated in all patient groups ("Fig 5C and 5D").

Next, we calculated correlations between the levels in serum, fibroblasts and muscle of miR-181a and miR-30c in UCMD patients (expressed as deltaCT) with various histopathological parameters measured in their corresponding muscle biopsies [18] as well as with the levels of
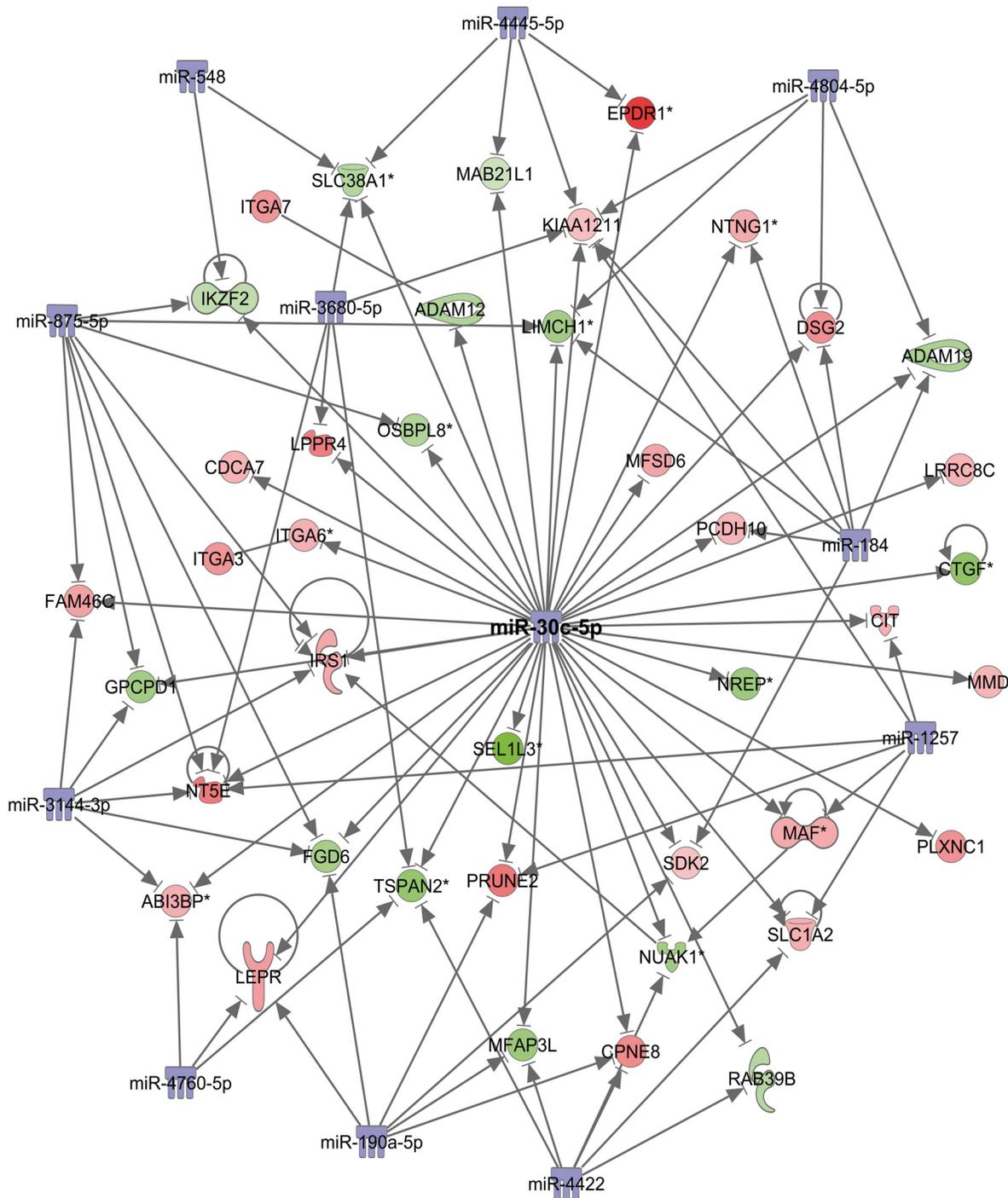
Fig 4. Ingenuity Pathway Analysis. Graphic representation of the network "cell cycle, skeletal and muscular system development". Nodes represent genes and lines show the relationship between genes. The intensity of the node color indicates the degree of the up-regulation (red) or down-regulation (green) of significant genes in the P-C comparison. Non-color nodes are added by the tool. For a detailed legend refer to http://ingenuity.force.com/ipa/articles/Feature_Description/Legend.

