Vascular Smooth Muscle Cell Phenotypic Changes in Patients With Marfan Syndrome


Objective—Marfan’s syndrome is characterized by the formation of ascending aortic aneurysms resulting from altered assembly of extracellular matrix microfibrils and chronic tissue growth factor (TGF)-β signaling. TGF-β is a potent regulator of the vascular smooth muscle cell (VSMC) phenotype. We hypothesized that as a result of the chronic TGF-β signaling, VSMC would alter their basal differentiation phenotype, which could facilitate the formation of aneurysms. This study explores whether Marfan’s syndrome entails phenotypic alterations of VSMC and possible mechanisms at the subcellular level.

Approach and Results—Immunohistochemical and Western blotting analyses of dilated aortas from Marfan patients showed overexpression of contractile protein markers (α-smooth muscle actin, smoothelin, smooth muscle protein 22 alpha, and calponin-1) and collagen I in comparison with healthy aortas. VSMC explanted from Marfan aortic aneurysms showed increased in vitro expression of these phenotypic markers and also of myocardin, a transcription factor essential for VSMC-specific differentiation. These alterations were generally reduced after pharmacological inhibition of the TGF-β pathway. Marfan VSMC in culture showed more robust actin stress fibers and enhanced RhoA-GTP levels, which was accompanied by increased focal adhesion components and higher nuclear localization of myosin-related transcription factor A. Marfan VSMC and extracellular matrix measured by atomic force microscopy were both stiffer than their respective controls.

Conclusions—In Marfan VSMC, both in tissue and in culture, there are variable TGF-β-dependent phenotypic changes affecting contractile proteins and collagen I, leading to greater cellular and extracellular matrix stiffness. Altogether, these alterations may contribute to the known aortic rigidity that precedes or accompanies Marfan’s syndrome aneurysm formation. (Arterioscler Thromb Vasc Biol. 2015;35:960-972. DOI: 10.1161/ATVBAHA.114.304412.)

Key Words: actin ■ aortic aneurysms ■ aortic stiffness ■ extracellular matrix ■ focal adhesion ■ myocardin ■ RhoA ■ TGF-β

Marfan’s syndrome (MFS) is a connective tissue disorder with pleiotropic manifestations affecting mainly the ocular lens, long-bone overgrowth, and dilatation of the proximal aorta, which leads to risk of death by aortic dissection and rupture.1 MFS is caused by mutations in the gene encoding fibrillin-1, which is the major component of extracellular microfibrils and acts as a scaffolding protein for elastin deposition and the formation of elastic fibers. It is widely accepted that MFS arises from a combination of interference with the normal assembly of microfibrils and enhanced transforming growth factor-beta (TGF-β) activation and signaling.2 Despite the identification of distinct TGF-β signaling pathways involved in the pathogenesis of Marfan aneurysm,3,4,5 the mechanisms contributing to aneurysm development remain an open study field.

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In healthy mature blood vessels, vascular smooth muscle cells (VSMC) proliferate extremely slowly, display little synthetic activity, and express a unique set of contractile components, namely α-smooth muscle actin (α-SMA), smooth muscle myosin heavy chain, smooth muscle protein 22 (SM22α or transgelin), and calponin.6–11 Their expression leads to a characteristic contractile or differentiated phenotype, which regulates blood vessel diameter and blood flow. However, vascular remodeling during normal development or in pathogenic conditions induces VSMC to acquire a synthetic and proliferative state, which alters their capacity to generate extracellular matrix (ECM) proteins, such as collagens, or the expression of the contractile machinery, or both.6–8 Therefore, VSMC are plastic cells that may undergo reversible phenotypic changes in response to local growth factors, reactive oxygen species, mechanical forces, and alterations in ECM.12–26 Their collective interactions and signaling interplay determine the final phenotypic characteristics of VSMC in tissue. TGF-β and its family members are among the most potent soluble factors that promote and maintain the VSMC contractile phenotype by upregulating smooth muscle structural genes and at the same time reducing VSMC proliferation and migration.6,14,17,18 However, TGF-β has bifunctional effects because it can also increase VSMC proliferation and migration.19–21 Nonetheless, it is believed that VSMC differentiation and proliferation are not necessarily mutually exclusive, which would facilitate VSMC response and adaptation to a wide variety of pathophysiological conditions. Deregulation in VSMC differentiation occurs in cardiovascular pathologies, such as atherosclerosis and intimal hyperplasia, as well as in aortic aneurysms.25–26 but its occurrence and relevance in the pathogenesis and progression of human Marfan aortic aneurysms has not been explored in detail.

