Monozygotic twins discordant for recessive dystrophic epidermolysis bullosa phenotype highlight the role of TGF-β signalling in modifying disease severity

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Recessive dystrophic epidermolysis bullosa (RDEB) is a genodermatosis characterized by fragile skin forming blisters that heal invariably with scars. It is due to mutations in the COL7A1 gene encoding type VII collagen, the major component of anchoring fibrils connecting the cutaneous basement membrane to the dermis. Identical COL7A1 mutations often result in inter- and intra-familial disease variability, suggesting that additional modifiers contribute to RDEB course. Here, we studied a monozygotic twin pair with RDEB presenting markedly different phenotypic manifestations, while expressing similar amounts of collagen VII. Genome-wide expression analysis in twins’ fibroblasts showed differential expression of genes associated with TGF-β pathway inhibition. In particular, decorin, a skin matrix component with anti-fibrotic properties, was found to be more expressed in the less affected twin. Accordingly, fibroblasts from the more affected sibling manifested a profibrotic and contractile phenotype characterized by enhanced α-smooth muscle actin and plasminogen activator inhibitor 1 expression, collagen I release and collagen lattice contraction. These cells also produced increased amounts of proinflammatory cytokines interleukin 6 and monocyte chemoattractant protein-1. Both TGF-β canonical (Smads) and non-canonical (MAPKs) pathways were basally more activated in the fibroblasts of the more affected twin. The profibrotic behaviour of these fibroblasts was suppressed by decorin delivery to cells. Our data show that the amount of type VII collagen is not the only determinant of RDEB clinical severity, and indicate an involvement of TGF-β pathways in modulating disease variability. Moreover, our findings identify decorin as a possible anti-fibrotic/inflammatory agent for RDEB therapeutic intervention.

INTRODUCTION

Dystrophic epidermolysis bullosa (DEB) is a group of inherited skin diseases caused by mutations in the gene COL7A1 (OMIM 120120) encoding for type VII collagen (C7), which forms anchoring fibrils that ensure adhesion of the basement membrane zone (BMZ) to the upper dermis in stratified epithelia. Patients affected by DEB have reduced or absent C7 expression in the skin and show abnormal or absent anchoring fibrils resulting in tissue separation below the lamina densa of the BMZ.

DEB can be inherited either as autosomal dominant (DDEB) or recessive (RDEB) trait. The clinical spectrum is extremely broad, ranging from localized acral blistering or even nail dystrophy only to generalized lesions affecting skin and mucous

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membranes lined by stratified epithelia. In particular, the severe
generalized RDEB variant (RDEB-sev gen) is characterized by
early development of pseudosyndactyly and mitten deformities
of hands and feet, constant severe oral and oesophageal involve-
ment, and high risk of developing squamous cell carcinomas
(SCC) (>90% by age 55 years) (1,2). Between these two extrem-
ities, a range of other phenotypes are recognized in which skin
blistering and scarring can be generalized or predominantly
localized to extremities, but are milder than in RDEB-sev-gen,
do not lead to pseudosyndactyly, and mucosal involvement is
less frequent and severe (3).
Correlation between the type of mutation and the specific fea-
tures of disease condition indicates that premature termination
codons (PTCs) in both alleles of COL7A1 usually result in lack of
C7 expression and absence of anchoring fibrils which lead to
the RDEB-sev gen subtype. On the other hand, compound het-
erozygosity for a missense or a splice-site mutation in at least one
allele allows the synthesis of reduced levels of mutant C7 and
leads to the spectrum of milder RDEB forms.
Although mutations in the COL7A1 gene are the major deter-
minants of DEB clinical symptoms, it has been shown that the
same genetic alterations in COL7A1 can be associated with
highly variable clinical signs in siblings affected with RDEB
(4,5). The heterogeneous phenotypes observed in unrelated
patients carrying the same mutations also support this hypothesis
(6,7). It has been proposed that a single nucleotide polymorph-
ism (SNP) in the promoter of the MMP1 gene, which codes for
the matrix metalloproteinase 1, is a modifier of clinical severity
in RDEB (8). Although MMP1 SNP was shown to influence
COL7A1 gene expression, the correlation between the high-risk
allele and disease severity was not confirmed in subsequent
studies (9,10). These findings suggest that MMP1 SNP is not the
only disease modifier in RDEB and that other modifying
factors, not directly related to the abundance of C7, may influ-
ence disease manifestations.
Downstream consequences of the COL7A1 mutations have the
greatest impact on disease morbidity and mortality. Although
skin fragility in RDEB is caused directly by disruption of C7, the
major disease burden results from ulcer formation and unremit-
ting attempts to tissue repair, in which the normal processes of
granulation tissue formation, re-epithelialization and extracellu-
lar matrix (ECM) remodelling are compromised. This in turn
leads to delayed wound healing accompanied by chronic inflam-
mation, fibrosis, scarring and possibly increased risk of develop-
ing SCC (2,11–13). Of note, individuals with RDEB can
develop SCC regardless of C7 expression, further indicating
that additional factors have a role in modulating the downstream
mutational consequences complicating this disease (14).
Cytokines expressed at the wound site play a pivotal role in
promoting and coordinating the activity of different cell types,
thus allowing efficient healing in physiological conditions
(15,16). Very little is known about which inflammatory cells,
molecular pathways and cytokine constellation are functioning
in fragile DEB skin, whether deregulated cytokine production
may influence the clinical outcome and how we can counteract
possible undesired consequences of their action.
With the aim of identifying molecules modulating disease
clinical outcome in DEB patients, we here analysed a monozy-
otic (MZ) twin pair with RDEB showing significant phenotypic
variations. MZ twins do not always have identical DNA
sequences; however, they share almost 100% of their genetic
polymorphisms. Thus, they represent a valuable model to
examine differences in gene activity associated with variable
phenotypes (17).
We first verified whether C7 deposition at the BMZ was com-
parable between MZ twins and then performed a genome-wide
expression array on cultured fibroblasts to detect molecules
differentially expressed in the two twins. Our results point to
the existence of protective modifier gene products that counter-
act the TGF-β pathway.

