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Mutations in the collagen XII gene define a new form of extracellular matrix-related myopathy

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Bethlem myopathy (BM) [MIM 158810] is a slowly progressive muscle disease characterized by contractures and proximal weakness, which can be caused by mutations in one of the collagen VI genes (*COL6A1*, *COL6A2* and *COL6A3*). However, there may be additional causal genes to identify as in ~50% of BM cases no mutations in the COL6 genes are identified. In a cohort of ~24 patients with a BM-like phenotype, we first sequenced 12 candidate genes based on their function, including genes for known binding partners of collagen VI, and those enzymes involved in its correct post-translational modification, assembly and secretion. Proceeding to whole-exome sequencing (WES), we identified mutations in the *COL12A1* gene, a member of the FACIT collagens (fibril-associated collagens with interrupted triple helices) in five individuals from two families. Both families showed dominant inheritance with a clinical phenotype resembling classical BM. Family 1 had a single-base substitution that led to the replacement of one glycine residue in the triple-helical domain, breaking the Gly-X-Y repeating pattern, and Family 2 had a missense mutation, which created a mutant protein with an unpaired cysteine residue. Abnormality at the protein level was confirmed in both families by the intracellular retention of collagen XII in patient dermal fibroblasts. The mutation in Family 2 leads to the up-regulation of genes associated with the unfolded protein response (UPR) pathway and swollen, dysmorphic rough-ER. We conclude that the spectrum of causative genes in extracellular matrix (ECM)-related myopathies be extended to include *COL12A1*.

INTRODUCTION

Bethlem myopathy (BM) [MIM 158810] is a slowly progressive muscle disease characterized predominantly by contractures, rigidity of the spine, skin abnormalities and proximal weakness (1). The only genetic cause identified to date for BM is dominant, or more rarely recessive mutations (2,3) in one of the collagen VI genes (*COL6A1*, *COL6A2* and *COL6A3*). Mutations in these genes also cause Ullrich congenital muscular dystrophy (UCMD) [MIM 254090], a more severe, neonatal or early childhood onset phenotype, with significant weakness, frequent respiratory impairment, proximal joint contractures, distal joint hyperlaxity (4,5) and, recently in one family, a myosclerosis myopathy phenotype. Based on the northern region of England population, the estimated prevalence for BM is 0.77/100 000 and that for UCMD is 0.13/100 000 (6).

Despite its milder phenotype overall, BM may present in infancy with hypotonia, congenital contractures, hip dysplasia, club foot and torticollis (7). Other patients present in childhood with delayed motor milestones, contractures or evidence of mild weakness (7). In some patients, symptoms may go unnoticed until adolescence or adulthood. Distal laxity may be evident initially; however, all individuals eventually develop joint contractures including contractures of the long finger flexors, wrists, elbows, hips, knees and ankles (1,7–10). Scoliosis or rigid spine may be present (7). Skin abnormalities including keloid

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formation, 'cigarette paper scarring' and follicular hyperkeratosis are common (4). Some or all of these features may overlap with other conditions including Emery–Dreifuss muscular dystrophy, congenital muscular dystrophies, limb girdle muscular dystrophies, *FHL1*-related myopathies and some forms of Ehlers–Danlos syndrome (11). Serum creatine kinase (CK) levels in BM are usually normal to mildly elevated ($<5\times$). Electromyography testing and muscle histology is normally consistent with a myopathic process (1,8). Standard collagen VI immunohistochemistry is typically normal (10,12), but collagen VI expression in cultured skin fibroblasts is often abnormal, with a sensitivity of $>78\%$, a negative predictive value of $>83\%$ and a specificity of $>75\%$ (12). We, and others, have reported that not all patients with a phenotype resembling BM have mutations in one of the collagen VI genes suggesting genetic heterogeneity underlying the pathogenesis of these myopathies (5,12,13).

We have identified ~24 patients with a BM-like phenotype but no mutations in the collagen VI genes. In this study, we first excluded candidate genes related to collagen VI processing by Sanger sequencing and then identified causative mutations in *COL12A1* in two families by whole-exome sequencing (WES). Both families carried dominant mutations with a phenotype that overlaps BM but with a non-typical pattern of muscle involvement as determined by MRI. In one of the families, we implicate ER stress due to accumulation of collagen XII in the ER as part of the pathomechanism.

RESULTS

To identify novel causal variants in the panel of 24 BM-like patients, direct sequencing of other genes hypothesized as candidates for the non-collagen VI overlapping BM phenotype was analysed by Sanger sequencing ('candidate panel', Table 1). *BGN*, *DCN*, *PLOD1*, *PLOD2*, *PLOD3*, *P4HA1*, *P4HA2*, *P4HA3*, *P4HB* and *COL5A1* were negative for pathogenic variations. WES identified heterozygous mutations in *COL12A1* in two families, which co-segregated with disease in an autosomal dominant fashion. *COL12A1* was then screened by Sanger sequencing in the remaining members of the cohort, but no additional disease-causing mutations were found.

The five patients who were seen in our centre all presented in childhood with a history of generalized muscle weakness. Patient 1b showed first symptoms as a newborn with hypotonia, torticollis, kyphosis and distal hyperlaxity. All adult *COL12A1* mutant patients reported that their muscle strength improved during their teenage years. Patient 2a, who is the most severely affected patient of our cohort (Table 1, Figs 1 and 2), reported of poor sporting prowess at school, finishing last in races and an inability to squat, yet worked in a physically demanding role as a miner for >15 years. Muscle strength started to deteriorate again in his late 30s. His two sons also presented with general weakness in childhood but experienced improvement of symptoms during adolescence. They both developed profound atrophic scaring of the skin of their back, shoulders and neck (Fig. 1). The three oldest patients developed finger flexion contractures with a typical Bethlem sign (Fig. 1). T1-weighted axial muscle MR images of the leg muscles revealed severe atrophy of the rectus femoris muscle in Family 1 and selective involvement of the femoral quadriceps, the adductor and the medial gastrocnemius muscles in the most severely affected patient in Family 2 (Fig. 1), a pattern of involvement not in concordance with that seen in BM. In Family 1, a mutation was identified, which causes the substitution of a conserved glycine residue of the Gly-X-Y motif in the triple-helical domain; NM004370; c.G8357A: p.Gly2786Asp (Supplementary Material, Fig. S1). Mutations to glycine residues of the triple-helical domain are common in disorders of collagen, for example, triple-helical glycine mutations in the collagen VI genes have been shown to block its proper assembly and secretion (14). This variant is absent from both the NHLBI Exome Sequencing Project (EVS) (15) and 1000genomes databases (16) and causes the substitution of a non-polar neutral amino acid with a negatively charged acidic residue. In Family 2, the mutation NM004370 c.C5893T: p.Arg1965Cys was identified (Supplementary Material, Fig. S1B). Arg1965 is a conserved residue (Supplementary Material, Fig. S1D), with the substitution replacing a basic polar and positively charged residue for a non-polar neutral residue, which leads to an unpaired cysteine. This mutation is present at low frequency (0.016%) in the NHLBI Exome Sequencing Project (EVS) for the African American population, but not in the European American population, and since the phenotype can be mild and resolve with age, it is possible that this disease-causing variant will be annotated as a polymorphism in the variation databases.