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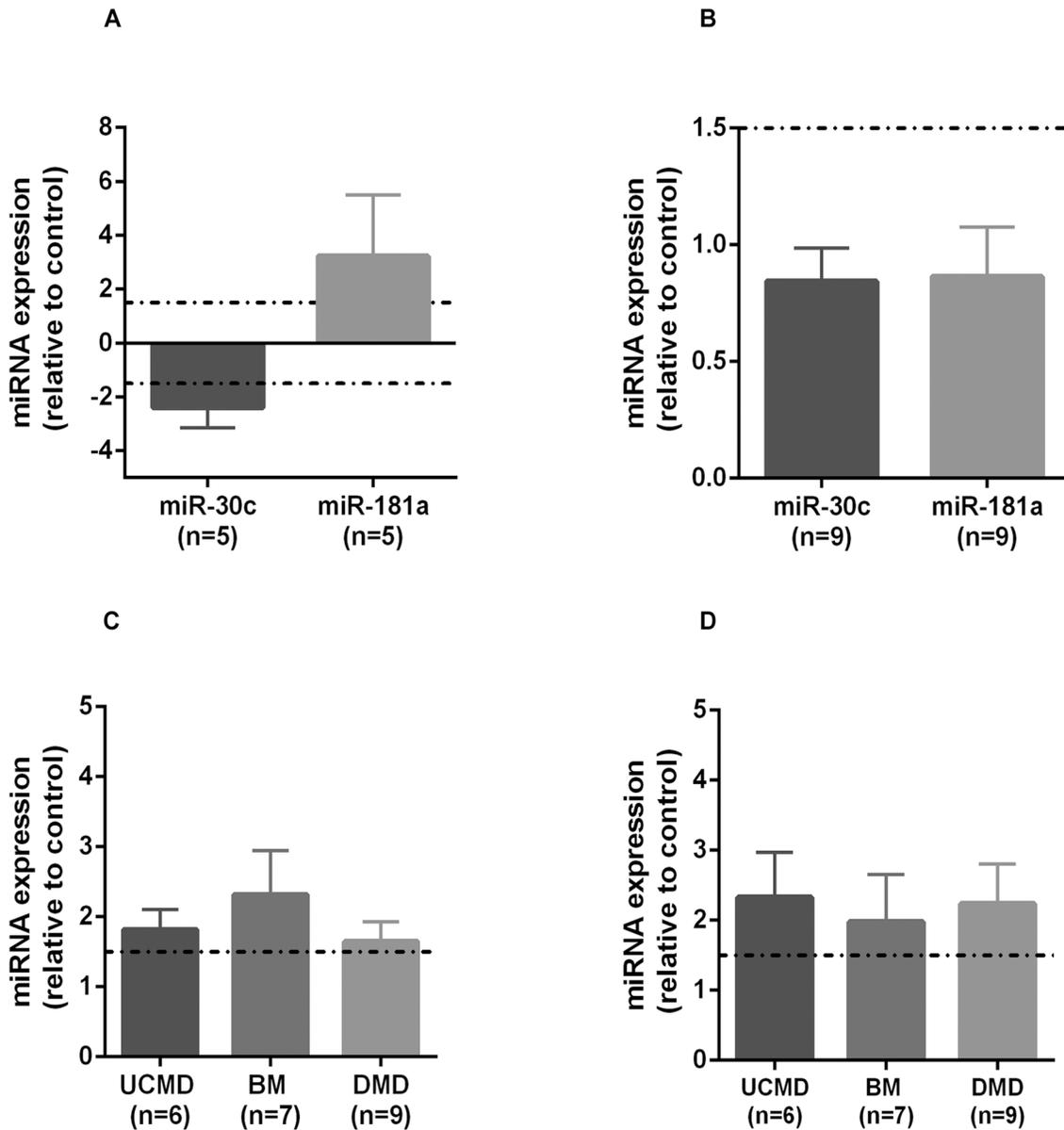
Fig 5. miRNAs expression analysis. Real-time PCR was used to measure relative expression of miR-181a and miR-30c in skeletal muscle (A) and fibroblasts (B) from UCMD patients and in serum samples from UCMD, BM and DMD patients for miR-181a (C) and miR-30c (D). miRNA expression level was normalized against U6 miRNA. Results were calculated relative to control samples and are represented as mean and standard error.

adiponectin and leptin in their serum, which are related to adiposity and metabolism. We selected those correlations achieving R value above 0.7 and p value below 0.05 (Table 6). A significant negative correlation was established between levels of miR-30c in serum and circulating adiponectin levels. At the histopathological level we found that miR-30c in UCMD muscle negatively correlated with fibre size and positively correlated with the % of pax7 positive myonuclei (an indication of the satellite cell population). UCMD miRNA_180 serum levels presented a strong negative correlation to the extent of fibrosis (measured as the % area occupied by collagen, [24]) and a positive correlation with circulating leptin levels.

Table 6. Correlation analysis between miR-30c and miR-181a levels and for various pathological and biochemical parameters.

Variable	Variable	p	R
miR-181a _serum	Leptin_serum	0.03892198	0.73211393
miR-181a_serum	% fibrosis_ Skm	0.01730787	-0.79922952
FiberSize_Skm	miR-30c_Skm	0.04966404	-0.70745107
Mean_fiberdiam_Skm	% adipose_Skm	0.0287597	0.75963608
Min_fiberdiam_Skm	Leptin_serum	0.01555091	-0.80665245
Min_fiberdiam_Skm	miR-30c_Skm	0.04472653	-0.718339
%MHCe_Skm	%MHCn_Skm	0.00713975	0.85265406
%MHCe_Skm	%fibrosis_ Skm	0.01877006	0.79340433
%MHCn_Skm	%fibrosis_ Skm	0.02623684	0.7673713
%Pax7_Skm	miR-30c_Skm	0.01820804	0.79560857
%Pax7_Skm	%fibrosis_ Skm	0.03621514	-0.73896665
Adiponectin_serum	miR-30c _serum	0.00277097	-0.89366847
Leptin_serum	miR-30c_fibroblasts	0.04631627	0.71475759

R: Pearson correlation coefficient; % fibrosis: percentage area occupied by collagen; %adipose: percentage area occupied by adipocytes; Skm: skeletal muscle biopsy; fiberdiam: fiber diameter; min: minimum; %MHCe:percentage of fibers immunoreactive for the embryonic isoform of myosin heavy chain; %MHCn:percentage of fibers immunoreactive for the neonatal isoform of myosin heavy chain; %Pax7:percentage of myonuclei positive for Pax7 immunolabelling.