RESULTS
Clinical and molecular findings in RDEB twins
The twins presented generalized skin blisters and oral cavity
involvement since birth. Finger and toe nails were also affected
since infancy leading to early nail loss. During childhood, they
started to complain dysphagia with the development of oesopha-
geal strictures which required one endoscopic dilation. Disease
severity was similar in both twins till early adolescence, when
one sibling presented a marked, apparently spontaneous,
improvement of both skin and oral manifestations. On examination
at the age of 33 years, the twin with a milder phenotype presented
skin blistering lesions and atrophy restricted to trauma exposed
sites (hands, feet, elbows, knees, pretibial area), whereas gener-
alized blisters, erosions and ulcerations, and marked inflam-
mation affected the majority of the body surface of the twin with
more severe clinical manifestations (Fig. 1A). Both twins also
presented increased caries and loss of all nails, while hair were
not affected. Based on the clinical data, the more affected twin
had widespread blistering typical of a severe RDEB generalized-
other phenotype, while the clinical manifestations of the milder
one, primarily confined to the extremities, were consistent with a
localized form (1,3).
The twins were screened for COL7A1 mutations and found to
be compound heterozygous for recessive mutations c.8117delC
and c.4965C>T, as previously reported (18). The former muta-
tion results in a frame-shift, which leads to a PTC, mRNA
decay and absent protein, or to truncated unstable polypeptides.
The latter creates a new donor splice site in exon 53 leading to a
shorter aberrant mRNA carrying a PTC. However, it also allows
splicing at the canonical donor site resulting in the synthesis of
minimal amount of full-length mRNA and C7 polypeptides
(19). In addition to blood, these COL7A1 mutations were con-
firmed in the genomic DNA purified from cultured keratinocytes
and fibroblasts isolated from twin skin biopsies. The true MZ twin
status was confirmed by microsatellite genotyping at nine tetranu-
cleotide short tandem repeat loci and the amelogenin locus (data
not shown). In addition, these individuals share the same status
(heterozygous 1G/2G) of functional SNP rs1799750 in the
MMP1 promoter, and the same sequence encompassing the pro-
mother region and the first exon of TGFBI gene, where functional
SNPs rs1800469 (homozygous CC), rs1982073 (homozygous TT)
and rs1800471 (homozygous GG) are located.
In agreement with the predicted consequences of the COL7A1
mutations, immunofluorescence analysis of twin skin biopsies
revealed a strongly reduced linear staining of C7 along the cuta-
neous BMZ, compared with control skin of healthy donors
(Fig. 1B). The intensity of the residual signal was roughly
comparable between twins, although it appeared slightly weaker in the skin of the less affected one. Staining intensity for laminin-332, collagen XVII and β4-integrin subunit, which are components of the cutaneous BMZ mutated in other EB types, was also comparable in the two twins (Supplementary Material, Fig. S1).

**Figure 1.** Clinical, immunohistochemical and biochemical characterization of the MZ twins with RDEB. (A) The twin with more severe phenotype shows diffuse blisters and erosions, while in his sibling skin lesions are restricted to trauma exposed body area (hands, elbows, knees, feet). (B) Immunostaining of the dermal–epidermal junction of uninvolved skin of the same body site showing highly reduced and similar C7 staining in both twins (bars: 50 μm; e, epidermis; d, dermis). (C) Western blot of keratinocyte cell lysates confirms the highly reduced expression of C7 in both twins when compared with a healthy control (C). Note that the C7 abundance is slightly higher in the lysates of the severely affected twin cells. (D) Western blot of concentrated conditioned medium from twin fibroblasts cultured in the presence of 50 μg/ml ascorbate, which improves secretion of collagens. A very faint signal is detectable in samples from both twins (S, twin with a severe phenotype; M, twin with a mild phenotype).
Western blot analysis of proteins extracted from primary keratinocytes using an anti-C7 polyclonal antibody confirmed that twins have strongly reduced levels of normal-sized C7 protein when compared with cells from a healthy control and indicated that the twin manifesting a more severe phenotype even produced slightly increased amounts of C7 compared with his sibling (Fig. 1C). Western blot of concentrated supernatants from cultured twin fibroblasts corroborated the data obtained with keratinocyte cell extracts (Fig. 1D). Taken together, these findings demonstrate that variations in disease severity between the MZ twins occur regardless of C7 abundance.

Comparison of gene expression profiles of twins’ cultured fibroblasts reveals differentially expressed genes influencing TGF-β signalling

To identify disease modifying mechanisms and/or factors with a potential impact on sibling-to-sibling phenotypic variability, total RNA purified from subconfluent primary fibroblasts isolated from non-lesional skin biopsies from the same body site was used for microarray hybridization. A total of 159 probesets were found to be up-regulated and 137 were down-regulated in the twin with milder phenotype when compared with his sibling, with a fold change > 2.0 and a false discovery rate (FDR) < 0.05 (Supplementary Material, Fig. S2 and Tables S1 and S2).

Gene set enrichment analysis (GSEA) was then used to find significantly perturbed gene sets extracted from literature, biological pathways and Gene Ontology categories. Among the gene sets significantly enriched (FDR < 0.05) in the twin with more aggressive phenotype, we observed that there were several genes involved in “muscle contraction” (Supplementary Material, Table S3). Specifically, fibroblasts of the twin with the more severe phenotype displayed increased mRNA levels of alpha-smooth muscle actin (α-SMA/ACTA2) and plasminogen activator inhibitor-1 (PAI-1/Serin), two genes that are expressed by contractile and profibrotic fibroblasts following TGF-β activity (Table 1) (20,21). Consistently, among the genes most up-regulated in the less affected twin, the proteoglycan decorin (DCN) was found in four out of four array probes (Table 1). DCN is an ECM component that binds TGF-β and inhibits its action (22). Interleukin-7 (IL-7), another TGF-β inhibitor but acting intracellularly (23), was also found to be up-regulated in the less affected twin (Table 1). Other genes related to TGF-β activity and fibrosis were also identified as differentially regulated in the twins, specifically the genes encoding for matrix metalloproteinase-3 (MMP-3), zinc finger E-box-binding homeobox 1 (zeb-1), dickkopf-related protein 2 (DKK2), TGF-β receptor II (TGF-βRII) and BMP-2-inducible protein kinase (BMP2K) (Table 1). Microarray data were validated by quantitative reverse transcriptase PCR (qRT-PCR), which confirmed the differential expression of all the above-reported genes (Fig. 2A).

Protein expression from these genes was also tested. Western blot analysis confirmed different levels of both intracellular (40 kDa core polypeptide) and released (~100 kDa proteoglycan) DCN in the twins’ fibroblasts (Fig. 2B). The former was identified in the cell supernatant following glycosaminoglycan digestion with chondroitinase, which reduces DCN size from 100 to 40 kDa (Fig. 2B, lower panel). Significantly increased amounts of released IL-7 and MMP3 were also found by ELISA in the supernatant of the twin with milder phenotype (S, 1.41 pg/10⁶ cells; M, 11.77 pg/10⁶ cells for IL7 and S, 1.28 ng/10⁶ cells; M, 8.84 ng/10⁶ cells for MMP3) (Fig. 2C).

Further confirming gene array data, α-SMA and PAI-1 expression levels, detected by western blot, were higher in the cell extracts of the twin with worse clinical manifestations (Fig. 2D). Immunofluorescence analysis on subconfluent cultured fibroblasts indicated that α-SMA was expressed in an increased percentage of cells in the more affected twin compared with cells from the less affected one (S, 12.8 ± 4.1%; M, 5.9 ± 3.1%) (Fig. 2E). Despite the strong difference observed in the transcription of Wnt signalling inhibitor DKK2, western blot of cell extracts indicated similar levels of intracellular proteins in the twins (Supplementary Material, Fig. S3). Also, the TGF-βRII level of expression was not significantly different in the protein extracts of the twins despite the differences observed in the transcripts (Supplementary Material, Fig. S3).