Both variants were predicted to be highly damaging using Mutation Taster, SIFT and Polyphen2 *in silico* prediction tools (17–19).

To consider the effect of these mutations at the protein level, we performed immunofluorescence (IF) analysis of collagen XII in dermal fibroblast derived from ventral forearm skin biopsies. In normal cells, extracellular collagen XII was abundant and appeared as a linear, organized matrix (Fig. 3A); however, in both Patient 1b (Fig. 3B) and Patient 2a (Fig. 3C), collagen XII was much less abundant, disorganized and showed significant intracellular retention. The collagen 12 epitope could not be detected unless after cell permeabilization, indicating intracellular retention of collagen 12 in both cases. Collagen 6 IF in dermal fibroblasts did not show any abnormalities in both cases (Table 1), nor was there any abnormality in the collagen XII immunofluorescent staining pattern in a BM patient with a common mutation in *COL6A1* (Fig. 3G). Western blotting for collagen XII in the cell layer showed normal levels of collagen XII in fibroblasts from both Family 1 and Family 2 (H). To investigate whether other ECM components could be affected in the context of collagen XII mutation, we performed immunofluorescent staining in fibroblasts from Family 1 and Family 2 for tenascin-X and collagen IV, and both showed a subtle reduction in intensity in both patients, compared with the normal control (Supplementary Material, Fig. S2).

Mutations, which lead the protein to have an unpaired cysteine residue, can cause endoplasmic reticulum stress due to protein misfolding and aggregation in many autosomal dominant diseases including those caused by other collagens such as Ehlers–Danlos syndrome (20).

To investigate whether the p.Arg1965Cys mutation in Family 2 caused ER stress, ER morphology was analysed by transmission electron microscopy (TEM) of skin biopsy material. Patient 2a showed a clear dilation and swelling of the ER consistent with mutant protein accumulation and ER stress (Fig. 4B and

Table 1. Clinical data of 24 patients with BM-phenotype overlap

Patient	Sex	Onset and motor function ability		First symptoms	Age last seen (years)	Functional ability (last seen)	Main clinical features									Candidate genes analysed
		Early mobility	Age onset (years)				Contractures	Skin findings	Joint laxity	Cardiac involvement	Respiratory/FVC percent predicted	CPK	EMG	Muscle biopsy	Fibroblast collagen VI IF	
1a	F	Normal	Childhood	Poor at sports in childhood	42	Mild proximal weakness and neck	LFF	Normal	Joint hyperlaxity, hip dislocation	Normal	Normal	93	NR	NR	Normal	LMNA, 'CP'
1b	F	Normal	Birth	Generalized hypotonia as infant	6	Mild neck weakness, minimal proximal	Torticollis and kyphosis newborn LFF, elbow, knees	Hyperkeratosis pilaris	Hypotonic generalized hyperlaxity, left hip subluxation	Normal	Normal	747	NR	Myopathic, fibrosis, type 1 fibre grouping	Normal	'CP'
2a	M	NR	Childhood	Last in races, could not squat until 14 years	48	Unable to rise from chair, proximal weakness, scapular winging	LFF, TA, wrist flexion, knee	Hypertrophic scar	NR	Normal	Normal	680–973	Myopathic	Myopathic, fibrosis; laminin B1 reduced	Normal	LMNA DM2/PROMM, FSHD, 'CP'
2b	M	Normal	adolescence	Poor at sports and struggling with stairs	22	Proximal muscle weakness	Rigid spine	Hypertrophic and atrophic scaring	Normal	Normal	Normal	1310	NR	NR	Normal	None
2c	M	Normal	adolescence	Poor at sports and struggling with stairs, pectus excavatum	27	Proximal muscle weakness, trendelenburg gait	LFF, rigid spine	Hypertrophic and atrophic scaring	Normal	Normal	79%	824	NR	NR	Normal	None
3	M	Normal	5–6	Unable to keep up with peers; mild eye closure, neck flexion, and prox. > dist. Weakness	22	Facial weakness resolved; generalized weakness, scapula winging	LFF, TA, elbow, rigid spine	Possible atrophic scar	NR	Partial RBBB aortic root mildly dilated	64% sitting, 60% supine	500	NR	Myopathic, fibrosis	Normal	FHL1, LMNA, 'CP'
4	M	Normal	Childhood	Contractures	43	30s noticed decrease stamina; proximal weakness	LFF, TA, elbow, hip, rigid lumbar spine	Normal	NR	RBBB; pacemaker	NH; 53% sitting 39% supine	230	Mixed myopathic/neurogenic	Myopathic, prominent fatty replacement	Normal	LMNA, 'CP'
10	M	Normal	2	Unable to run, fell frequently, difficulty climbing; abnormal gait, right scapula winged	50	Proximal weakness	LFF, TA, elbow, jaw, neck	Psoriasis	Hypotonic infant	Normal	NH; 40% sitting, 24% supine	992	Myopathic	Myopathic, 50% fat replacement, fibrosis, occasional ringed and vacuolated fibres, patchy laminin B1 reduced	Normal	LMNA, FKRP, 'CP'
13	F	Delayed walking	Birth	Childhood mobility difficulties	49	Mild facial and proximal weakness	LFF, birth foot abnormality, elbow	NR	Hypotonic infant	Normal	Normal	Normal	NR	Myopathic, fibrosis	Normal	LMNA, FSHD, 'CP'
16	F	Walked age 2 years	Birth	Poor anti-gravity birth, but not 'floppy'	5	2 years: walks with walking frame	LFF	Normal	Elbows and knees	Normal	NH	67	NR	Myopathic, type 1 fibre predominance	Normal	SMN, 'CP'
17a	M	Normal	Childhood	4 years: toe walking; 23 years: proximal weakness	60	53 years: wheelchair dependent; Firm woody consistency of all muscles; generalized weakness	LFF, TA, elbows, hamstring, knees, hips, rigid spine	Generalized lipodystrophy	NR	Normal	NH; 23% sitting, 18% supine	1301	Inconclusive	Myopathic, myosclerosis	Normal	LMNA, 'CP'
17b	F	Normal	2	Falls with difficulty getting off floor; 5 years: difficulty getting up stairs	47	Generalized wasting; ambulant short distance, unable to rise from floor	LFF, TA, elbows	Hyperkeratosis pilaris, easy bruising, pinched nose	NR	Normal	NH; 50% sitting, 30% supine	395	Myopathic	Myopathic, fibrosis	Normal	FHL1, 'CP'
17c	F	Normal	40	Difficulty running and holding objects above head	57	Struggles getting up from floor; symmetrical limb weakness	LFF, elbows, shoulders, Hips, hamstrings, neck	Slow healing wounds, easy bruising	Recurrent jaw dislocations	NR	Normal	1891	Myopathic	Myopathic	Normal	'CP'