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Effect of Ascorbic Acid (AA)

Although gene expression analysis in UCMD skin fibroblasts has not been reported before there is a previous study on the effect of AA on gene expression in healthy human skin fibroblasts [17]. We found that 104 out of the 294 genes identified by Duarte et al. were also altered in our analysis (C_{AA}-C comparison).

In our study treatment of skin fibroblasts with ascorbic acid induced changes in gene expression both in UCMD and control cells. However, the number of genes whose expression significantly changed in response to AA in UCMD fibroblasts was almost half than in controls. When we compared the list of genes in both cases (PAA-P and CAA-C comparisons) we found that 307 genes were common for UCMD and control cells, 267 genes were unique for the UCMD group and 777 were unique for the control group. In order to ascertain how UCMD and control cells respond differently to AA we performed gene enrichment analysis using DAVID and looked *at the GO* terms that only changed in either control cells or in UCMD cells (36 and 35 GO categories respectively). Further grouping of those gene ontologies revealed that genes that changed after AA treatment in control cells only were mainly related to regulation of wound healing and angiogenesis, steroid and estrogens metabolism and response to vitamin and vitamin biosynthesis. In contrast those that changed in UCMD cells only were related to the cell-cycle and cell division and extracellular matrix organisation.

Given that AA treatment was able to induce important changes in gene expression we aimed to identify those genes that reversed their expression by applying AA in UCMD fibroblasts (PAA-P) with respect to those that changed purely by the effect of collagen VI mutations (P-C). We identified a set of only 40 genes that reverted sign (from down-regulated to up-regulated and vice versa) (Table 7) upon AA treatment in UCMD cells. Importantly, AA was able to change the expression of those genes to levels comparable to those found in untreated C cells. That relatively short list of genes included significantly enriched functional terms such as extracellular matrix organization and tissue regeneration. Using covariance analysis we found

Table 7. Genes that revert their expression in patients cells after AA treatment.

Gene	FC P$_{AA}$—P	FC P -C
HLA-DRA	-2.0799	2.3098
KRTA P1-5	-2.1133	2.1484
EVI2A	-1.3430	2.1085
TNFRSF11B *	-1.4997	2.0947
KRT19	-1.3663	1.9729
DUSP1	-1.3470	1.9561
SEMA3B	-1.4063	1.8835
GRIA1	-2.3154	1.8560
HMGA1	-1.3774	1.7728
FOS	-1.7687	1.7060
*TIMP3	-1.3988	1.6497
SLC47A1	-1.3180	1.6351
GFRA1	-1.5015	1.6309
FXYD5	-1.4225	1.5697
*TNXB	-1.5475	1.7728
TRIL	2.2708	-2.2650
*ELN	1.6928	-2.1487
LCTL	1.3947	-2.1322
*TNC	1.4138	-2.0623
C1QTNF3	2.2280	-2.0488
OGN	2.3625	-2.0416
ARHGAP28	1.5783	-1.9912
C5orf13	1.4576	-1.9893
*COL1A1	1.6571	-1.9391
LRRC15	1.7660	-1.8854
*DPT	2.1617	-1.8734
FGD6	1.4797	-1.8577
GNB4	1.6055	-1.8471
ANGPTL1	1.5504	-1.8420
ARRDC4	1.4529	-1.8416
CSRP2	1.4479	-1.8146
*ADAM19	1.3157	-1.7809
COL10A1	2.4221	-1.7746
ACVR2A	1.5374	-1.7553
PDGFD	1.7902	-1.7188
VCAN	1.4575	-1.6998
IGF1	1.5920	-1.6075
NPNT	1.5610	-1.5337
IGSF10	1.4776	-1.4192

*Genes associated with the ECM (GO:0043062~extracellular structure organization)