**Table 1. TGF-β-related genes differentially expressed by MZ twins**

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<tr>
<th>Probeset</th>
<th>Gene symbol</th>
<th>Description</th>
<th>Log2ratio</th>
<th>FDR</th>
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<tr>
<td>200974_at</td>
<td>ACTA2/α-SMA</td>
<td>Actin, alpha 2, smooth muscle, aorta</td>
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<td>0.0413</td>
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<tr>
<td>202628_s_at</td>
<td>SERPINE1/PAI-1</td>
<td>Serpin peptidase inhibitor, member 1</td>
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<td>0.0063</td>
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<tr>
<td>202627_s_at</td>
<td>SERPINE1/PAI-1</td>
<td>Serpin peptidase inhibitor, member 1</td>
<td>1.17</td>
<td>0.0083</td>
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<tr>
<td>219546_at</td>
<td>BMP2K</td>
<td>BMP2 inducible kinase</td>
<td>-1.02</td>
<td>0.0204</td>
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<tr>
<td>201893_x_at</td>
<td>DCN</td>
<td>Decorin</td>
<td>-1.04</td>
<td>0.0057</td>
</tr>
<tr>
<td>208944_at</td>
<td>TGFBR2</td>
<td>TGF, beta receptor II (70/80 kDa)</td>
<td>-1.07</td>
<td>0.0135</td>
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<tr>
<td>210875_s_at</td>
<td>ZEB1</td>
<td>Zinc finger E-box binding homeobox1</td>
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<td>0.0337</td>
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<td>212764_at</td>
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<td>Zinc finger E-box binding homeobox1</td>
<td>-1.24</td>
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<td>Decorin</td>
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<tr>
<td>206693_at</td>
<td>IL7</td>
<td>Interleukin 7</td>
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<td>205828_at</td>
<td>MMP3</td>
<td>Matrix metalloproteinase 3</td>
<td>-4.23</td>
<td>0.0031</td>
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<tr>
<td>219908_at</td>
<td>DKK2</td>
<td>Dickkopf 2 homolog (X. laevis)</td>
<td>-5.67</td>
<td>0.0202</td>
</tr>
</tbody>
</table>

For each probe set the official gene symbol, the gene name and the log2 of the expression ratio severe versus mild are shown (FDR < 0.05; fold-change > 2).
Fibroblasts of the more affected twin manifest a profibrotic and proinflammatory phenotype

As shown above, fibroblasts of the twin with worse phenotype expressed higher levels of α-SMA and PAI-1, which are both markers of myofibroblast differentiation induced by TGF-β1. Genearray hybridization proved that the TGFβ1 gene was up-regulated in the severe twin fibroblasts, with a fold increase (1.94) just below the cutoff (≥2.0) (see NCBI Gene Expression Omnibus repository number GSE51056). We, therefore, tested the TGF-β1 concentration in the fibroblast supernatants by ELISA, which detects both the latent precursor and the active
form without latency-associated peptide (24). The results showed a significantly increased amount of released cytokine in cell cultures from the twin with more severe clinical manifestations (S, 1.14 ± 0.14 ng/10^6 cells; M, 0.53 ± 0.15 ng/10^6 cells) (Fig. 3A). In parallel, sequencing of the promoter region and exon 1 of the TGF-β1 gene in DNA samples from both twin fibroblasts excluded the presence of de novo mutations and showed identical genotypes at SNPs that are known to be functionally associated with gene transcription rate and protein production (data not shown) (25,26). These findings suggest that TGF-β1 expression differences between the twins were not determined by alterations of the gene sequence. Since COL7A1 transcription is stimulated by the TGF-β1/Smad signalling (27), these data may also explain why C7 expression appears to be slightly increased in skin and skin cells of the more affected twin.

The most prominent response of dermal fibroblasts to TGF-β activity is increased deposition of ECM components (28). Western blot analysis on fibroblast supernatants showed increased levels of released collagen I in the twin with more severe phenotype (Fig. 3B), further indicating that an enhanced TGF-β1 activity associates with disease severity. The contractile activity of twins’ cells was then analysed by a fibroblast-populated collagen lattice contraction assay. After 48 h of culture, fibroblasts from the more affected twin were able to contract the collagen gel to an average 46.1 ± 7.7% of the initial gel area; the same number of cells from the twin with milder phenotype barely contracted the collagen gel in the same period of time (collagen area after 48 h: 95.6 ± 7.6% of initial gel area) (Fig. 3C).

Fibroblasts express diverse cytokines that can act in an autocrine and paracrine way playing a central role as fibrosis mediators (16). A cytokine antibody array analysis was therefore performed on media conditioned by twins’ fibroblasts. This analysis revealed significantly higher levels of two pro-inflammatory cytokines, IL-6 and monocyte chemoattractant protein-1 (MCP-1), in the twin with a more severe phenotype (Fig. 3D). These cytokines are induced by TGF-β (29,30) and promote fibroblast transition to myofibroblasts (31).

These findings demonstrate that fibroblasts of the twin with worse phenotype exhibit various characteristics of cells that promote fibrosis and inflammation.

Constitutive TGF-β signalling activation in the fibroblasts of the more affected twin

The diverse fibroblast phenotype found in the two twins further supported the contribution of TGF-β action in the discordant clinical outcome of the twins. TGF-β canonical signalling is mediated by activation of Smad transcription factors; however, other mediators, prominently the mitogen-activated protein kinases (MAPKs), are also activated in response to TGF-β stimulation (32). To better dissect TGF-β activation status in the fibroblasts of the two twins, the Smad and MAPK signalling mediators were analysed by western blot, in basal condition or following TGF-β1 administration (Fig. 4A). A TGF-β1 dose–response was performed and 0.25 ng/ml, the minimal concentration found to be able to elicit a biological effect (not shown), was chosen to unveil differences in the response to TGF-β1 in the fibroblasts from the two twins.

Cells of the more affected twin expressed a higher amount of phosphorylated-(p-)Smad2, p-p38 and p-ERK1/2, in basal conditions. Specifically, in our experimental conditions, p-Smad2 was undetectable or barely detectable in the cell extracts of the twin with a milder phenotype, while increased levels of this isoform were always observed in samples from the more affected twin.