Continued

Table 1. *Continued*

Patient	Sex	Onset and motor function ability		First symptoms	Age last seen (years)	Functional ability (last seen)	Main clinical features									Candidate genes analysed
		Early mobility	Age onset (years)				Contractures	Skin findings	Joint laxity	Cardiac involvement	Respiratory/FVC percent predicted	CPK	EMG	Muscle biopsy	Fibroblast collagen VI IF	
7a	F	Normal	3	Started falling	43	Neck flexion and upper limb weakness, calf hypertrophy	LFF, TA, elbow	Prominent scar	Recurrent patellar dislocation	Normal	Normal	589	Myopathic	Myopathic	Normal	LMNA,
7b	M	Normal	50	Difficulty lifting arms above head	71	Difficulty walking, neck weakness, easy fatigue; Calf hypertrophy	LFF, TA, elbow	Prominent scar	NR	Normal	NH; 56% sitting, 44% supine	800–2654	Myopathic	Myopathic, fibrosis, type 1 fibre predominance	Abnormal	LMNA, 'CP'
5	F	Normal	4	Frequent falls, struggled with stairs, unable to jump	30	Generalized weakness, wheelchair	LFF, TA, elbow, wrist extension	Hypertrophic scar hyperkeratosis pilaris	NR	2:1 AV conduction block; pacemaker	57% sitting, 43% supine	823	NR	NR	Abnormal	LMNA 'CP'
7	F	Normal	School age	Difficulty running, difficulty climbing	38	Proximal, neck flexion weakness, scapular winging; calf hypertrophy	LFF, TA, elbow	Hypertrophic scar hyperkeratosis pilaris	NR	Normal	Normal	253–500	Myopathic	NR	Abnormal	'CP'
9	M	Normal	50	Scapular winging, proximal weakness. Calf hypertrophy	60	Neck flexion/ extension and proximal weakness	LFF, wrist, rigid lumbar spine	Hyperkeratosis pilaris, Palmar surface thick skin	NR	Normal	Normal	1110	NR	Myopathic	Abnormal	FKRP, CAPN3, 'CP'
11	F	NR	6	Could not keep up with peers, difficulty getting up from floor	29	Proximal weakness, scapular winging; wasting of upper arms and shoulders	LFF, TA, elbows, wrists, knees, rigid spine	keloid	NR	Normal	59% sitting, 48% supine	588	Myopathic	Myopathic, type 2 fibre predominance, fibrosis; laminin B1 reduced	Abnormal	LMNA, 'CP'
12	M	NR	Unknown	56 years: right > left winging, minimal shoulder weakness	62	NR	LFF, elbows, forearm supination, wrists, neck	Hardened, leathery like	NR	Normal	Normal	214	Myopathic	Myopathic, laminin B1 reduced	Abnormal	LMNA, 'CP'
15	F	Delayed motor development	6–8 month	Difficulty getting up stairs and from floor	59	50 years: wheelchair for outdoor use; proximal weakness	LFF, TA, elbows	Hyperkeratosis pilaris	NR	NR	NH; 29% sitting, 23% supine	52	Myopathic	NR	Abnormal	LMNA, 'CP'
19	F	Walked age 18 months	Childhood	Difficulty walking; 10 years: difficulty rising floor and stairs	53	Generalized weakness; 50s: wheelchair for outdoor use	LFF, TA, hips	Prominent scar, hyperkeratosis pilaris	Hypotonic infant CHD	Normal	58% sitting, 50% supine	Elevated	Mixed myopathic/ neurogenic	Fibrosis	Abnormal	LMNA, FKRP 'CP'
20	F	Walked age 19 months	1 year	Fell frequently, poor at sports	19	12 years: calves wasted, neck flexion/extension weakness, scapular winging, generalized weakness	LFF, TA, elbows, wrists, shoulders, hips, spine, scoliosis	Atrophic scar, hyperkeratosis pilaris	CHD, generalized hypotonia	Normal	58% sitting, 52% supine	654	NR	Myopathic, fibrosis	Abnormal	LMNA, SEPN1 'CP'
21	M	Walked age 2 years	Birth	Unable to keep up with peers	41	Neck flexor, proximal weakness	Birth torticollis, LFF, TA, elbows, hips, knees, rigid spine	Hyperkeratosis pilaris	CHD	NR	59% sitting, 41% supine	340	NR	Myopathic, fibrosis, type 1 fiber predominance	Abnormal	LMNA, 'CP'

Onset and motor function ability of 24 patients with BM-phenotype overlap but without mutations in the *COL6A* genes.
NR, not recorded; UL, upper limb; AV, atrioventricular; CHD, congential hip dysplasia; LFF, long finger flexion; NH, nocturnal hypoventilation; NR, not recorded; RBBB, right bundle branch block; TA, tendon achilles; CP, candidate panel.