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that PAA samples (patients cells treated with AA) grouped more closely to control cells (C) than untreated patient cells (P) cells indicating that AA acid can modify gene expression towards the basal (untreated C cells) phenotype for some genes. An example for some of those genes is shown in "Fig 6".
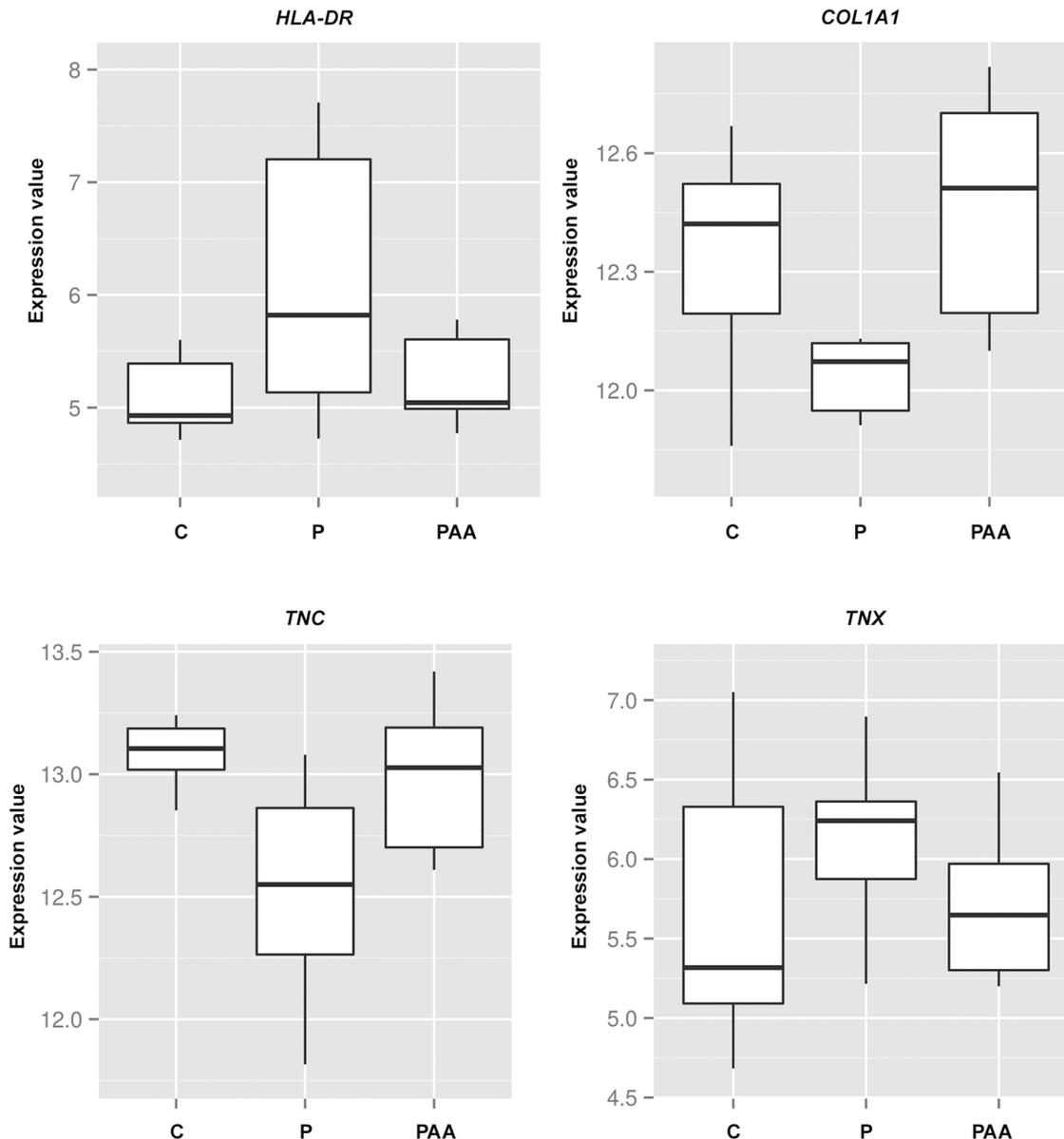
Fig 6. Co-variance analysis was used to plot the expression values of a selection of genes of interest in untreated patient cells (P), control cells (C) and patient cells treated with AA (P$_{AA}$). Median and ranges are represented. A. HLA-DRA, B. COL1A1, C. TNC, D. TNX.

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Discussion

Fibroblasts are the main source of collagen VI in skin and muscle, two of the main tissues affected in patients with mutations in COL6A genes, and therefore are the primary disease cell type. This is the first report of global gene expression profiling of collagen VI deficient dermal fibroblast. We have shown that collagen VI mutations, with almost 600 genes differentially regulated, affect several aspects of fibroblasts biology, mainly:

1. Their interaction with the surrounding environment via changes in the expression of membrane receptors and ECM components and their relationships. This would result in modified collagen fibril formation and adhesion which in turn regulates multiple signaling pathways.

2. Changes in the expression of genes involved in coagulation, inflammation and angiogenesis which are key stages of wound healing.

3. Gene expression regulation involving vitaminC and miRNAs.

ECM organization and ECM-cell interaction

Collagen fibrillogenesis.   The connective tissue features of collagen VI deficiency are independent of the skeletal muscle dysfunction and are directly related to the role of collagen VI in the organization and maintenance of the extracellular matrix in the affected tissues (skin, tendon, ligaments). Ultrastructural abnormalities in collagen fibrils have been reported in collagen VI deficient human and both wounded and unwounded mouse skin [30], [31], [3]and in the tendons of col6a3 deficient mice [32]. Therefore, some of the changes that we describe here may also be taking place in the tendon ECM.

For example, tenascin C in adult tendon concentrates at the myotendinous and ostetendinous junctions, its gene expression is regulated by mechanical loading and some *TNC* polymorphisms have been associated with risk of Achilles tendinopathies [33]. It would be worth investigating if the *TNC* gene is also down-regulated in tenocytes. In contrast *TNX* mRNA was over-expressed in UCMD fibroblasts as previously reported by us and others in UCMD muscle [34], [24]. Collagen VI and tenascin- X are both necessary for correct collagen fibril formation *in vitro* [35]. Specifically, tenascin X regulates the quantity of collagen fibrils whereas collagen VI regulates the rate at which those are formed. Thus, an imbalance in collagen VI and tenascin X expression levels may be contributing to impaired collagen fibrillogenesis and in changes in tendon structure and function. Thus, our data may help towards understanding the development of contractures which cause considerable disability.