**Figure 3.** Profibrotic and proinflammatory phenotype of fibroblasts from the twin with a more severe phenotype. (A) ELISA detecting total TGF-β1 protein level in fibroblast conditioned medium, collected 24 h after starving. Values are expressed as ng of protein × 10^6 cells ± SD (**P < 0.01). (B) Western blot for collagen type I protein on medium conditioned by comparable number of fibroblasts in the two samples, collected 24 h after starving. (C) Collagen lattice contraction assays. Images were taken at 0 and 48 h time points. Percentage of contraction found to be able to elicit a biological effect (not shown), was chosen to unveil differences in the response to TGF-β1 in the fibroblasts from the two twins.
twin. Following treatment with TGF-β1, both fibroblast strains strongly increased p-Smad2 to similar levels, while p-p38 and p-ERK1/2 levels increased only in the cells of the twin with a milder phenotype. In the cells of the more affected twin, p-ERK1/2 and p-p38 levels surprisingly decreased following TGF-β1 treatment.

To further investigate fibroblast ability to respond to TGF-β1, collagen lattice contraction assay was performed in the presence or absence of 0.25 ng/ml TGF-β1 (Fig. 4B). Exogenous addition of TGF-β1 induced lattice contraction in preparations from both twins, although the reduction in area, when compared with contraction in basal conditions, was significant only in lattices containing cells of the twin with a milder phenotype (S, from 46.1 ± 7.7 to 34.3 ± 3.5%; M, from 95.6 ± 7.6 to 65.1 ± 6.8%). However, even in the presence of exogenous TGF-β1, the contractile activity of the fibroblasts from the less affected twin...
remained less marked than that showed in basal condition by the fibroblasts of the twin with a more severe phenotype.

**DCN overexpression mitigates the profibrotic and proinflammatory phenotype of fibroblasts from the more affected twin**

DCN, one of the molecules found to be up-regulated in the less affected twin by gene expression profiling, manifests potent antifibrotic properties (32–35). Therefore, we decided to investigate whether DCN can mitigate the profibrotic phenotype of the fibroblasts from the twin with severe clinical manifestations. Released DCN accumulates in the ECM where it traps TGF-β thus preventing its biological activity (22). We therefore examined the expression of DCN by immunofluorescence in the twins’ skin. A substantial increase in expression of this protein was observed as a diffuse staining in the dermis of the twin with milder clinical manifestations when compared with skin sections from the twin with more severe phenotype (Fig. 5A and B). DCN expression was also investigated by western blot of proteins purified from ECM deposited by cultured twins’ fibroblasts. An increased amount of DCN was detected in the ECM extracts of the twin with milder clinical manifestations (Fig. 5C), thus suggesting that the less contractile/proinflammatory phenotype of these cells might be due to the inhibitory activity of DCN on TGF-β.

To confirm whether an increase in DCN expression could antagonize the contractile phenotype of RDEB fibroblasts of the more affected twin, the recombinant core protein (200 nm) was added to the collagen mixtures containing fibroblasts before lattice gelification (Fig. 6A). In the presence of DCN, lattices contracted significantly less when compared with those without addition, both in basal condition (100.8 ± 5.2% of initial area in DCN-treated versus 40.2 ± 8.7% in DCN-untreated) and following treatment with TGF-β (0.25 ng/ml) (55.7 ± 8.5 versus 30.3 ± 4.1%). Therefore, without TGF-β stimulation, DCN was able to almost completely prevent the fibroblast contractile activity.

To further test the anti-fibrotic function of DCN in RDEB, the contractile fibroblasts of the more affected twin were transfected with a vector containing the full-length DCN cDNA (pcDNA3-DCN). Transfected cells that overexpressed DCN were then analyzed for gel contraction ability, and Smad2 expression (Fig. 6B and C). Collagen lattices prepared with DCN overexpressing cells contracted significantly less than lattices prepared with cells transfected with an empty pcDNA3 vector (85.0 ± 7.1% of initial area versus 49.0 ± 9.9%) (Fig. 6B). Moreover, DCN overexpression obtained with plasmid transfection was also able to inhibit, although not significantly, the contraction of lattices stimulated with 0.25 ng/ml of TGF-β1 (41.5 ± 17.7 versus 17.5 ± 6.4%). Consistently, western blot analysis of proteins purified from lattices populated with DCN-transfected fibroblasts revealed decreased p-Smad2 levels (Fig. 6C), indicating that cell contraction was prevented through inhibition of TGF-β-signalling.

Taken together, these data indicate that increased DCN expression, either delivered as exogenous protein or expressed from a recombinant transfected vector, is able to mitigate the profibrotic phenotype of dermal fibroblasts from the more affected twin.

**DISCUSSION**

The best phenotype predictor in RDEB is the amount of C7 in the skin. This, in principle, is determined by the particular combination of COL7A1 mutations affecting the genome of the patients. However, many observations attest that the genotype–phenotype correlation rules are not absolute. For example, siblings can present very distant clinical phenotypes. They share the same COL7A1 genotype, yet their skin may express different C7 amounts (4). Genetic and epigenetic modifiers are believed to play a role; nevertheless, knowledge in this matter is quite limited thus far (8–10). In addition, emerging evidences attest that C7 amount is not the only determinant of disease severity. First, a proteome profiling of primary fibroblasts from RDEB and normal individuals showed that the C7 deficiency impacts on the expression of a significant number of secreted proteins required for maintenance of cutaneous basement membranes and ECM (36). Second, wound healing in patients with severe RDEB can proceed even in the absence of C7 regeneration, as it was observed in a clinical trial using intradermal allogeneic fibroblast or vehicle alone injections (37) and after allogeneic bone-marrow transplantation therapy (38). These examples point to additional, yet unclear, non-C7-based mechanisms able to stimulate the regenerative capacity of damaged skin (39), and are relevant to the discordant co-twins studied here. Indeed, the more affected twin presents ulcerations and a marked inflammation, yet C7 abundance in his skin and skin cells is similar or even slightly increased compared with his sib. Therefore, RDEB MZ-twins provide the best demonstration that C7 amount in the skin is not the only predictor of disease severity.

MZ-twins should share 100% of their genetic polymorphisms. However, it is now evident that point mutations and copy number variations can occur post-zygotically, explaining genetic effect on the phenotype (40,41). Somatic mosaicism in either one of the twins may thus be a possible explanation for phenotypic divergence. However, we found neither significant difference in the expression of C7 protein in both uninvolved skin and primary skin cells of the co-twins, nor we detected back or second site mutations in the genome of these cells after COL7A1 re-sequencing. In addition, epigenetic differences, which may influence transcription and expression of proteins without changes in DNA sequence, may be responsible for disease discordance (42). Intriguingly, as twins’ phenotype started to diverge during adolescence, it could be hypothesized that environmental modifications have targeted the cell epigenome, e.g. by altering DNA methylation or chromatin status. These changes are stably inherited through subsequent cell generations and may influence cell behaviour (43). Indeed, MZ twin pairs accumulate differences in global DNA methylation and histone modification over time and across different tissue types (44,45).