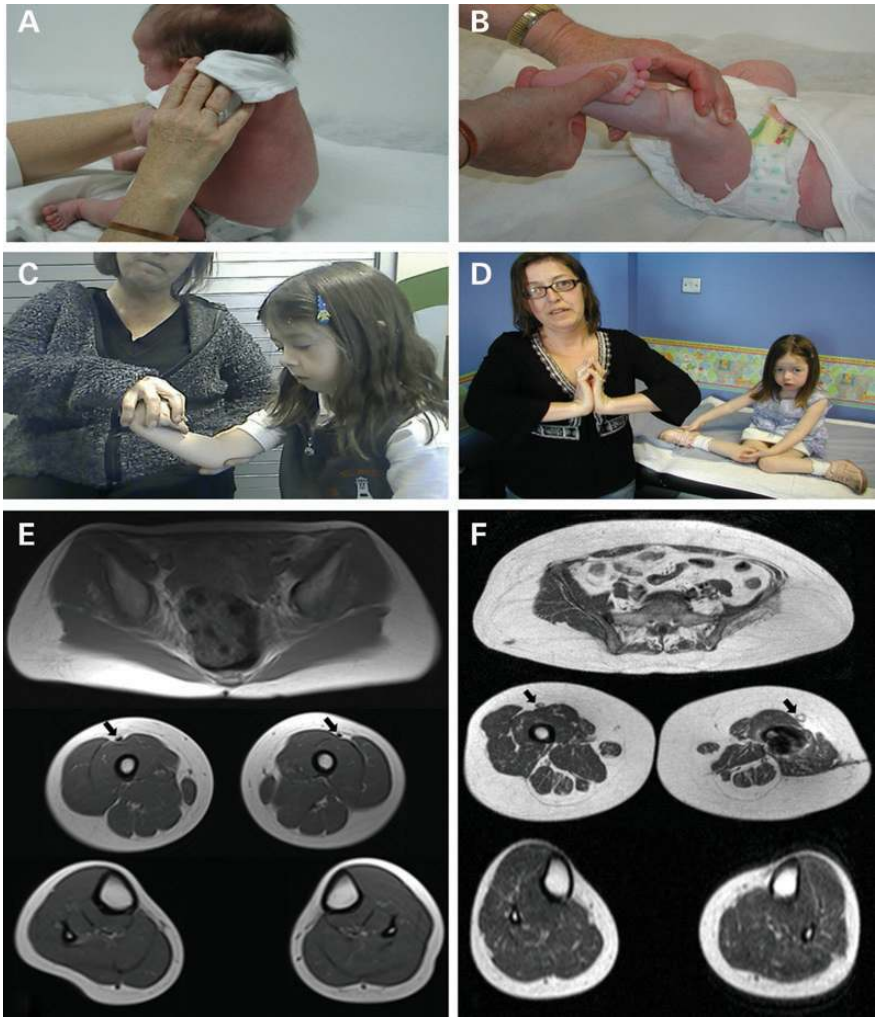

Figure 1. Patient 1b presented as a newborn with kyphosis (**A**) and distal hyperlaxity (**B**), which continued through childhood (**C**). Her mother (Patient 1a) displayed finger flexion contractures (**D**). T1-weighted axial MR images of the leg muscles showed profound atrophy of the rectus femoris muscles (black arrows) in Patient 1b (**E**) and Patient 1a (**F**), who also shows atrophy of the left hip muscles caused by a left hip replacement. There were no other specific signs of pathology.

C) compared with normal control in which the ER appeared to be smaller and more linear (Fig. 4A). Dilated ER was also apparent on TEM of affected individuals from the same family (Patients 2b and 2c; data not shown). To consider whether accumulation of misfolded collagen XII protein in ER caused the activation of the unfolded protein response (UPR) pathway, the expression of 84 key UPR genes were analysed by qRT–PCR. Figure 4D shows that for Patient 2a, the most severely affected member of Family 2, there was a significant and general up-regulation across the panel of 84 UPR genes, implying that the UPR pathway was strongly activated in this individual. The sons of this patient, 2b and 2c, also showed a clear up-regulation of UPR pathway genes, but to a lesser extent. Using fibroblast-derived RNA, the expression of three key genes of the UPR pathway were expressed relative to the normal control and compared between Family 1 and Family 2. CHOP (4E) and PERK (4F) showed significant ($P < 0.05$) up-regulation, whereas GRP78/BiP (4G) showed a trend towards up-regulation in Family 2 bearing the Arg1965Cys mutation, but not in Family 1, consistent with our hypothesis that Gly2786Asp does not invoke the UPR pathway.

DISCUSSION

Genetic heterogeneity in our cohort with a clinical phenotype resembling BM appeared to be ∼50%, but additional causal loci had not been identified. We first sought additional causal genes in our group by a hypothesis-driven candidate gene approach, which focussed on those genes involved in collagen VI processing. In our cohort, 10 of the 24 patients had abnormal collagen VI immunofluorescent staining in dermal fibroblasts. In these patients with a secondary dysfunction of collagen VI, it can be hypothesized that the binding partners of collagen VI, biglycan (21) and decorin (22), which regulate proper attachment of collagen VI to the basal lamina, could be candidates for causing disease, but both were negative. The post-translational modification enzymes of the lysyl hydroxylase family of enzymes (*PLOD1, PLOD2* and *PLOD3*) and the prolyl 4-hydroxylase family (P4HA1, P4HA2, P4HA3 and P4HB) were also negative for mutations. Due to phenotypic overlap with Ehlers–Danlos syndrome (23,24), COL5A1 mutations were also ruled out. By applying WES, we identified mutations in the *COL12A1* gene in two families with a myopathy