In this study, taking into account the role of TGF-β signaling in the pathogenesis of MFS, we have characterized the cellular phenotype of human Marfan VSMC both in aortic tissue and in cell culture. We confirm previous reports of intrinsic overactivation of TGF-β signaling in VSMC within the medial layer of MFS aortic tissue, as compared with healthy subjects. Concomitantly, expression of contractile proteins is enhanced and collagen I accumulate strongly. VSMC isolated from Marfan patients and maintained in culture also showed these phenotypic changes, which were reduced by the pharmacological inhibition of TGF-β signaling. Alterations in actin stress fibers and increased RhoA signaling were also evident in Marfan VSMC in culture, which correlates with increased VSMC and ECM stiffness. All these alterations are discussed in reference to the signaling and mechanical dysfunctions occurring in the dilated aorta of Marfan patients.

### Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbr.</th>
<th>Definition</th>
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<tr>
<td>αSMA</td>
<td>smooth muscle actin type alpha</td>
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<tr>
<td>CNN1</td>
<td>human calponin-1 gene</td>
</tr>
<tr>
<td>COL1A1</td>
<td>human collagen, type 1, alpha 1 chain gene</td>
</tr>
<tr>
<td>CTGF</td>
<td>human connective tissue growth factor gene</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>ELN</td>
<td>human elastin gene</td>
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<tr>
<td>FA</td>
<td>focal adhesions</td>
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<tr>
<td>MFS</td>
<td>Marfan’s syndrome</td>
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<tr>
<td>MYOC</td>
<td>human myocardin gene</td>
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<tr>
<td>PAI-1</td>
<td>human plasminogen activator inhibitor type-1 gene</td>
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<tr>
<td>SM22α</td>
<td>smooth muscle protein 22 alpha or transgelin</td>
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<tr>
<td>SMTN</td>
<td>human smoothelin gene</td>
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<tr>
<td>SRF</td>
<td>human serum response factor gene</td>
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<tr>
<td>TAGLN</td>
<td>human transgelin gene</td>
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<td>VSMC</td>
<td>vascular smooth muscle cells</td>
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### Results

#### Human Marfan Aortic Aneurysms Overexpress Both Contractile and Secretory Phenotypic Markers

We first assessed the basal level of TGF-β signaling activation in patients with Marfan syndrome and control samples. To this end, pSMAD2 levels were measured by immunohistochemistry in dilated (lesion) and distal visually nondilated (nonlesion) zones of Marfan aortic aneurysms. For each aortic tissue sample, we examined the organization of elastic fibers (an example is given in upper panel in Figure 3A). Aneurysmal aortic segments showed nuclear accumulation of pSMAD2 (Figure 1) than healthy aortas and even than nondilated zones. These results indicate that TGF-β signaling is enhanced in aortic aneurysms of Marfan patients.

Because TGF-β is a potent inducer of the phenotypic differentiation of VSMC, we examined the expression of phenotypic markers of the contractile and secretory or synthetic VSMC phenotypes. The former were represented by SMA, calponin-1, smoothelin, and SM22α/transgelin; the latter by collagen I. Immunostaining showed increased expression of calponin-1 and smoothelin in dilated zones of the tunica media of Marfan aneurysms. SMA and SM22α also increased albeit not significantly. In adjacent nondilated zones, only smoothelin was significantly enhanced (Figure 2A). Immunoblotting of protein extracts from the tunica media (excluding intima and adventitia layers) showed that calponin-1 and SM22α protein levels significantly increased in the dilated and nondilated segments of the Marfan aorta. Smoothelin also increased in the aneurysmal aorta, but its expression varied between samples (Figure 2B). Thus, both technical approaches indicate that Marfan aortic aneurysms variably overexpress contractile protein markers. On the contrary, collagen I content increased only in the dilated segment of Marfan aortas, which invariably correlated with the extensive breakage of elastic fibers (Figure 3A). Curiously, in the dilated zone, stronger collagen I staining was usually observed in the tunica media that limits with the intima and adventitia layers, but in nondilated zones and in healthy aortas, it was only seen in the tunica media adjacent to the intima (Figure 3A). Taken together, these data indicate that VSMC of the tunica media of dilated (lesion) zones of Marfan aortic aneurysms variably overexpress both contractile protein markers and collagen I.

VSMC explanted from human Marfan aortic aneurysms show TGF-β-dependent phenotypic changes accompanied by enhanced RhoA signaling and actin cytoskeleton and focal adhesion rearrangements.
To explore the molecular mechanisms responsible for the phenotypic changes observed in Marfan aneurysm aortic tissue, we cultured VSMC explanted from the tunica media. We first tested whether cultured Marfan VSMC also showed the overactivation of TGF-β signaling pathway seen in human aortic aneurysms (Figure 1). We observed (1) enhanced