Whatever mechanism may be involved, MZ twins have fewer variables than other siblings. We have chosen to compare gene expression in primary human fibroblasts because these cells are involved in RDEB pathophysiology. Indeed, alterations in the homeostasis of stromal compartment play an important role in determining disease severity of RDEB patients in particular with respect to SCC aggressiveness (12). Particularly, unremitting healing and scarring, major features of DEB patients
are strongly dependent on fibroblast function. Since the MZ twins manifest differences related to healing impairment with persistent inflammation, it was conceivable that fibroblast behaviour could play a central role in modulating disease severity. There are good reasons to believe that alterations of TGF-β signalling significantly contribute to the phenotypic differences observed in the MZ twins. This pathway appears to be highly involved in RDEB pathogenesis: (i) as mentioned above, an increase in secreted TGF-β was observed in human dermal fibroblasts derived from C7-negative RDEB skin compared with normal fibroblasts (36); (ii) TGF-β1 promotes wound healing (15,46), however, excessive TGF-β1

**Figure 5.** DCN expression in the extracellular matrix released by fibroblasts of the MZ twins. (A) DCN immunostaining on twins’ skin sections (green). Nuclei are stained with DAPI (blue) (bars:100 μm; e, epidermis; d, dermis). S, twin with a severe phenotype; M, twin with a mild phenotype. (B) Three-dimensional surface plot images as determined by ImageJ software analysis (left, upper and lower plots). Quantification of fluorescence intensity in skin sections from the twin with severe (S) or mild (M) phenotype is reported on the right. Values are expressed as mean obtained by evaluating 10 randomly selected fields (*P < 0.05). (C) DCN protein level in extracts of fibroblast-deposited extracellular matrix. Equal amount of total protein extracts was loaded for the two samples. S, twin with a severe phenotype; M, twin with a mild phenotype.
signalling is detrimental for healing outcome leading to abnormal ECM deposition and scar formation (20); (iii) hypomorphic col7a1 mice with reduced C7 levels develop a phenotype matching severe RDEB, characterized by early blistering of the skin and mucous epithelia, nail dystrophy and mitten deformities, which associate with an ongoing inflammatory response, TGF-β accumulation, increased number of myofibroblasts and dermal matrix remodelling (47). Of relevance, it has been recently reported that these mice show increased TGF-β expression and activation at wound sites and manifest delayed skin repair (13). Based on this evidence, inhibition of TGF-β pathway has been hypothesized to prevent phenotypic complications in these mice and is thought to be a potential target of intervention in the human disease.

Figure 6. DCN is able to mitigate the profibrotic phenotype of the fibroblasts from the more affected twin. (A) Collagen lattice contraction assay performed with fibroblasts of the twin with worse clinical manifestations (S) suspended at 2 × 10^5 cells/ml into a solution containing or not 200 mM human recombinant core DCN protein, treated or not with 0.25 ng/ml TGF-β1. Images were taken 48 h after TGF-β1 treatment. Percentage of contraction referred to initial lattice area for different experimental conditions is reported in the histogram (***P < 0.01; **P < 0.005). (B) Collagen lattice assay to analyse contraction ability of fibroblasts from the twin with severe phenotype (S) transfected with a plasmid expressing the core DCN protein (pcDNA3-DCN +) or with an empty plasmid (pcDNA3-DCN −). Percentage of contraction referred to initial lattice area for different experimental conditions is reported in the histogram (P < 0.05). (C) Phosphorylated (p-) Smad2 protein expression in total lysates obtained from lattices containing fibroblasts of the severe (S) twin transfected with the plasmid expressing human DCN (pcDNA3-DCN +) or an empty plasmid (pcDNA3-DCN −). Data ± SD are expressed as ratio between phosphorylated and total corresponding protein (P < 0.05).

Having found diverse TGF-β signalling activation in the fibroblasts of the twins, we wondered whether fibroblasts of the less affected one were able to respond to TGF-β stimulation. The ability of the fibroblasts from the less affected twin to contract lattices following TGF-β1 treatment indicated that these cells are responsive to TGF-β. Surprisingly, TGF-β1 treatment exerted an inhibitory effect on the phosphorylation of both p38 and ERK in the more affected twin. Considered that the MAPK pathway is a route of signalling common to diverse effectors, a possible explanation for this finding is that different factors simultaneously act through this signalling pathway, and stimulation with exogenously added TGF-β overloads the system determining an inhibitory effect.

As for cytokine array, the finding that IL-6 and MCP-1 are up-regulated in the twin with more severe phenotype is in
line with enhanced TGF-β signalling in the fibroblasts of this twin. Indeed, IL-6 and MCP-1 are induced following TGF-β stimulation in many cell types, including fibroblasts, and promote monocyte recruitment and inflammation in the stroma (29,30,31). It has been speculated that an autocrine loop may exist in which TGF-β-mediated increased expression of MCP-1 and IL-6 contributes to differentiation of fibroblasts into myofibroblasts which in turn amplify TGF-β proinflammatory and profibrotic effect (48).

Among the genes found to be up-regulated in the twin with milder phenotype, there was IL-7 which acts by inducing Smad7, an inhibitory Smad that interferes with TGF-β/Smad signalling (23). This cytokine was proved to have antifibrotic properties and has been tested as a potential therapeutic compound for pulmonary fibrosis (23). As such, IL-7 could be taken into consideration as a possible modifier gene in the twins.

One of the genes more significantly up-regulated in the twin with milder phenotype is the proteoglycan DCN. Of note, DCN was also found to be more expressed in the skin of this sibling in vivo, and to be released at higher levels and to accumulate in the ECM in vitro. DCN inhibits TGF-β activity by binding it, thus preventing interaction with TGF-β receptors (22,49,50). If TGF-β activation plays a pathological role in RDEB patient clinical manifestations, DCN may be regarded as a modifier gene, able to mitigate TGF-β effects.

TGF-β1 protein release was increased about 2-fold in the fibroblasts of the twin with more severe clinical manifestations. Since TGF-β has been shown to inhibit DCN synthesis in dermal fibroblasts (51), one can speculate that such autocrine loop might contribute to worsen the disease in the more affected twin.

In our hands, DCN, either exogenously administered or over-expressed following cDNA transfection, was able to mitigate the fibrotic phenotype of the more affected twin fibroblasts. Based on this, DCN appears as a potential therapeutic candidate to prevent, or at least mitigate, disease complications in patients affected with RDEB. To this regard, DCN therapeutic properties have been proved in preclinical studies using animal models for other pathologies associated with excessive scarring, such as myocardial infarction (34,52), glomerulonephritis (53,54), atherosclerosis (55) and corneal fibrosis (56).

Finally, it would be interesting to analyse DCN expression in a panel of RDEB patient skins to determine whether a correlation between expression levels and disease severity does exist. However, this implies the availability of a prospective collection of homogenous samples since patient age, habits and site of skin biopsy can all represent factors that influence DCN expression. In conclusion, the TGF-β signalling alterations observed in the MZ-twin model unveil a molecular pathway relevant to the clinical phenotypic variability in RDEB. In addition, our findings disclose previously unrecognized roles of DCN as anti-fibrotic and anti-inflammatory molecule in RDEB.