Furthermore, bioinformatics analysis of gene expression data using correlation networks predicted a modified disease ECM scenario which is likely to contribute to the ultrastructural and functional changes seen in skin but could maybe also be extrapolated to muscle.

Cell adhesion and integrins.   Cell adhesion and migration are fundamental for the formation and maintenance of tissues and underlie many processes such as morphogenesis and wound healing. High affinity adhesion is predominantly provided by integrins and CD44 [36]. We have demonstrated that adhesion of UCMD fibroblasts to laminin is increased and that the main laminin-binding integrins are over-expressed. Although integrin α3 is mainly regarded as a laminin receptor there are several reports of its binding to collagens [37]. In pulmonary fibrosis, a model of aberrant wound healing, integrin-α3 induces fibrosis via E-cadherin and TGF-B receptor [27]. Over-expression of this integrin in UCMD cells may predispose them to acquire a fibrotic profile. Integrin alpha 3 may thus provide a therapeutic target for attenuation of fibrosis without the downside effects of using non-specific TGF-B blockers. Over-expression of integrin-α7 has been described in dystrophin deficiency but not in the context of collagen VI deficiency [38]. Although it is mainly expressed in muscle cells it can be induced in other cell types including fibroblasts [39]. We did not find statistically significant changes in the expression of any of the 4 classical collagen binding integrin alpha subunits (α1, α2, α10 and α11).

Integrin mediated cell signaling modulates many aspects of cell behaviour including cell cycle, apoptosis, cell size, polarity and motility, gene expression and the response to growth factors and their activity as it is the case for TGF-B [40]. In a recent report Accorsi et al. reported up-regulation of integrin α5 and αV in muscle from laminin-α2 deficient mice [41]. Thus, integrin dysregulation and extracellular remodeling appear to be common features of collagen VI and laminin-2 deficient CMD contributing to the fibrotic and inflammatory pathology

observed in both diseases. Our previous gene expression studies support the notion that both forms of CMD share common physiopathological mechanisms [24].

Wound healing

Wound healing is an intricate process and is likely that the disarranged pattern of gene expression that we describe here disrupts its time course and final outcome resulting in abnormal scaring. For example, components of the complement cascade (C3 and C5) can directly accelerate wound healing by increasing inflammatory and fibroblast cell recruitment, collagen deposition and organization and wound strength [8]. The changes in expression of genes involved in angiogenesis and inflammation may also help explain other features seen un UCMD patients such as malar rash which is a form of erythema (redness of the skin) that appears over the cheeks and nose which is related to inflammation and increased vasodilation of superficial capillaries and is also observed in Lupus erythematosus patients.

Role of miRNAs

Physical interaction network analysis revealed that miR-30c and miR-181a may have an important role in the regulation of gene expression in the context of UCMD. Both miRNAs were significantly increased in serum from all patients studied and correlated with different pathological and biochemical variables such as fibrosis. Therefore they could represent novel surrogate markers of muscular dystrophy in addition to the already described distromirs [42,43]. Furthermore, they could also provide novel targets to ameliorate pathology. For example, expression of miR-30c is repressed by miR-29b which in turn removes repression of connective tissue growth factor (CTGF) contributing to fibrosis [28].

Differential effect of AA

Vitamin C (ascorbic acid) is important for the maintenance of healthy skin because of its action as antioxidant and on collagen production at the transcriptional and post-translational levels [44]. We have shown that Vitamin C treatment has different effects in healthy and collagen VI deficient fibroblasts and this could underlie some of the skin features seen in patients. The molecular basis for this difference warrants further investigation. Vitamin C (ascorbate or ascorbic acid) enters the cell via simple diffusion and active transport. The main transporters are Sodium-dependent-vitamin C transporters SVCT1 and 2 (encoded by the *SLC23A1* and *SLC23*A2 genes) and the glucose transporters Glut-1 and Glut-3 (encoded by the *SLC2A1* and *SLC2*A3 genes). We did not find significant changes in the expression of any of those 4 genes in patient cells relative to control cells. Interestingly, the expression of a set of altered functionally relevant genes was reverted towards a normal level after ascorbic acid addition, which suggests that vitamin C may have beneficial effects on some of the aspects of UCMD pathology although this needs further investigation and demonstration in vivo. Clinically, vitamin C supplementation improves wound healing in patients with pressure ulcers and Vitamin C pretreatment accelerates wound closure in wound mouse models [45], [46]. Vitamin C is used in the treatment of mitochondrial myopathies together with other vitamins and co-factors [47] and its use has been assessed in Charcot-Marie-Tooth disease type 1 A (CMT1A) patients where it was found to be safe but without clinical benefit [48], [49].

Conclusions

In summary, in this study we followed a systems biology approach, a global research strategy able to integrate multi-level data and thus to address the challenge of understanding rare

disorders [50]. The differences observed between disease and healthy cells, not only at the gene expression level, but also at the functional, gene-gene connectivity and transcriptional regulation levels, allow us to propose a model for a "collagen VI myopathy-ECM pathway" which may serve as a starting-point for a global description of the disease and novel therapeutic options, to be explored on a larger set of patients.