**Figure 1.** Tissue growth factor (TGF)-β/SMAD signaling pathway in human Marfan aortic aneurysms. Representative pictures of immunohistochemistry staining of pSMAD2 in ascending aortas from 3 healthy donors and 3 Marfan patients where dilated and adjacent nondilated zones were obtained. Quantitative analysis of pSMAD2 immunostaining shows increased nuclear staining in dilated zones of Marfan aortic aneurysms compared with nondilated zones and healthy aortic tissue. Bars, 100 μm. Quantitative immunohistochemistry obtained from 6 controls and 8 Marfan aortas. *P≤0.05 and **P≤0.01 vs healthy aortas; $P≤0.05$ vs dilated aneurysmal zone.
basal pSMAD2 and pSMAD3 protein levels (Figure 4A) and nuclear accumulation of both pSMADs (Figure 4B); and (2) increased total TGF-β levels in the extracellular medium (conditioned medium; Figure 4C), which in turn increased the pSMAD2 signal response in hepatocarcinoma Hep3B cell line28 (Figure 4D). Moreover, the previous incubation of conditioned media with a neutralizing anti-TGF-β antibody, which was subsequently added to Hep3B cells to evaluate the nuclear translocation of SMAD2, indicated that Marfan-conditioned medium contained more active TGF-β than that
from control VSMC (For details see Results and Figures I–IV in the online-only Data Supplement). Overall, these results indicate that cultured VSMC from Marfan aortas usually maintain intrinsic overactivation of the TGF-β signaling pathway.

We next measured the expression of the aforementioned phenotypic markers. Marfan VSMC usually showed increased contractile protein levels (Figure 5A), which were reduced on treatment with the TGF-β receptor I inhibitor LY364947 (LY) (Figure 5A/+LY). LY strongly reduced the intrinsically high pSMAD2 and pSMAD3 protein levels seen in cultured Marfan VSMC (Figure 4A/+LY; Figure I in the online-only Data Supplement). To assess whether these phenotypic changes occurred at transcriptional level, mRNA expression levels were measured. Regarding the contractile protein markers, only calponin-1 (CNN1) transcript levels increased strongly in all Marfan cells examined (Figure 5B). In contrast, no transcriptional changes occurred for SMA (ACTA2) or smoothelin (SMTN), and the alterations were strikingly reduced for SM22α (TAGLN; Figure 5B). In Marfan cells, LY36497 reduced the transcriptional expression of all contractile markers examined.

Critical in VSMC differentiation is myocardin (MYOCD), which usually requires serum response factor (SRF) for the transcriptional transactivation of VSMC-specific genes. Marfan VSMC showed strong transcriptional upregulation of MYOCD but not of SRF, whereas both were sensitive to LY364947 (Figure 5B). We also evaluated the well-known TGF-β downstream gene targets plasminogen activator inhibitor type-1 (PAI1) and connective tissue growth factor (CTGF). Unexpectedly, CTGF expression was unchanged and PAI1 even decreased in Marfan VSMC. However, as observed for SRF, both were highly sensitive to LY36497. In contrast to clonal and other primary cell types, VSMC already show a variable baseline expression of TGF-β gene targets, which could mask the expected upregulation of these characteristic TGF-β downstream genes. To evaluate this possibility, control VSMC were treated with exogenous TGF-β, and MYOCD, SRF, PAI-1, and CTGF mRNA levels were compared. MYOCD expression increased much more than SRF, PAI1, or CGTF (5-fold versus 1.5-, 1.6-, and 1.9-fold, respectively; Figure V in the online-only Data Supplement), which indicates that myocardin is by far the preferential target of the TGF-β overactivation occurring in Marfan VSMC. As representative of TGF-β-regulated ECM proteins, we examined collagen I (COL1A1) and elastin (ELN) mRNA levels. Unlike ELN, COL1A1 was transcriptionally upregulated, but both were highly sensitive to LY364947 in Marfan cells (Figure 5C). In addition, Marfan cells produced a denser collagen ECM in culture (Figure 5D).
and their extracellular medium contained more collagen than control (Figure 5E). Taken together, these results show that human Marfan VSMC in culture retain the phenotypic expression changes occurring in aortic aneurysms and implicate myocardin as the main agent responsible.