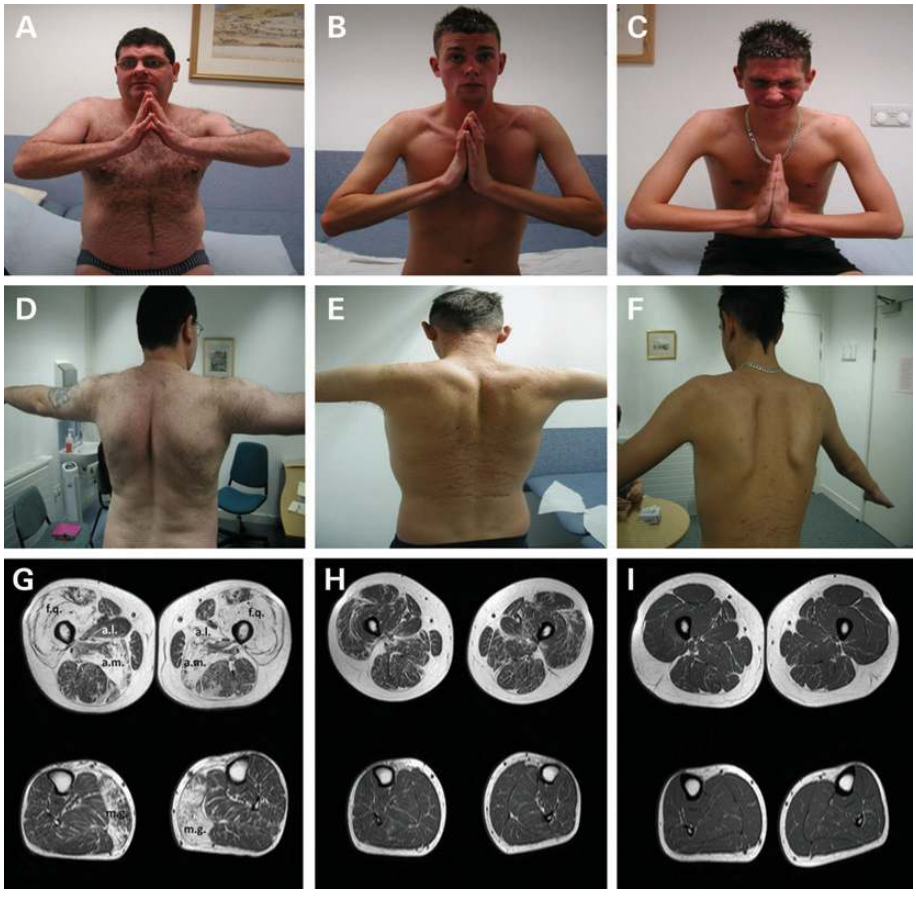

Figure 2. The two eldest patients in Family 2 [Patient 2a (**A**) and Patient 2b (**B**)] displayed finger flexion contractures, whereas in the youngest and most mildly affected patient, no finger flexion contractures were apparent (**C**). Scapular winging and prominent atrophic scarring of the skin was evident in Family 2 [Patient 2a (**D**), Patient 2b (**E**) and Patient 2c (**F**)]. In Family 2, the most severely affected patient (2a; **G**) showed prominent involvement of the femoral quadriceps (f.q.), adductor magnus (a.m.) and medial gastrocnemius (m.g.) muscles and asymmetric involvement of the adductor longus (a.l.) muscles. Muscle pathology in his oldest son (2b; **H**) was less pronounced and the muscles of the youngest son looked normal (2c; **I**).

which closely resembles, but is distinct from, classical BM. Patients presented with symptoms of distal hyperlaxity, muscle weakness, skin changes and joint contractures. Another similarity was the striking improvement in clinical course, which has also been described in collagen VI-associated BM (25). Despite shared clinical features in our cohort, there is clearly a high degree of genetic heterogeneity since only two families on our cohort were found to have collagen XII-myopathy, and further novel genes remain to be discovered. While we report here two families in which collagen 12 mutations are acting in a dominant negative fashion, in work published alongside this manuscript in the same issue of this journal (46) also demonstrate a null, recessive, mechanism in human and mouse.

Despite the phenotypic overlap, in our study, we were able to make a distinction between the currently reported patients with collagen XII mutations and collagen VI mutations by muscle MRI. MRI has emerged as an increasingly valuable diagnostic investigation for patients with suspected collagen VI-related disease, especially in the milder cases where diagnostic signs can be subtle (5,26–28). Whereas in patients with BM, the periphery of the muscles is more affected than the central part, and in the calf, one of the first signs is often a 'rim' of fatty infiltration between the soleus and gastrocnemius muscles; the only prominent finding on T1-weighted MR images in Family 1 was atrophy of the rectus femoris muscles. In Family 2, the pattern of muscle involvement correlated with disease severity, with the most severely affected individual, Patient 2a, showing a more pronounced pathology on MRI compared with his milder sons, 2b and 2c.

Collagen XII is a member of the FACIT (Fibril Associated Collagens with Interrupted Triple helices) group of ECM proteins and exists as homotrimers of alpha (XII) chains, which undergo a complex biosynthesis and assembly process both intra- and extra-cellularly. The FACIT collagens appear to function as regulators of fibrillar scaffolds by providing specific molecular bridges between fibrils and other ECM components. In the case of collagen XII, several lines of evidence have indicated that it could function as a regulator of collagen I fibrils, such as preferential co-expression of collagen XII and collagen I in chick embryos, the localization of collagen XII to the surface of collagen I fibrils by electron microscopy and their biochemical co-precipitation (29). Collagen XII modulates ECM deformability in human fibroblast cells, and biomechanical stress in murine osteoblastic MC3T3-E1 cells by stretch induces collagen XII gene expression, suggesting collagen XII