Supporting Information

S1 Table. List of differentially expressed genes for each comparison.
(XLSX)

S2 Table. Top-ten down- and up-regulated genes.
(DOCX)

S3 Table. Validation of microarray results by qRT-PCR.
(XLSX)

S4 Table. List of enriched Gene Ontology terms (GO_BPs) for each comparison.
(XLSX)

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Author Contributions

Conceived and designed the experiments: SGK CJM. Performed the experiments: SP TC MAR MP CJM. Analyzed the data: SP TC CJ CIO JDM EG AN JC SGK CJM. Contributed reagents/materials/analysis tools: CIO JDM EG AN JC. Wrote the paper: SP TC MAR CJ CIO JDM EG AN JC SGK CJM.

References

1. Allamand V, Brinas L, Richard P, Stojkovic T, Quijano-Roy S, Bonne G. ColVI myopathies: where do we stand, where do we go? Skelet Muscle. Inserm, U974, Paris, France. v.allamand@institut-myologie.org.; 2011; 1: 30. doi: 10.1186/2044-5040-1-30

2. Bonnemann CG. The collagen VI-related myopathies: muscle meets its matrix. Nat Rev. National Institutes of Health, PO Box 5801, Bethesda, MD 20824, USA. carsten.bonnemann@nih.gov; 2011; 7: 379–390. doi: 10.1038/nrneurol.2011.81

3. Kirschner J, Hausser I, Zou Y, Schreiber G, Christen H-J, Brown SC, et al. Ullrich congenital muscular dystrophy: connective tissue abnormalities in the skin support overlap with Ehlers-Danlos syndromes. Am J Med Genet A. 2005; 132A: 296–301. doi: 10.1002/ajmg.a.30443 PMID: 15690374

4. Collins J, Bonnemann CG. Congenital muscular dystrophies: toward molecular therapeutic interventions. Curr Neurol Neurosci Rep. Division of Neurology, Cincinnati Children's Hospital Medical Center, MLC 2015, 3333 Burnet Avenue, Cincinnati, OH 45229, USA. james.collins@cchmc.org; 2010; 10: 83–91. doi: 10.1007/s11910-010-0092-8

5. Ferreira LM, Gragnani A, Furtado F, Hochman B. Control of the skin scarring response. An Acad Bras Cienc. 2009; 81: 623–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/19722029 PMID: 19722029

6. Oono T, Specks U, Eckes B, Majewski S, Hunzelmann N, Timpl R, et al. Expression of type VI collagen mRNA during wound healing. J Invest Dermatol. 1993; 100: 329–34. Available: http://www.ncbi.nlm.nih.gov/pubmed/8440917. PMID: 8440917

7. Peltonen J, Kähäri L, Uitto J, Jimenez SA. Increased expression of type VI collagen genes in systemic sclerosis. Arthritis Rheum. 1990; 33: 1829–35. Available: http://www.ncbi.nlm.nih.gov/pubmed/2261003. PMID: 2261003

8. Sinno H, Prakash S. Complements and the wound healing cascade: an updated review. Plast Surg Int. 2013; 2013: 146764. doi: 10.1155/2013/146764 PMID: 23984063

9. Pfaff M, Aumailley M, Specks U, Knolle J, Zerwes HG, Timpl R. Integrin and Arg-Gly-Asp dependence of cell adhesion to the native and unfolded triple helix of collagen type VI. Exp Cell Res. 1993; 206: 167–76. doi: 10.1006/excr.1993.1134 PMID: 8387021

10. Burg MA, Tillet E, Timpl R, Stallcup WB. Binding of the NG2 proteoglycan to type VI collagen and other extracellular matrix molecules. J Biol Chem. La Jolla Cancer Research Center, The Burnham Institute, La Jolla, California 92037, USA.; 1996; 271: 26110–26116.

11. Kuo HJ, Maslen CL, Keene DR, Glanville RW. Type VI collagen anchors endothelial basement membranes by interacting with type IV collagen. J Biol Chem. 1997; 272: 26522–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/9334230. PMID: 9334230

12. Sabatelli P, Bonaldo P, Lattanzi G, Braghetta P, Bergamin N, Capanni C, et al. Collagen VI deficiency affects the organization of fibronectin in the extracellular matrix of cultured fibroblasts. Matrix Biol. 2001; 20: 475–86. Available: http://www.ncbi.nlm.nih.gov/pubmed/11691587. PMID: 11691587

13. Kawahara G, Okada M, Morone N, Ibarra CA, Nonaka I, Noguchi S, et al. Reduced cell anchorage may cause sarcolemma-specific collagen VI deficiency in Ullrich disease. Neurology. 2007; 69: 1043–9. doi: 10.1212/01.wnl.0000271386.89878.22 PMID: 17785674

14. Atkinson JC, Ruhl M, Becker J, Ackermann R, Schuppan D. Collagen VI regulates normal and transformed mesenchymal cell proliferation in vitro. Exp Cell Res. Department of Gastroenterology, Freie Universitat of Berlin, Universitatsklinikum Benjamin Franklin, Germany.; 1996; 228: 283–291. S0014-4827(96)90328-6 [pii] doi: 10.1006/excr.1996.0328

15. Ruhl M, Sahin E, Johannsen M, Somasundaram R, Manski D, Riecken EO, et al. Soluble collagen VI drives serum-starved fibroblasts through S phase and prevents apoptosis via down-regulation of Bax. J Biol Chem. Department of Medicine I, Klinikum B. Franklin, Free University of Berlin, Hindenburgdamm 30, 12200 Berlin, Germany.; 1999; 274: 34361–34368. Available: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10567413.