MATERIALS AND METHODS

Patients and ethics statement

A pair of 33-year-old Italian MZ twins affected by RDEB, previously diagnosed by clinical criteria, immunofluorescence antigen mapping and COL7A1 mutation screening, were analysed (18). Ethylenediamine tetraacetic acid (EDTA)-blood samples were obtained from the patients and a skin biopsy of an uninvolved area at the same position of the forearm was taken from each patient. Part of the biopsy was processed for cell cultures and part used for immunofluorescence analysis. Written informed consent was obtained from the two patients. Procedures were in accordance with the ethical standards of the Committee on Human Experimentation of Istituto Dermopatico dell’Immacolata-IRCCS. The study was conducted according to the Declaration of Helsinki principles.

Genetic analyses

Genomic DNA was extracted from blood and cultured skin cells using a commercial kit (Qiagen, Hilden, Germany). The entire COL7A1 coding region was analysed by a previously described procedure (18) using genomic DNA from cultured fibroblasts and keratinocytes (see below). Examination of the twins’ zygosity was performed using blood genomic DNA and the AmpFLSTR® Profiler® PCR Amplification Kit (Applied Biosystems®, Warrington, UK), which amplify nine tetranucleotide short tandem repeat loci and the amelogenin locus, as recommended by the manufacturer. Electrophoretic analysis was performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) with Performance Optimized Polymer 4 (POP4) using Genescan software (Applied Biosystems). The allele sizes were then analysed using Genotyper software (Applied Biosystems).

Genotype at SNP rs1799750 (-1607 1G/2G) in the MMP1 promoter was checked by PCR amplification and sequence analysis using primers (F) 5′-CCCTCTTGAACTCATGTTATG and (R) 5′-ACTTTCTCCCTTTATGGATTCC that amplify a 169 bp fragment (T<sub>ann</sub> 60°C). A 2115-bp fragment of the TGFBI gene encompassing a portion of the promoter region and the exon 1 with partial coding sequence was amplified with primers (F) 5′-GGTCCCAGGACAGCAGTTGG and (R) 5′-CGCAGCTTGAACAGATCTG (T<sub>ann</sub> 57°C) from DNA purified from fibroblasts, and then sequenced with appropriate internal primers.

Cell cultures

Primary fibroblast and keratinocyte cultures were established from biopsies taken from non-lesional skin of the same body site of two RDEB patients. Skin fibroblasts were grown in Dulbecco modified Eagle’s medium (DMEM) and Ham’s F10 medium (1:1) supplemented with 10% fetal bovine serum, 50 μg per ml penicillin and streptomycin, 4 mM L-glutamine and, for selected experiments, 50 μg/ml ascorbic acid. Cells were routinely subcultured and used for experiments at passages 6–8. Keratinocytes were cultivated on a feeder layer of immortalized 3T3-J2 murine fibroblasts, as described previously (57).

Immunofluorescence studies

Part of the skin biopsies from the patients was frozen in OCT. For detecting proteins of the dermal–epidermal junction, skin sections were air-dried, while for DCN immunodetection skin sections were fixed in ice-cold ethanol. The following primary antibodies were used: monoclonal anti-human type VII collagen,
1:100 dilution (LH7.2, Sigma Immunochemical, St Louise, MO, USA); monoclonal anti-human laminin-332, dilution 1:5 (clone GB3, a kind gift of Dr Meneguzzi, Inserm, Nice); monoclonal anti-human BP180, dilution 1:25 (clone IA8C, a kind gift of Dr Ovaribe, Nagoya University, Japan); monoclonal anti-human integrin β4, 1:40 dilution (clone 3E1, Chemicon, Merck Millipore, Billerica, MA, USA); monoclonal anti-human DCN, 1:1000 dilution (R&D Systems, Minneapolis, MN, USA). Biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA, USA) were then used followed by treatment with fluorescein (FITC)-conjugated streptavidin (UCS Diagnostics, Morlupo, Italy). Only for DCN immunodetection, a streptavidin-FITC-conjugated secondary antibody was used directly after the primary antibody (GE Healthcare Life Science, Little Chalfont, UK). Slides were mounted and analysed using a Zeiss-Axioskop microscope (Zeiss, Oberkochen, Germany). Nuclei were counterstained with DAPI (Vectashield Mounting Medium with DAPI, Vector Laboratories). To quantify DCN levels, the green channel intensity was analysed by three-dimensional surface plot with ImageJ 1.34 (http://rsb.info.nih.gov/ij) (58,59).

Primary fibroblasts were seeded on untreated glass cover slips and grown to sub-confluence. Cells were fixed with 3% paraformaldehyde/phosphate buffer saline (PBS), permeabilized with 0.1% Triton X-100 and unspecific stain blocked with 0.1% BSA/PBS, followed by incubation with the primary monoclonal anti-human-α-SMA antibody (Sigma-Aldrich, St Louis, MO, USA), 1 h at room temperature, 1:200 dilution. After washing with 1× PBS, cells were incubated with the FITC-conjugated secondary antibody for 1 h at room temperature. Cover slips were mounted and observed under microscope, and nuclei were counterstained as above. At least seven randomly selected fields were recorded with a digital camera (AxioCamMRc5, Zeiss) and fluorescent cells counted.

**Microarray analysis**

Total RNA was extracted by TRIzol (Life Technologies, Invitrogen, Gröningen, The Netherlands) from a triplicate of primary fibroblast cultures of the two RDEB twins at passage 6. Five micrograms of total RNA were reverse transcribed, converted into cRNA, fragmented, labelled and hybridized to Human Genome U133A 2.0 and U133Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA). Microarray data were processed by GCRMA (GC Robust Multi-Array Average) (60) using the Bioconductor affy (61) package of the R statistical programming language (62). GCRMA summarized data from the two array platforms were combined to create a final data set including 22277 common probes, quantile normalized and finally adjusted for batch effect using ComBat (63). Differences in gene expression were identified by moderated t-test using the limma (64) package for empirical Bayes statistics for differential expression. The resulting P-values were corrected for multiple testing by computing the FDR. Genes with a FDR < 0.05 and fold change >2 were selected as differentially expressed and retained for further analysis. GSEA was used to identify differentially regulated biological processes and pathways (65). Raw and normalized microarray data have been deposited in NCBI Gene Expression Omnibus (GEO) repository with the accession number GSE51056.