The expression of contractile protein markers by Marfan VSMC may affect actin cytoskeleton organization and its regulation. In this respect, Marfan cells showed more robust actin stress fibers, which were identified by their more compact organization and stronger fluorescence than control cells (Figure 6A). This morphological observation was confirmed biochemically by the higher filamentous (F)/globular (G)-actin ratio of Marfan cells compared with control cells (Figure 6B). LY364947 treatment abolished the actin stress fiber rearrangement (Figure 6A+LY). In addition, active RhoA (GTP-bound RhoA) levels increased in Marfan cells (Figure 6C). It is known that in response to RhoA signaling, the myocardin family member myocardin-related transcription factor is diverted to the nucleus and correlates with the differentiated VSMC phenotype. Unlike myocardin, myocardin-related transcription factor-A (MKLI) mRNA levels were similar in Marfan and control VSMC (Figure VIA in the online-only Data Supplement), but the nuclear accumulation of myocardin-related transcription factor-A was higher in Marfan than in control cells (Figure VIB in the online-only Data Supplement).
Figure 5. mRNA and protein expression levels of vascular smooth muscle cell (VSMC) phenotypic markers of human Marfan VSMC in culture. **A**, Representative Western blotting experiment of indicated contractile protein markers in control and Marfan VSMC in culture with or without the presence of LY364947 (LY; 10 μmol/L for 5 days). On the right, relative densitometry. Contractile markers are increased in Marfan cells compared with control cells. Quantitative results obtained from 2 controls and 4 Marfan VSMCs. N=2. *P≤0.05, **P≤0.01, and ***P≤0.001 Marfan vs control cells; #P≤0.05 and ##P≤0.01 LY-treated vs untreated control cells; $P≤0.05 and $$P≤0.01 LY-treated vs untreated Marfan cells. **B** and **C**, Relative mRNA levels for ACTA2 (α-smooth muscle actin), CNN1 (calponnin-1), TAGLN (transgelin or SM22α), SMTN (smoothelin), MYOCD (myocardin), SRF (serum response factor), PAL1 (plasminogen activator inhibitor type-1), and CTGF (connective tissue growth factor) (B) and COL1A1 (collagen 1) and ELN (elastin) (C) were analyzed by real-time RT-PCR. All data were first normalized to RPL32 mRNA and expression was calculated relative to corresponding levels in control cells. MYOCD, CNN1, and COL1A1 increase their expression in Marfan VSMC. In contrast, TAGLN and PAL1 expression levels were reduced, and the rest of examined genes remained unaltered LY treatment significantly reduced the gene expression of all contractile markers with the exception of SMA. Results obtained from 2–3 controls and 3 Marfan VSMC samples. N=4. *P≤0.05, **P≤0.01, and ***P≤0.001 Marfan vs control cells; #P≤0.05, #P≤0.01, and ###P≤0.001 LY-treated vs their respective nontreated Marfan cells. **D**, Confocal microscopy of the extracellular matrix (ECM) organization synthesized by Marfan and control cells after 3 days of postconfluence stained to collagen I. The collagen extracellular network of Marfan cells is more complex than that of control cells. Bar, 50 μm. Quantitative image analysis shows higher collagen I fluorescence signal in Marfan than in control cells. **E**, Solubilized collagen content in the extracellular medium measured with a colorimetric assay is higher in Marfan than control cells. Results shown in **E** and **F** obtained from 2 control and 3 Marfan VSMC samples. N=2. *P≤0.01 and ***P≤0.001.
Figure 6. Actin stress fibers organization, RhoA activation, cellular and extracellular matrix (ECM) stiffness, and focal adhesions in human Marfan vascular smooth muscle cell (VSMC). **A**, Actin stress fiber organization visualized with phalloidin-TRITC in the presence or absence of LY364947 (LY; 10 μmol/L for 5 days). Marfan VSMC shows more compact stress fibers compared with control cells. LY364947 treatment abolished this actin cytoskeleton rearrangement. Bar, 50 μm. **B**, Filamentous/globular (F/G) actin ratio analysis shows that Marfan cells contain more relative F-actin than control cells. **C**, Representative Western blotting analysis of RhoA GTPase expression. Marfan cells show a higher RhoA-GTP/RhoA ratio than control cells. **D**, Stiffness measured by AFM in different points (indicated by colored dots) over the cell (cellular stiffness) or over cell-to-cell junctions (ECM stiffness; see Material and Methods in the online-only Data Supplement for details) shows that Marfan cells and their ECM are stiffer than respective controls. **P≤0.01. **E**, Western blotting analysis of focal adhesion (FA) components: focal adhesion kinase (FAK) and paxillin and their respective active phosphorylated forms. Marfan VSMC express these components more than control cells. F, Representative immunofluorescence of the subcellular distribution of vinculin and paxillin (and its active phosphorylated form). Marfan VSMC show FA evenly distributed over the plasma membrane, whereas in control cells, they are restricted to the cell periphery. Bar, 50 μm. The density of FA in Marfan cells is also higher than in control cells. **P≤0.05. Results in **C** obtained from 3 control and 4 Marfan VSMC samples. N=2. Results in **D**, **E**, and **F** were from 3 control and 3 Marfan VSMC samples. N=3.
VSMC can regulate the contractile tone of the aorta through the differential expression of contractile proteins and dynamic changes in actin cytoskeleton organization and its regulatory signaling. Consequently, (sub)cellular processes that rely on them should also be perturbed. To this end, we tested the stiffness of VSMC and secreted ECM by measuring the Young (or elastic) modulus by atomic force microscopy. Marfan VSMC were stiffer than control cells (Figure 6D, upper panel). Similar results were also obtained with the secreted ECM (Figure 6D, lower panel). The density and plasma membrane distribution of focal adhesions (FA) is another important regulator of total aortic stiffness and stress. Marfan VSMC expressed more focal adhesion kinase and paxillin (and their respective active phosphorylated forms) than control cells (Figure 6E). Analysis of FA localization in the plasma membrane showed that in Marfan cells, they were widely distributed on the ventral cell surface in contrast to control cells, where they were largely restricted to the cell periphery (Figure 6F). Because the actin cytoskeleton organization as well as the density and the subcellular distribution of FA influence cell migration, we next examined the migration capacity of cells in culture. We observed that Marfan VSMC migrated less than control cells (Figure VII in the online-only Data Supplement). Overall, contractile phenotypic overexpression shown in Marfan cells is accompanied by increased GTP-bound RhoA levels that contribute to a more compact actin stress fiber, focal adhesion rearrangement, and reduced migration. These contractile-associated alterations together with overexpressed collagen I lead to a significant increase in VSMC and ECM stiffness.