16. Sarkar P, Randall SM, Muddiman DC, Rao BM. Targeted proteomics of the secretory pathway reveals the secretome of mouse embryonic fibroblasts and human embryonic stem cells. Mol Cell Proteomics. 2012; 11: 1829–39. doi: 10.1074/mcp.M112.020503 PMID: 22984290

17. Duarte TL, Cooke MS, Jones GDD. Gene expression profiling reveals new protective roles for vitamin C in human skin cells. Free Radic Biol Med. 2009; 46: 78–87. doi: 10.1016/j.freeradbiomed.2008.09.028 PMID: 18973801

18. Paco S, Ferrer I, Jou C, Cusí V, Corbera J, Torner F, et al. Muscle fiber atrophy and regeneration coexist in collagen VI-deficient human muscle: role of calpain-3 and nuclear factor-κB signaling. J Neuropathol Exp Neurol. 2012; 71: 894–906. PMID: 22975586

19. Jimenez-Mallebrera C, Maioli MA, Kim J, Brown SC, Feng L, Lampe AK, et al. A comparative analysis of collagen VI production in muscle, skin and fibroblasts from 14 Ullrich congenital muscular dystrophy patients with dominant and recessive COL6A mutations. Neuromuscul Disord. Dubowitz Neuromuscular Centre, Imperial College, Hammersmith Hospital, London, UK. c.jimenez@jc.ac.uk; 2006; 16: 571–582. doi: 10.1016/j.nmd.2006.07.015

20. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics. 2003; 4: 249–64. doi: 10.1093/biostatistics/4.2.249 PMID: 12925520

21. Breitling R, Armengaud P, Amtmann A, Herzyk P. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. FEBS Lett. 2004; 573: 83–92. doi: 10.1016/j.febslet.2004.07.055 PMID: 15327980

22. Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. Laboratory of Immunopathogenesis and Bioinformatics, Clinical Services Program, SAIC-Frederick Inc., National Cancer Institute at Frederick, Frederick, Maryland 21702, USA.; 2009; 4: 44–57. doi: 10.1038/nprot.2008.211

23. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. 2003; 13: 2498–504. doi: 10.1101/gr.1239303 PMID: 14597658

24. Paco S, Kalko SG, Jou C, Rodríguez MA, Corbera J, Muntoni F, et al. Gene expression profiling identifies molecular pathways associated with collagen VI deficiency and provides novel therapeutic targets. PLoS One. 2013; 8: e77430. doi: 10.1371/journal.pone.0077430 PMID: 24223098

25. Taylor CJ, Satoor SN, Ranjan AK, Pereira e Cotta MV, Joglekar MV. A protocol for measurement of noncoding RNA in human serum. Exp Diabetes Res. 2012; 2012: 168368. doi: 10.1155/2012/168368 PMID: 22811698

26. MacDonald EM, Cohn RD. TGFβ signaling: its role in fibrosis formation and myopathies. Curr Opin Rheumatol. 2012; 24: 628–34. PMID: 22918531

27. Kim KK, Wei Y, Szekeres C, Kugler MC, Wolters PJ, Hill ML, et al. Epithelial cell alpha3beta1 integrin links beta-catenin and Smad signaling to promote myofibroblast formation and pulmonary fibrosis. J Clin Invest. 2009; 119: 213–24. doi: 10.1172/JCI36940 PMID: 19104148

28. Abonnenc M, Nabeebaccus AA, Mayr U, Barallobre-Barreiro J, Dong X, Cuello F, et al. Extracellular matrix secretion by cardiac fibroblasts: role of microRNA-29b and microRNA-30c. Circ Res. 2013; 113: 1138–47. doi: 10.1161/CIRCRESAHA.113.302400 PMID: 24006456

29. Eisenberg I, Alexander MS, Kunkel LM. miRNAS in normal and diseased skeletal muscle. J Cell Mol Med. 2009; 13: 2–11. doi: 10.1111/j.1582-4934.2008.00524.x PMID: 19175696

30. Minamitani T, Ariga H, Matsumoto K. Deficiency of tenascin-X causes a decrease in the level of expression of type VI collagen. Exp Cell Res. 2004; 297: 49–60. doi: 10.1016/j.yexcr.2004.03.002 PMID: 15194424

31. Lettmann S, Bloch W, Maaß T, Niehoff A, Schulz J-N, Eckes B, et al. Col6a1 null mice as a model to study skin phenotypes in patients with collagen VI related myopathies: expression of classical and novel collagen VI variants during wound healing. PLoS One. 2014; 9: e105686. doi: 10.1371/journal.pone.0105686 PMID: 25158062

32. Pan T-C, Zhang R-Z, Markova D, Arita M, Zhang Y, Bogdanovich S, et al. COL6A3 protein deficiency in mice leads to muscle and tendon defects similar to human collagen VI congenital muscular dystrophy. J Biol Chem. 2013; 288: 14320–31. doi: 10.1074/jbc.M112.433078 PMID: 23564457