**Real-time RT-PCR**

RNA from samples used for microarray probe labelling and from samples obtained with additional sets of fibroblast cultures was tested. cDNA synthesis was performed using the Superscript™ III First Strand System (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s protocol. The SYBR Green PCR Master Mix (Applied Biosystem, Foster City, CA, USA) was then used according to the manufacturer’s instructions. The following gene-specific primers were designed using the Primer Express software (Applied Biosystems): dickkopf homolog 2 (DKK2) forward 5′- AAGGAGACCCCTGCTACGA-3′ and reverse 5′-GAAATGACGACGACGACAAAC-3′; matrix metallopeptidase 3 (MMP3), forward 5′-GCAAGGACCTCGTTT TCATT-3′ and reverse 5′-CTTTGGAATCAGTCGTC-3′; interleukin 7 (IL-7), forward 5′-AAAGTTTCAGAAAACCA AACAAAT-3′ and reverse 5′-TCCAAAACCTTGTGTTTGG GG-3′; DCN, forward 5′-TCTCTGATGACCGCAGCTTT-3′ and reverse 5′-GAGTGTGTCAGGGGGAAGA-3′; zinger finger E-box binding homeobox 1 (ZEB1), forward 5′-AACTGCT GGAGGATGACAC-3′ and reverse 5′-TCTTGCTCTCATCT GCCTGA-3′; BMP2, inducible kinase (BMP2), forward 5′-GCCCTTGAAATGTACACCA-3′ and reverse 5′-AGATTG ATAGATGAGACATTCCAGTC-3′; alpha smooth muscle actin (α-SMA) forward 5′-CCTATCCCCGGAGCTAACAG-3′ and reverse 5′-AGGCGTGTGTCCTCCTCTCT-3′; plasminogen activator inhibitor type 1 (PAI-1), forward 5′-ACGGGAA CCCATTCGAGAT-3′ and reverse 5′-CCCTTCCTGATTTCG TCAGC-3′; transforming growth factor-beta receptor 2 (TGF-βR2), forward 5′-CAAGCAGCTCTGAGATC-3′ and reverse 5′-TGATGAGCCGACGATATTACA-3′. Expression levels were calculated by the relative standard curve method. Gene-specific PCR products were measured by means of the ABI PRISM 5700 detection system (Perkin-Elmer, Norwalk, CT, USA) and normalized to the glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) amplification with specific primers (forward 5′-GAAGGTGAGGTCGGAGCT-3′ and reverse 5′-GAAGATGTTGATGGGATTTC-3′). Quantification was performed using the comparative CT method (66). Experiments were repeated three times, giving comparable results.

**ELISA**

Human TGF-β1, IL-7 and MMP-3 Quantikine HS ELISA Kits (R&D Systems) were used following manufacturer’s protocol to determine levels of TGF-β1, IL-7 and MMP-3 in serum-free conditioned media, in 24 h, from primary fibroblast cultures of the twins. Plates were analysed in a Microplate reader 3550-UV (BioRad Laboratories, Hercules, CA, USA). Samples were assayed in triplicate for each patient.

**Western blotting analysis**

To analyse C7 expression in cultured skin cells, twin and control primary keratinocyte extracts were prepared on ice with RIPA buffer [Tris – HCl pH 8.50 mm, NaCl 150 mm, sodium deoxycholate 0.5%, sodium dodecyl sulphate (SDS) 0.1%, NP40 1%, NaF 50 mm, sodium orthovanadate 10 mm, protease inhibitor cocktail from Roche (Basel, Switzerland)]. Concentrated conditioned medium was obtained from an equal number of twin
and control primary fibroblasts grown in the presence of 50 μg/ml ascorbic acid. Protein extracts and conditioned media were electrophoresed on 7–10% SDS polyacrylamide gels (SDS–PAGE) and transferred to PVDF membranes (Amersham Pharmacia Biotech, Little Chalfont, UK). Filters were soaked in 5% non-fat dry milk or 5% BSA/TTBS (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 0.05% Tween 20). Western blots were performed using a rabbit polyclonal anti-C7 antibody (EMD Millipore/Calbiochem, Darmstadt, Germany) diluted 1/500 in TTBS + 5% milk. Mouse monoclonal anti-tubulin (dilution 1:1000, Calbiochem, Darmstadt, Germany) diluted 1/500 in TTBS and primary antibodies: rabbit anti-phospho Smad2 (Ser 465/467), rabbit anti-phospho TGF-β receptors I and II, all from Cell Signaling Technology (Danvers, MA, USA); rabbit anti-Phospho p38 MAPK (Thr180/Tyr182), rabbit anti-phospho MAPK, rabbit anti-TGF-β receptors I and II, all from Cell Signaling Technology (Danvers, MA, USA); rabbit anti-Phospho p38 MAPK, rabbit anti-phospho TGF-β receptors I and II, all from Cell Signaling Technology (Danvers, MA, USA); rabbit anti-Phospho p38 MAPK, rabbit anti-phospho TGF-β receptors I and II, all from Cell Signaling Technology (Danvers, MA, USA); rabbit anti-Phospho p38 MAPK, rabbit anti-phospho TGF-β receptors I and II, all from Cell Signaling Technology (Danvers, MA, USA); rabbit anti-Phospho p38 MAPK, rabbit anti-phospho TGF-β receptors I and II, all from Cell Signaling Technology (Danvers, MA, USA).

Primary fibroblasts, serum-starved for 24 h, were treated or not with TGF-β1 (R&D Systems) at different concentrations ranging from 0.25 to 2 ng/ml. At different time points, cells were washed with ice-cold PBS and lysed in 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM NaF and protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany). For the analysis of total cell extracts, 1% SDS was added to this buffer. Proteins were loaded on a SDS–PAGE, transferred to nitrocellulose (Hybond-C, GE Healthcare) and incubated for 1 h in 1× Western Blocking reagent (Roche Diagnostic). Proteins were detected using the following primary antibodies: rabbit anti-phospho Smad2 (Ser 465/467), rabbit anti-phospho ERK1/2 (Thr202/Tyr204), rabbit anti-phospho p38 MAPK (Thr180/Tyr182), rabbit anti-p38 MAPK, rabbit anti-TGF-β receptors I and II, all from Cell Signaling Technology (Danvers, MA, USA); rabbit anti-Smad 2/3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-ERK 1/2 (Gene Tex Inc., San Antonio, TX, USA); mouse anti-α-SMA (Sigma-Aldrich), mouse anti-PAI-1 (R&D Systems) and rabbit anti-DKK2 (Abcam, Cambridge, UK). Rabbit polyclonal anti-GAPDH antibody (dilution 1:500, Santa Cruz Biotechnology) was used as loading control.