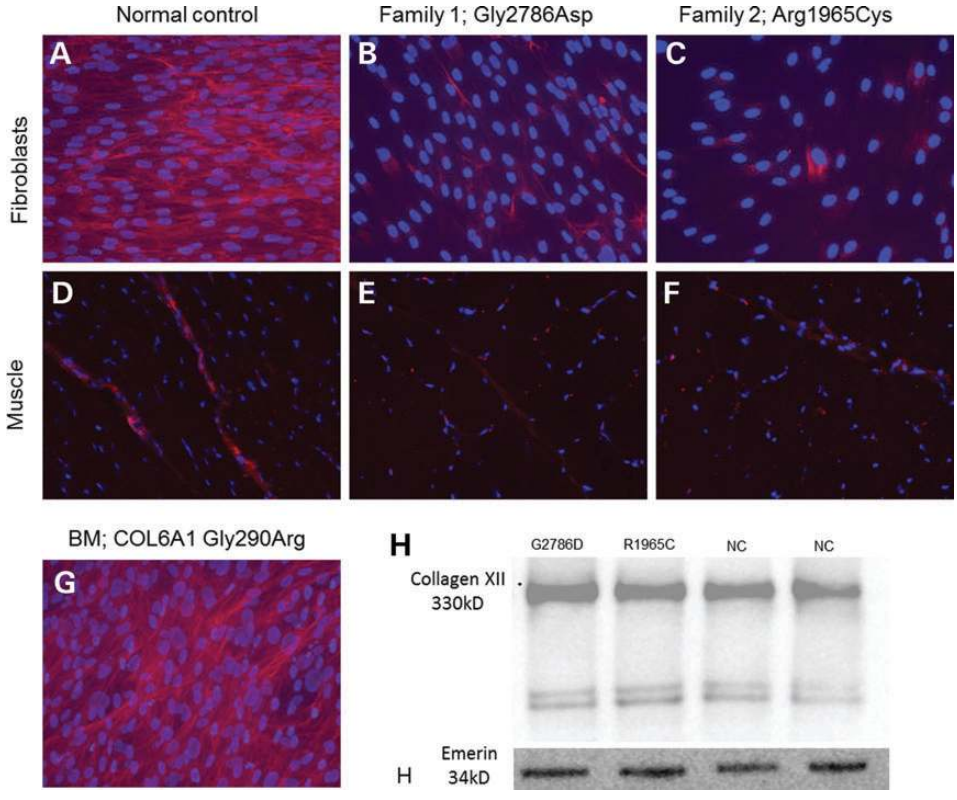

Figure 3. Dermal fibroblast from Patient 1b (**B**) and Patient 2a (**C**) showed significant intracellular retention of collagen 12, compared with normal control fibroblasts (**A**), which showed abundant, linear and matrical organization of extracellular collagen 12. Immunofluorescence analysis of muscle biopsy sections revealed collagen 12 labelling of the perimysium with very weak staining around the muscle fibre (endomysium) in normal muscle (**D**). Collagen 12 labelling was weaker at the fascia surrounding the muscle fibre bundle in both Patient 1a (**E**) and Patient 2a (**F**). In a BM patient with a COL6A1 Gly290Arg mutation, collagen XII is indistinguishable from the normal control (**G**). Images taken at ×20 magnification. Immunoblot for collagen XII from lysed human fibroblasts (**H**): Family 1, Patient 2a (lane 1; Gly2786Arg); Family 2, Patient 2a (lane 2; Arg1965Cys) and in normal fibroblasts (lanes 3 and 4). Emerin was used as loading control.

can modulate the biomechanical properties of ECM (30,31). It is intriguing to speculate whether the phenotypic overlap seen in BM, which is caused by mutations in collagen VI, and in collagen XII-myopathy could be as a result of modulation of the biomechanical properties of collagen VI fibrils through their shared binding partner, the small proteoglycan decorin (32,33), although organization of the collagen VI matrix was not grossly abnormal in dermal fibroblasts from these patients, nor the collagen XII labelling pattern in a BM patient with a mutation in COL6A1. Interestingly, collagen XII also binds the large, ECM protein tenascin-X, which is responsible for Ehlers–Danlos syndrome, a connective-tissue disorder consisting of skin and joint hyperextensibility with phenotypic overlap with the collagen VI-related diseases (34). We observed a subtle reduction in tenascin-X and collagen IV by immunofluorescence in collagen XII-myopathy patient fibroblasts versus normal control; however, the pathomechanistic and diagnostic relevance of this remains difficult to interpret.

In Family 1, a mutation was identified, which causes the substitution of a conserved glycine residue of the Gly-X-Y motif in the triple-helical domain; NM004370; c.G8357A: p.Gly2786Asp. The heterozygous substitution of conserved glycine residues in collagen molecules is a common mutation mechanism in BM and other disorders of collagen (14,35,36). When a dominant negative effect is exerted, the mutated collagen alpha chains will participate in trimer assembly but subsequent folding of the triple-helical domain will be impaired. Most deleterious are those missense changes that introduce residues with bulkier side chains, as was the case in Family 1 with the replacement of glycine with aspartic acid (37). The effect of the triple-helical domain misfolding is further augmented by excessive post-translational modification, since the posttranslational reactions are normally terminated by folding of the protein into the triple-helical conformation (37).

In Family 2, the mutation NM004370 c.C5893T: p.Arg1965Cys was identified. The introduced cysteine is located in the FN3–15 domain, whereas the other cysteines either flank the four von Willebrand Factor A domains or are located at the C-terminal where the homotrimer assembly is initiated. The unpaired cysteine is in the middle of the unassembled branches and may change the homotrimer conformation. Unpaired cysteine residues are a substructure that triggers the recognition of misfolded or mutated proteins, along with exposed hydrophobic regions and immature glycans. When folding or assembly intermediates expose, unpaired cysteines, ER resident chaperones or oxidoreductases interact with them, and as a consequence, they are retained in the ER or retrieved from the Golgi, a process concomitant with activation of the UPR pathway (38,39). The precise mechanism for this retention is unknown, but a possible explanation is that the unpaired sulfhydryl group interacts with other components of the ER chaperone, thereby preventing efficient trafficking. Significant up-regulation of the UPR pathway in Family 2 suggested indeed that this cellular