Discussion

The major findings of the present study are that aortic aneurysms from Marfan patients show overexpression of contractile protein markers and collagen I, which to a large extent are associated with TGF-β signaling overactivation. VSMC explanted from Marfan aneurysms and kept in culture retain the canonical TGF-β signal pathway, as reported in VSMC derived from human abdominal aortic aneurysms. The high levels of active TGF-β could be attributed to the reduced TGF-β receptor capacity of the defective extracellular assembly of fibrillin-1 microfibrils or to epigenetic modifications, or both. In any case, they are not caused by the transcriptional increase of TGF-β itself, TGF-β receptors, or other downstream molecular components of the signaling pathway. Although cultured Marfan VSMC show intrinsically enhanced TGF-β activation, they still respond to exogenous TGF-β. This indicates that Marfan cells can still modulate the signaling response to the cytokine, more for SMAD2 than for SMAD3. This result is consistent with immunohistochemical findings reported in MFS and bicuspid aortic valve aortic aneurysms and cultured VSMC. However, in contrast to earlier reports that PAL1 was upregulated in aneurysmal aortae of different pathogenesis (which includes Marfan syndrome samples), in the Marfan cultured VSMC studied here its expression decreased (see below). Moreover, in the previous study, no significant differences in active TGF-β levels between aneurysmal and control conditioned media were detected, which again contrasts with our findings. However, these authors do not distinguish between the diverse pathogenesis of the aneurysms examined, and we here evaluated only VSMC-conditioned media from Marfan aortic aneurysms. On the other hand, the difference between SMAD2 and SMAD3 responses could be related to the connective tissue remodeling response because SMAD3 directly affects myofibroblastic differentiation and vascular fibrosis, In sum, as seen in Marfan aortic tissue, the enhanced canonical TGF-β/SMAD pathway in human VSMC in culture validates their use in the study of molecular impairments occurring in MFS and related diseases.

Pathophysiological Significance of Phenotypic Changes of VSMC in Marfan Syndrome

Regulation of the VSMC phenotype is a complex, multifactorial process involving TGF-β and other pathways, whose primary molecular mechanism(s) could be multiple and concurrent. For instance, some microRNAs directly regulate the phenotype of VSMC. Here we show that tunica media of Marfan aortic aneurysms and explanted VSMC overexpress a variety of contractile markers, which could be triggered by the overexpression of myocardin, which induces the expression of contractile differentiation markers in a TGF-β-dependent manner. Regulation is usually performed through SRF-dependent gene transcription, but Marfan cells do not show changes in SRF expression, the basal levels of which are much higher than those of myocardin. In any case, we cannot rule out that myocardin may also act independently of SRF. Therefore, myocardin seems as one of the main TGF-β signaling pathways involved in the pathogenesis of MFS and related diseases.
targets that trigger the overexpression of contractile proteins in Marfan VSMC.

Interestingly, contractile protein upregulation occurs in parallel to (and not in contraposition to) that of collagen I. At first glance, the acquisition of a contractile profile would be expected to antagonize the secretory response necessary for aortic tissue remodeling. However, coexpression of characteristic protein markers of secretory and contractile phenotypes are not necessarily mutually exclusive, and they could take place simultaneously in response to local requirements and to the presence of both stimulatory and inhibitory factors or mechanical stretching.6,14 The overexpression of collagen I is a paradigm in fibrotic disease in which TGF-β is a key fibrogenic cytokine.51 Under chronic TGF-β activation, both Marfan aortic tissue (in dilated zones) and cultured VSMC show higher expression of collagen I. In the aneurysmal aortic wall, this local fibrotic-like response could be aggravated by the fact that aortic dilatation is accompanied by VSMC apoptosis (also seen in our samples; not shown),52–54 which together with the known increased activity of matrix metalloproteinases and elastolysis promotes destructive remodeling and scarring of the tunica media. Such destruction in turn leads to a sustained vicious cycle of ongoing fibrilogenesis, impairing the necessary coordination between contractile and elastic forces, which, respectively, depend on cells and the ECM. We predict that in MFS, primary injury in the extracellular elastic component of tunica media aortic wall concomitantly to chronic TGF-β signaling induces VSMC to express not only more contractile molecular machinery but also more collagen I, thus contributing to aortic stiffness55–59 and subjacent mechano-transduction properties of vascular cells32,33,60 (see below). Although we do not know when these changes become pathological, they may begin during development and perhaps associated with the different embryonic origin and TGF-β sensitivity of VSMC in ascending aorta.51