To detect released collagen I, culture medium of primary fibroblasts serum-starved for 24 h was collected, and a given volume normalized for cell number was loaded on a SDS–PAGE, transferred to nitrocellulose (Hybond-C, GE Healthcare) and incubated for 1 h in 1× Western Blocking reagent (Roche Diagnostic). Proteins were detected using the following primary antibodies: rabbit anti-phospho Smad2 (Ser 465/467), rabbit anti-phospho ERK1/2 (Thr202/Tyr204), rabbit anti-phospho p38 MAPK (Thr180/Tyr182), rabbit anti-p38 MAPK, rabbit anti-TGF-β receptors I and II, all from Cell Signaling Technology (Danvers, MA, USA); rabbit anti-Smad 2/3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit anti-ERK 1/2 (Gene Tex Inc., San Antonio, TX, USA); mouse anti-α-SMA (Sigma-Aldrich), mouse anti-PAI-1 (R&D Systems) and rabbit anti-DKK2 (Abcam, Cambridge, UK). Rabbit polyclonal anti-GAPDH antibody (dilution 1:500, Santa Cruz Biotechnology) was used as loading control.

For released DCN detection, culture medium of over confluent primary fibroblasts serum-starved for 24 h was dialysed against 50 mM Tris/HCl, 50 mM sodium acetate, pH 8.0, and treated with 0.1 unit of chondroitinase ABC (Sigma) at 37°C for 2 h. Untreated DCN and DCN treated with chondroitinase ABC were separated on SDS–PAGE. For immunoblot analysis, the proteins were then transferred to nitrocellulose (Hybond-C, GE Healthcare), incubated for 1 h in 1× Western Blocking reagent (Roche Diagnostic) and treated with a mouse monoclonal antibody against human DCN (R&D Systems) for 1 h. Detection was performed using an appropriate horseradish-peroxidase-coupled secondary antibody (GE Healthcare). Antibody binding was detected by the ECL plus detection system (GE Healthcare). Relative intensity of signals was quantified using a GS-710 densitometer (Bio-Rad Laboratories). Detection of ECM-associated DCN was performed as described (67). Briefly, confluent fibroblast monolayers were incubated overnight with PBS/20 mM EDTA at 4°C and washed with PBS/1% Triton X-100. This treatment leaves the ECM intact, free of cell debris and firmly attached to the well. Detached fibroblasts were counted. ECM was then lysed in SDS buffer (50 mM Tris–HCl pH 7.5, 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 5 min. Immunoblot was performed as described above loading volumes of ECM protein extracts normalized for fibroblast number.

### Cytokine protein arrays

Study of multiple cytokine expression levels was carried out on serum-free conditioned media from primary fibroblast cultures of the two twins, using RayBio® Human Cytokine Antibody Arrays 6 and 7 (RayBiotech Inc, Norcross, GA, USA) following manufacturer’s protocol. The array filters were exposed using Amersham Hyperfilm ECL (GE Healthcare) and relative intensity of signals was quantified using a GS-710 densitometer (Bio-Rad Laboratories). By comparing the signal intensities, relative expression levels of cytokines between the two patients were calculated.

### DCN expression plasmid construction and transfection

To prepare the full-length DCN expression construct, RT-PCR of total mRNA purified from normal human fibroblasts was performed using Superscript III enzyme (Invitrogen) and oligo-dT as primer. Then, the resulting cDNA was amplified with the Expand High Fidelity Taq Polymerase System (Roche Molecular Diagnostics) using primers (F) 5′-ATCGGATCCATGAAG GCCACTATCATCCCTC and (R) 5′-ATCTCTGAGTTACTT ATAGTTCCGAGTTG, which bear BamH1 and Xhol restriction sites at their extremities, respectively. The resulting cDNA fragment spanning the 1080-bp long DCN open reading frame (Ensembl transcript n. ENST0000052754) was digested and subcloned into the BamH1/Xhol-restricted pcDNA3 (Invitrogen) expression vector. DCN identity into this vector (pcDNA3-DCN) was checked by sequencing.

Fibroblast transfection with pcDNA3-DCN was performed using the Amaxa Nucleofector System (Lonna, Cologne, Germany) with program P-016. The protocol suggested by the manufacturer was optimized as follows: cultured cells were detached from plates using 0.25% trypsin–0.05% EDTA (Gibco BRL, Milan Italy), re-suspended in PBS containing 0.2% BSA and counted. Then, 1 × 10^6 cells were collected by centrifugation for 5 min at 800 rpm, re-suspended in 100 μl of transfection reagent R (Nucleofector Cell Line Kit R) containing 4 μg of either recombinant or empty plasmid, placed in a cuvette for the electroporation and immediately plated. After 48 h recovery, transfected cells were used to prepare lattices as described below. In parallel, DCN overexpression in transfected cells was confirmed by western blot using the above antibody.

### Collagen lattice contraction assay

Collagen solution was produced by mixing acidic-soluble type I collagen (Symatese Biomaterials, Chaponost, France) 3 mg/ml, a 5-fold concentration of DMEM and a buffer solution (0.05 M NaOH, 2.2% NaHCO₃, 200 mM HEPES) in the ratio 7:2:1. Collagen solution was mixed with cell suspension in serum-free
medium, plated in six-well cell culture cluster (Costar; Corning, New York, USA) and gelled at 37°C for 30 min. The final concentration of collagen was 2.1 mg/ml with a cell density of 4 x 10^4 cells/ml. Two millilitres of serum-free DMEM was then poured onto the gel to prevent the surface from dehydrating. After 12 h of incubation, the gel was detached from each well and left floating. Surface area of gel samples was measured at detachment (time 0) and after 24 and 48 h. Gel area was calculated on acquired images by computer-assisted morphometric analysis (Axiovision, Zeiss). The contraction of the gel was expressed as percentage of initial lattice area following the formula: A2/A1 x 100, where A1 is the initial gel area and A2 the area at the observed interval. Three culture plates were used for each experimental group.

To analyse lattice contraction in response to TGF-β1, floating gels were treated with 0.25 ng/ml of recombinant human TGF-β1 (R&D Systems).

To test the ability of DCN to counteract the contractile phenotype, gelification was performed without or with 200 nM recombinant human DCN (R&D Systems), in the presence or not of TGF-β1 (0.25 ng/ml), using 2 x 10^3 cells/ml.

In different experiments, pcDNA3-DCN or pcDNA3 transfected fibroblasts were used to prepare lattices (cell density = 2 x 10^3 cells/ml) and treated or not with TGF-β1 (0.25 ng/ml). Forty-eight hours after TGF-β1 treatment, lattices were collected, washed with ice-cold PBS and lysed in 50 mM Tris – HCl, pH 7.4, 150 mM NaCl, 0.5% deoxycholate, 1% SDS, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM NaF and protease inhibitor cocktail (Roche Diagnostic). Lysates were homogenized, centrifuged 10 min at 10000g and used to perform western blot for phosphorylated Smad2 as described above. Experiments were performed at least two times.

Statistical analysis

All numerical data are presented as means ± standard deviation (SD) unless otherwise indicated. Experiments were performed at least in triplicates and were repeated at least two times. Statistical evaluation was performed using an unpaired Student’s t-test. The mean values were considered significantly different when the probability of the differences of that magnitude fell below 5% (P < 0.05).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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