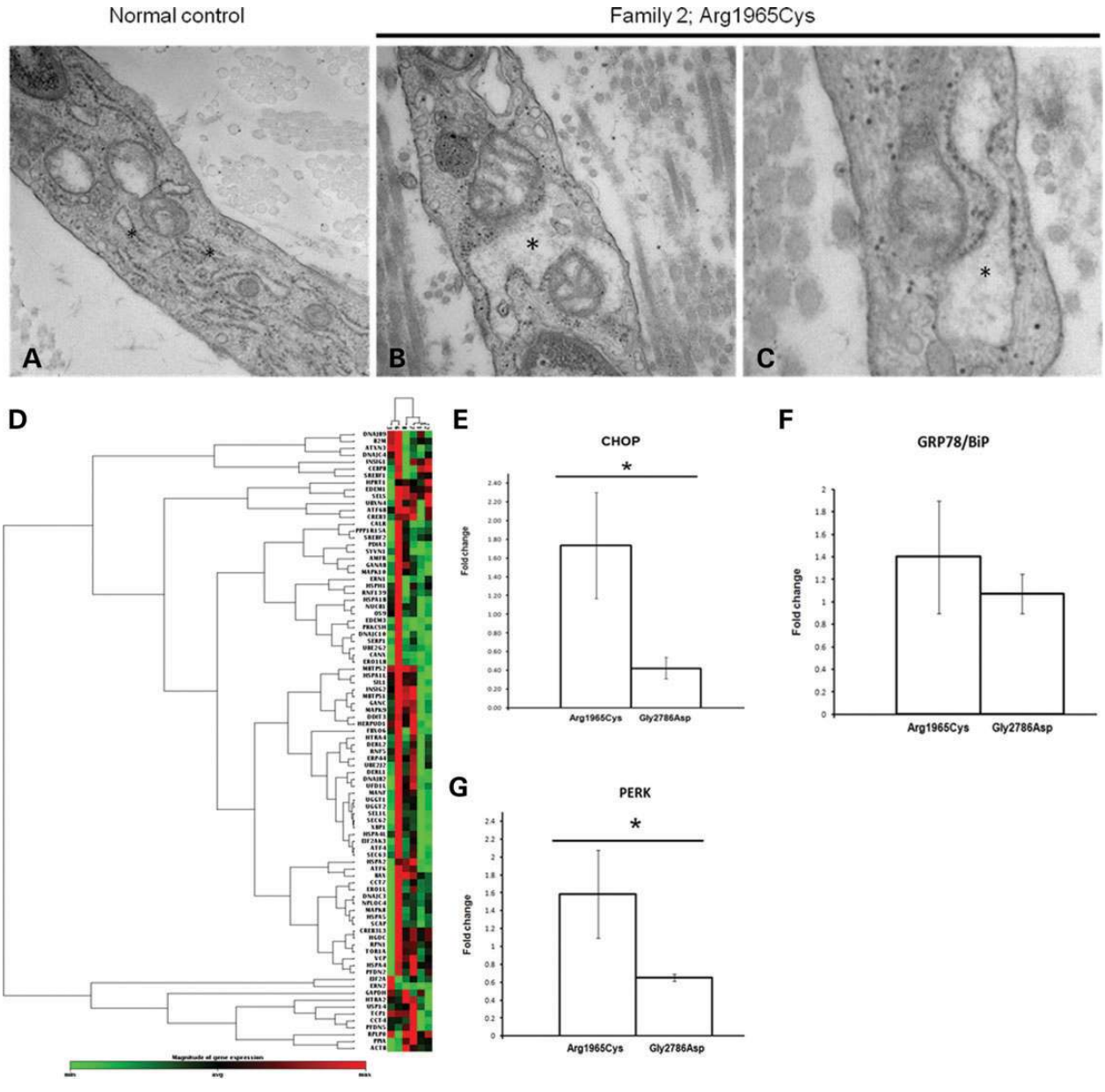

Figure 4. Transmission electron microscopy of skin biopsy sections showed that Patient 2a had grossly swollen and dilated endoplasmic reticulum (**B** and **C**) compared with a healthy individual, in which the ER appeared to be thinner and with a tubular appearance (**A**). The ER is highlighted by an asterisk. (**D**) A clustergram depicting the relative expression levels of 84 key genes of the UPR pathway when comparing mRNA from normal control fibroblasts (1–3) versus Family 2 (6–8) shows significant up-regulation for Patient 2a (lane 6) and moderate up-regulation in this pathway for his mildly affected sons, 2b (lane 7) and 2c (lane 8). The fold change in expression of three key UPR pathway genes (CHOP; **E**, GRP78/BiP; **F** and PERK; **G**) normalized to NHDFc are shown for Family 2 and Family 1. The *P*-values are calculated based on a Student's *t*-test, $^{*}P < 0.05$.

pathology was acting in this family. Immature proteins may also form aggregates that are excluded from vesicles exiting from the ER (40), and this aggregation can lead to dilated, swollen ER, which we have confirmed in Family 2 by TEM. Constitutive activation of the UPR pathway due to constant supply of misfolded proteins due to genetic mutation leads to chronic ER stress, which in turn induces the cell to activate apoptosis (41). Both Family 1 and Family 2 have mutations, which result in an absence or non-assembly of collagen XII in the ECM. Both also showed improvement in clinical course from childhood to adulthood. However, Individual 2a, the oldest patient in either family has latterly displayed disease progression and is now heavily reliant on walking aids. It is interesting to speculate whether the additional effect of this pro-apoptotic pathomechanism beyond the absence of collagen XII protein is the cause of this deterioration. Further work and close clinical follow-up will be needed to delineate the precise pathomechanisms acting in these families.

This study highlights the ability of next-generation sequencing approaches, in this case WES, to identify causal mutations in new disease genes. Our patient cohorts, and the distinctions between them, form a valuable resource for the identification of as yet known disease-causing genes. It is hoped that the identification

of other proteins involved in these overlapping phenotypes will extend our understanding of the pathophysiology of this group of diseases and provide us with more logical ways to develop treatment. In the longer term, understanding all of the mechanisms that can produce a similar phenotype may indicate novel pathways in the development of pathology and thereby indicate novel targets for the development of therapeutic interventions. The treatment potential for these diseases can only be assessed with complete knowledge of the underlying genetic causes.

MATERIALS AND METHODS

Patients and methods

A database of undiagnosed patients maintained by the Northern Genetics Service, in Newcastle upon Tyne, UK, was screened for patients with a BM-like diagnosis as classified by a group of experienced neuromuscular-trained clinicians (KB, VS, HL and AS). A total of 24 patients were identified with features overlapping classical BM but without mutations in *COL6A1, COL6A2* and *COL6A3* (Table 1). The overlapping features included multiple joint contractures (including long finger flexion contractures), joint laxity, muscle weakness/motor difficulties, presence of the characteristic skin abnormalities, mildly elevated CK and myopathic findings on muscle biopsy and EMG studies. A retrospective case review was done focusing on their clinical history, exam and diagnostic evaluations. This study was approved by the local ethics committee, and all subjects gave the appropriate permissions and consents.

Genetic analysis

We performed WES in five members of the cohort that were selected as those most overlapping with classical BM in terms of clinical features and represented the most homogeneous subset of the larger cohort. An additional selection criterion was the availability of parental DNAs. Genomic DNA was fragmented to ~150 bp using adaptive focused acoustics (Covaris, Illumina Paired-End Sample Preparation kit). Agilent SureSelect Human All Exon Kit v2 was used in conjunction with the SureSelect Target Enrichment System for Illumina Paired-End Sequencing Library (v2.0.1). An Illumina Genome Analyser IIx was used to sequence the captured fragments, with an output of 75-bp paired-end reads. Alternatively, genomic DNA was subjected to size-selection and coding regions purified using the SureSelect system before sequencing on an Illumina HiSeq platform using the TruSeq system (Eurofins). Raw sequencing reads were aligned to the consensus genome (hg19), sorted and converted to a BAM file using Mosaik [version 1.1.21; http://bioinformatics.bc.edu/marthlab/Mosaik (9 May 2012, date last accessed)]. The BAM file was indexed and variants called using SAMtools (42). The alignments were optimized for indel calling and indels called using dindel (43). The resulting list of variants were visualized and assessed using the UCSC Genome Browser (44). Putative disease-causing variants were verified and segregated where possible by PCR amplification and Sanger sequencing. Genomic DNA was extracted from blood samples by automated DNA extraction on the M48 BioRobot using the MagAttract DNA blood Mini M48 kit (Qiagen 951336) as part of the routine service performed by the Northern Region Genetics Service molecular laboratory and amplified using Moltaq PCR kit (Molzym P-010-1000). *COL6A1*, *COL6A2*, *COL6A3*, *BGN*, *DCN*, *PLOD1*, *PLOD2*, *PLOD3*, *P4HA1*, *P4HA2*, *P4HA3*, *P4HB* and *COL5A1* were screened for gene mutations at the RNA and DNA level as previously described (12). Primer sequences are available on request. All sequencing was performed using bi-directional fluorescent sequencing on an ABI 3730 XL 96 capillary sequencer, with BigDye Version 3.1 chemistry.