33. Juneja SC, Veillette C. Defects in tendon, ligament, and enthesis in response to genetic alterations in key proteoglycans and glycoproteins: a review. Arthritis. 2013; 2013: 154812. doi: 10.1155/2013/154812 PMID: 24324885

34. Higashi K, Higuchi I, Niiyama T, Uchida Y, Shiraishi T, Hashiguchi A, et al. Abnormal expression of proteoglycans in Ullrich's disease with collagen VI deficiency. Muscle Nerve. 2006; 33: 120–6. doi: 10.1002/mus.20449 PMID: 16258947

35. Minamitani T, Ikuta T, Saito Y, Takebe G, Sato M, Sawa H, et al. Modulation of collagen fibrillogenesis by tenascin-X and type VI collagen. Exp Cell Res. 2004; 298: 305–15. doi: 10.1016/j.yexcr.2004.04.030 PMID: 15242785

36. Schmidt S, Friedl P. Interstitial cell migration: integrin-dependent and alternative adhesion mechanisms. Cell Tissue Res. 2010; 339: 83–92. doi: 10.1007/s00441-009-0892-9 PMID: 19921267

37. Borza CM, Pozzi A, Borza D-B, Pedchenko V, Hellmark T, Hudson BG, et al. Integrin alpha3beta1, a novel receptor for alpha3(IV) noncollagenous domain and a trans-dominant Inhibitor for integrin alphavbeta3. J Biol Chem. 2006; 281: 20932–9. doi: 10.1074/jbc.M601147200 PMID: 16731529

38. Hodges BL, Hayashi YK, Nonaka I, Wang W, Arahata K, Kaufman SJ. Altered expression of the alpha7-beta1 integrin in human and murine muscular dystrophies. J Cell Sci. 1997; 110 (Pt 2: 2873–81. Available: http://www.ncbi.nlm.nih.gov/pubmed/9427295.

39. Smith AN, Willis E, Chan VT, Muffley LA, Isik FF, Gibran NS, et al. Mesenchymal stem cells induce dermal fibroblast responses to injury. Exp Cell Res. 2010; 316: 48–54. doi: 10.1016/j.yexcr.2009.08.001 PMID: 19666021

40. Lowell CA, Mayadas TN. Overview: studying integrins in vivo. Methods Mol Biol. 2012; 757: 369–97. doi: 10.1007/978-1-61779-166-6_22 PMID: 21909923

41. Accorsi A, Mehuron T, Kumar A, Rhee Y, Girgenrath M. Integrin dysregulation as a possible driver of matrix remodeling in Laminin-deficient congenital muscular dystrophy (MDC1A). J od Neuromuscul Dis. 2015; 2: 51–61. doi: 10.3233/JND-140042

42. Alexander MS, Kunkel LM, Cell S, Children B. "Skeletal Muscle MicroRNAs: Their Diagnostic and Therapeutic Potential in Human Muscle Diseases". J Neuromuscul Dis. 2015; 2: 1–11. doi: 10.3233/JND-140058

43. Zaharieva IT, Calissano M, Scoto M, Preston M, Cirak S, Feng L, et al. Dystromirs as serum biomarkers for monitoring the disease severity in Duchenne muscular Dystrophy. PLoS One. 2013; 8: e80263. doi: 10.1371/journal.pone.0080263 PMID: 24282529

44. Park HJ, Ock SM, Kim HJ, Park HJ, Lee YB, Choi JM, et al. Vitamin C attenuates ERK signalling to inhibit the regulation of collagen production by LL-37 in human dermal fibroblasts. Exp Dermatol. 2010; 19: e258–64. doi: 10.1111/j.1600-0625.2010.01070.x PMID: 20163451

45. Guo S, Dipietro LA. Factors affecting wound healing. J Dent Res. 2010; 89: 219–29. doi: 10.1177/0022034509359125 PMID: 20139336

46. Park N-Y, Lim Y. Short term supplementation of dietary antioxidants selectively regulates the inflammatory responses during early cutaneous wound healing in diabetic mice. Nutr Metab (Lond). 2011; 8: 80. doi: 10.1186/1743-7075-8-80

47. Valero T. Mitochondrial biogenesis: pharmacological approaches. Curr Pharm Des. 2014; 20: 5507–9. Available: http://www.ncbi.nlm.nih.gov/pubmed/24606795. PMID: 24606795

48. Passage E, Norreel JC, Noack-Fraissignes P, Sanguedolce V, Pizant J, Thirion X, et al. Ascorbic acid treatment corrects the phenotype of a mouse model of Charcot-Marie-Tooth disease. Nat Med. 2004; 10: 396–401. doi: 10.1038/nm1023 PMID: 15034573

49. Lewis RA, McDermott MP, Herrmann DN, Hoke A, Clawson LL, Siskind C, et al. High-dosage ascorbic acid treatment in Charcot-Marie-Tooth disease type 1A: results of a randomized, double-masked, controlled trial. JAMA Neurol. 2013; 70: 981–7. doi: 10.1001/jamaneurol.2013.3178 PMID: 23797954

50. Villoslada P, Steinman L, Baranzini SE. Systems biology and its application to the understanding of neurological diseases. Ann Neurol. 2009; 65: 124–39. doi: 10.1002/ana.21634 PMID: 19260029