Changes in the VSMC phenotype are also reported in other aortic aneurysms21,22 in a murine model of MFS.62 Our results are largely coincident with those obtained after the proteomic analysis of the tunica media of human Marfan aneurysms,63 which report strong upregulation of calponin1, filamin A, and vinculin among other contractile proteins. However, this coincidence does not extend to transgelin (SM22α), which in the study by Pilop et al63 was downregulated, but that in our Marfan aortic aneurysms showed variability and even reduced at transcriptional level in cultured Marfan cells. Moreover, VSMC explanted from heterozygous TGF-β1 knockout mice concomitantly to chronic TGF-β1 signaling induces VSMC to express not only more contractile molecular machinery but also more collagen I, thus contributing to aortic stiffness55–59 and subjacent mechano-transduction properties of vascular cells32,33,60 (see below). Although we do not know when these changes become pathological, they may begin during development and perhaps associated with the different embryonic origin and TGF-β sensitivity of VSMC in ascending aorta.51

Pathophysiological Relevance of the Alterations in the Organization and Regulation of the Actin Cytoskeleton and Focal Adhesions in Marfan VSMC

Many of the contractile markers studied here participate in pathways that regulate the plasticity and organization of the actin cytoskeleton to obtain contractile properties. Concomitantly to the acquisition of a more contractile phenotype, human Marfan VSMC show (i) an increase in RhoA-GTP levels, accompanied by more robust actin stress fibers and greater cellular stiffness; (ii) enhanced FA components with altered distribution in the plasma membrane; and (iii) a reduced migration capability. The increase in F/G-actin ratio (indicative of more filamentous actin) and the more robust actin cables in Marfan VSMC are crucial to the generation of mechanical tension.64,65 Together with the overexpression of contractile proteins, this increases the stiffness of the VSMC themselves.32,66 which affects the contractile tone of the Marfan ascending wall.55–59 The more robust stress fiber organization of Marfan cells most likely result from the higher activation of RhoA, which is critical to the regulation of contractility and differentiation of VSMC through the activation of the noncanonical TGF-β signaling pathway.67,68 RhoA also mediates in the assembly of FA signaling complexes (adhesomes).55,65 The fact that Marfan VSMC and ECM are both stiffer than their respective controls likely contribute to alterations in FA and therefore to mechano-transduction responses.60 Importantly, the aortic wall of thoracic aortic aneurysm patients with ACTA2 and MYH11 mutations also shows upregulation of TGF-β signaling.69,70 This points to a functional link between TGF-β and VSMC contractility alterations71 that would involve actin-based modifications, which is indeed what we observe in cultured Marfan VSMC.

Finally, a unique feature of the present study is the comparison between dilated and nondilated zones of human Marfan aortic aneurysms, which aimed to identify exclusive markers of the aneurysmal area. In general, immunohistochemical and Western blotting results indicate that the acquisition of a more contractile phenotype is inherent not only to the Marfan aortic aneurysm, but also to the adjacent nondilated zones, where disruptive molecular events take place without apparent histopathologic damage, but with known biomechanical consequences, such as increased aortic stiffness.57–60,73 The response of collagen I seems to be different. Its overexpression perturbs the tissue organization of the Marfan aortic wall, indicating an adaptive fibrotic response to the elastic fiber disruption in response to TGF-β and biomechanical alterations, which
renders the aortic wall relatively inextensible. Whether this accumulation of fibrillar collagen accelerates the development of aortic aneurysms, or conversely, constitutes an initial beneficial response to hemodynamic stress and tissue deformation, is unclear. The more intense staining for collagen I in the media that limits intima and adventitia layers may represent a local response of VSMC (and myofibroblasts) to reinforce the aortic wall and to compensate, at least partially, for the severe loss of elasticity in the dilated aortic zone.

In conclusion, we report that Marfan VSMC in culture and in aortic tissue undergo phenotypic changes in which TGF-β plays a significant role. The overexpression of contractile machinery, accompanied by a rise in collagen I expression and secretion, increases intrinsic cellular and ECM stiffness, which together contribute to the aortic rigidity that usually precedes or accompanies aneurysm formation in MFS.

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Disclosures

None.

References


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In Marfan’s syndrome, human vascular smooth muscle cell undergo a phenotypic change, expressing both more contractile proteins and collagen I. Such changes are favored by the chronic activation of tissue growth factor-β. Marfan vascular smooth muscle cell in culture show significant RhoA activation followed by nuclear translocation of myocardin-related transcription factor-A, robust actin stress fiber organization, and alterations in focal adhesion components and their subcellular distribution. As a result of changes in the contractile machinery, intrinsic vascular smooth muscle cell stiffness increases. At the same time, there is a fibrotic-like response represented by collagen I over-expression that also renders the secreted extracellular matrix more rigid. The interplay between all these molecular alterations likely contributes to aortic tissue rigidity and subsequent aneurysmal formation and progression in Marfan’s syndrome. Molecular or pharmacological intervention directed at any of these subcellular alterations that could revert these phenotypic changes or the increase in vascular smooth muscle cell stiffness could lead to new therapies for Marfan’s syndrome.