Muscle and skin biopsies

With appropriate consent, skin biopsies were taken from the ventral forearm, digested with collagenase and fibroblasts cultured in Ham's F-10 (Gibco 22390) supplemented with 20% heat inactivated foetal bovine serum (PAA Laboratories A11-043), fungizone (Gibco 15290-018), penicillin–streptomycin (Gibco 15070-063) and L-glutamine (Gibco 25030-032). Muscle biopsies snap-frozen in isopentane cooled in liquid nitrogen were mounted in OCT (R. A. Lamb LAMB/OCT) and cut into 6 μm sections and mounted onto SuperFrost® Plus slides. Slides were wrapped in cling film and stored at −80°C prior to immunolabelling. Muscle histology was assessed by H&E staining. All muscle biopsies were processed for immunohistochemistry and multiplex western blot. Immunostaining of unfixed frozen tissue for both procedures was performed using antibodies relating to diagnosis of LGMD as previously described (45).

Fibroblast culture

Fibroblasts derived from skin biopsies were grown in Dulbecco's modified Eagle's medium (Sigma D6429) supplemented with 10% foetal bovine serum (Sigma F7524), 1% penicillin–streptomycin (Sigma P0781) in 5% CO_2 at 37°C. Fibroblasts were seeded onto uncoated BD Biosciences glass 8-well culture slides (VWR 53106-306) and grown to confluency, upon which the medium was changed to include 50 μg/ml L-ascorbic acid phosphate (Sigma A4544). Normal human dermal fibroblast cells derived from foreskin (NHDFc Promocell C-12300) were used as a control.

Immunofluorescence of dermal fibroblast cultures and muscle sections

Skin biopsies were performed on patients with BM and on patients with overlapping features as part of their diagnostic evaluation. Fibroblast culturing and immunohistochemistry analysis was undertaken as previously reported (12), using a rabbit polyclonal to collagen XII (KR75; gift from Manual Koch, Cologne; 1:3000) with an Alexa 594-conjugated goat anti-rabbit secondary antibody (Molecular probes, A-11037; 1:1000). For immunolabelling of collagen IV (1:70, raised in rat. Gift from Dr Tom van Agtmael) and tenascin-X (1:100, raised in guinea pig. Gift from Manuel Koch), the protocol was as above apart from fixation of cells for 10 min in ice-cold methanol, and an antigen retrieval step by treatment with 0.05 m HCl/0.05 M KCl. Muscle sections were fixed in ice-cold methanol for 5 min, before blocking in 5% goat serum in PBS for 1 h. KR75 (1:1000) was applied overnight at 4°C. Alexa 594-conjugated goat anti-rabbit secondary antibody (Life Technologies; A-11037, 1:1000) was applied at room temperature for 90 min, before mounting in Vectorshield with DAPI mounting media (Vectorlabs; H-1200).

Western blotting

Proteins were extracted from confluent 6-well plate (BD Biosciences) after incubation of cells for 24 h in serum-free media. Protein extracts from cell layer were prepared using RIPA buffer containing Triton X-100 (Sigma) and protease inhibitor cocktail (Roche). Cell lysates were centrifuged at 1400 rpm for 15 and 2 μl of each supernatant combined with 5 μl 4× LDS loading buffer (Invitrogen) containing 10% beta mercaptoethanol (Sigma) and were heated for 5 min at 90°C before loading onto a 3–8% Tris–acetate gel (Invitrogen). Membranes were blocked with 5% BSA (Sigma–Aldrich) for 1 h before incubation with primary antibodies; anti-human collagen XII (gift of Prof. Manuel Koch, 1:2000) and anti-Emerin (Novocastra 6120-07, 1:50) overnight. Membranes were incubated with secondary antibodies, goat anti-rabbit, HRP conjugated (Invitrogen G21234, 1:3000) and goat anti-mouse, HRP conjugated (Invitrogen G21040, 1:3000) for 1 h. Imaging was performed on a Bio-spectrum imaging analyser device (UVP, Upland, CA, USA) using Vision Works v7.0.2.

Electron microscopy

Tissue was rinsed in 0.1 M sodium cacodylate buffer, post-fixed in 1% OsO_4(Agar Scientific) for 1 h and dehydrated in sequential steps of acetone (25, 50, 75 and 100% twice) prior to impregnation in resin (TAAB Lab Equipment) and polymerized at 60°C for 24 h. Semi-thin survey sections of 1 μm were cut and stained with 1% toluidine blue in 1% borax. Ultrathin sections of 70 nm were cut using a diamond knife on a Leica EM UC7 ultramicrotome. Sections were mounted on Pioloform-filmed copper grids prior to staining with 1% aqueous uranyl acetate and lead citrate (Leica) and viewed on a Philips CM100 Compustage Transmission Electron Microscope. Images were taken at a magnification of ×46 000.

Unfolded protein response pathway PCR array

RNA was extracted from dermal fibroblast cultures using the Qiagen RNeasy Micro Kit (74004). cDNA was synthesized using the Qiagen RT^2 First Strand Kit (330401). qRT–PCR was performed by a technical service offered by Qiagen using Human Unfolded Protein Response PCR Array (PAHS-089Z), which includes 84 UPR pathway relevant genes, plus 5 housekeeping genes (*B2M*, *HPRT1*, *PRL13A*, *GAPDH* and *ACTB*) and 3 RNA and PCR quality controls. Three biological replicates of normal human dermal fibroblast cells (NHDFc Promocell C-12300) were used as a control. The fold change is calculated based on the replicate $2^{-\Delta C_t}$ values for each gene in the control group and treatment groups. *P*-values are calculated based on a Student's *t*-test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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