**Significance**


Vascular Smooth Muscle Cell Phenotypic Changes in Patients With Marfan Syndrome
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SUPPLEMENTAL MATERIAL

VASCULAR SMOOTH MUSCLE CELL PHENOTYPIC CHANGES IN PATIENTS WITH MARFAN SYNDROME


From the University of Barcelona School of Medicine (Barcelona, Spain) (E.C-M., T.M., C.S-P., J.S., D.G., Y.M., V.H., E.S., J.J.U., D.N., I.F., G.E.); Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) (Barcelona, Spain) (G.E.); Institut de Nanociència i Nanotecnologia (IN²UB) (Barcelona, Spain) (G.E.); Institut de Bioenginyeria de Catalunya (IBEC) (Barcelona, Spain) and CIBER de Enfermedades Respiratorias (CIBERES) (D.N.); Heart Institute (InCor), University of São Paulo School of Medicine (São Paulo, Brazil) (T.M.); Institut d’Investigació Biomèdica de Bellvitge (IDIBELL) (L’Hospitalet de Llobregat, Barcelona) (E.C.-M., J.L.-L., L.C., E.B., I.F.); Centro de Biología Molecular Severo Ochoa (CSIC-UAM, Madrid) (O.B., F.R.-P.); Hospital de Bellvitge-IDIBELL (L’Hospitalet de Llobregat, Barcelona) (C.G.-C., E.C., D.T.); Hospital Clinic i Provincial (Barcelona) (M.C.); Hospital 12 de Octubre (Madrid) (A.F.); Johns Hopkins University and Howard Hughes Medical Institut (Baltimore, USA) (H.D.D.)

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SUPPLEMENTAL RESULTS

Vascular smooth muscle cells isolated from ascending aorta aneurysms from Marfan patients maintain constitutive overactivation of the TGF-β-SMAD signaling pathway

VSMC were seeded to the same cellular density (200,000 cells) and maintained in culture for 2 and 7 days for an initial comparative study (see C_{2d} and C_{7d} lines in supplemental Fig. I). Marfan VSMC showed higher pSMAD2 and pSMAD3 protein levels both at 2 (Fig. 4A; supplemental Fig. I) and at 7 days (supplemental Fig. I) in culture compared with control cells. These results demonstrate that the canonical TGF-β signaling pathway is intrinsically activated in human Marfan VSMC in culture. To confirm that this is attributable to the postulated autocrine up-regulation of the TGF-β signaling pathway, Marfan VSMC were treated with LY364947 (LY), a specific inhibitor of the TGF-β receptor I (TGFβRI/ALK5)\(^1\,2\). pSMAD2 and pSMAD3 levels were greatly reduced after LY treatment (LY lines in Fig. 4A and supplemental Fig. I). The addition of exogenous TGF-β (2 ng/mL; see Tβ line in supplemental Fig. I) enhanced the already constitutively high pSMAD2 but not pSMAD3, whose response was abrogated after LY addition (LY+Tβ lines in supplemental Fig. I).

Once SMADs2/3 are phosphorylated in the cytoplasm, they are quickly translocated to the nucleus. Marfan cells showed a stronger nuclear immunofluorescence signal than control cells (Fig. 4B). We also examined the characteristic induced nuclear localization of SMAD2 and SMAD3 after the treatment with TGF-β. Control cells showed an unstained (black) nucleus, which became positive for both SMADs after the addition of TGF-β (supplemental Fig. II). Cultured Marfan VSMC already showed a basal nuclear staining for both SMADs (compare control and Marfan cells in left panels in supplemental Fig. II). When Marfan cells were subsequently stimulated with TGF-β, the basal nuclear fluorescence for SMAD2 increased, but this was not the case for SMAD3 (+TGF-β panels in supplemental Fig. II). These results are consistent with the observation that the exogenous stimulation with TGF-β produced an additional increase in pSMAD2, but not in pSMAD3 protein levels in Marfan VSMC (compare C_{2d} and Tβ lines in supplemental Fig. I), whose levels were already very high even in basal culture conditions. Taken together, these findings strongly indicate that cultured Marfan VSMC show chronic activation of the canonical TGF-β signaling pathway. To explore whether this could primarily be attributed to changes in the mRNA levels of TGF-β itself, its receptors (TβRII) or downstream signaling effectors, we performed real-time RT-PCR analysis. Marfan cells showed no significant transcriptional changes in TGF-β or TGFβRI, but SMAD2, which decreased in the three Marfan cell lines and TβRII, also altered transcription in one out of the three Marfan cell lines examined (supplemental Fig. IIIA). No differences were observed in the expression of the co-SMAD4, inhibitory SMADs 6 and 7, the adaptor SARA or the type III TGFβ receptor β-glycan (supplemental Fig. IIIB), which taken together indicate that the constitutive over-activation of the TGF-β pathway in cultured Marfan VSMC is not primarily caused by the transcriptional increase of essential molecular components of the TGF-β pathway. The decrease observed in SMAD2 gene expression is likely caused by some autoregulatory mechanism to counteract the chronic activation by high active TGF-β levels present in the extracellular medium of Marfan cells, which were measured both by ELISA and the conditioned medium added to hepatocellular carcinoma Hep3B cells, which are highly sensitive to TGF-β.\(^3\)

We also carry out a comparative analysis of LY36497 with a neutralizing anti-TGF-β1 antibody to confirm that, besides more total content of TGF-β (Fig. 4C), Marfan conditioned medium also contained more active TGF-β as results already shown in Fig. 4D demonstrate. In particular, neutralizing anti-TGF-β1 antibody blocked the nuclear translocation of SMAD2 triggered by Marfan conditioned medium when it was added to Hep3B cells (supplemental figure IV). This experiment was done in parallel with...
LY36497, whose results were the same as those with anti-TGF-β1 antibody and, therefore, validate the use of LY36497 as a suitable tool to explore the involvement of TGF-β signaling pathway as previously reported.\textsuperscript{2,3,4} However, it cannot be discarded a partial inhibition of other signaling pathways such as p38 and MAPK, although LY36497 inhibits them with much less efficacy (200 fold).\textsuperscript{4,5}

\textbf{SUPPLEMENTAL REFERENCES}


Supplemental Figure I. Overactivation of the TGF-β-SMAD signaling pathway in VSMC explanted from human Marfan aortic aneurysms (part II): additional capacity of cells to respond to exogenous TGF-β. Representative western blotting experiments and densitometry analysis of pSMAD2 and pSMAD3 in one control and two Marfan VSMC lines (MFC1 and MFC2) after 2 and 7 days of culture (C_{2d} and C_{7d}, respectively). Marfan cells show endogenous higher pSMAD2 and pSMAD3 levels compared with control cells. LY364947 treatment (LY lines; 10 µM for 2 days) abolished SMADs activation. In Marfan VSMC, exogenous added TGF-β (Tβ lines) produced an additional response in pSMAD2 levels but not in pSMAD3. pSmad3 (1) and pSmad3 (2) respectively represent a short and long film expositions; the latter done to visualize bands in control cells.
**Supplemental Figure II.** Overactivation of the TGF-β-SMAD signaling pathway in VSMC explanted from human Marfan aortic aneurysms (part III): basal and TGF-β-induced nuclear translocation of SMAD2 and SMAD3. Immunoflorescence staining of SMAD2 and SMAD3 in cultured control and Marfan VSMC (MFC1, MFC2 and MFC3) under basal culture conditions and after the addition of exogenous TGF-β. Unlike control cells, Marfan VSMC already show nuclear staining of both SMAD proteins in the nucleus. In control VSMC, TGF-β addition produced the expected nuclear localization of both SMADs, but in Marfan cells, nuclear staining increased only for SMAD2 but not for SMAD3.
Supplemental Figure III. Transcriptional expression of the TGF-β, its receptors and signaling components in cultured Marfan VSMC. (A) Quantitative and (B) semi-quantitative RT-PCR analysis of mRNA isolated from explanted VSMC from Marfan patients (MFC1, MFC2 and MFC3) and healthy donors (controls). Marfan VSMC consistently show no changes in the expression of any TGF-β signaling components genes except SMAD2, whose expression levels are significantly reduced compared with control cells. Results in (A) obtained from two control and three Marfan VSMC. N=4. Statistical significance: ***p ≤ 0.001.
Supplemental Figure IV. Conditioned extracellular medium from Marfan VSMC contain high levels of bioactive TGF-β. Comparative analysis between LY364947 and neutralizing anti-TGF-β1 antibody to inhibit the nuclear translocation of SMAD2 in Hep3B cells induced by the conditioned extracellular medium from control and Marfan VSMC. Hep3B cells were incubated with the conditioned medium from control (CO1 and CO4) or Marfan (MFC1 and MFC4) VSMC in the absence or presence of LY36497 (10 mM) or neutralizing anti-TGF-β antibody (100 µg/ml). Preliminary experiments showed that this concentration of anti-TGF-β antibody was the most effective to totally inhibit the nuclear translocation of SMAD2 caused by the addition of TGF-β (2 ng/ml) to Hep3B cells cultured in absence of FBS (not shown). Note that the conditioned medium from control VSMC causes no or little nuclear localization of SMAD2 compared with baseline Hep3B, which the latter was blocked by LY and anti-TGF-β antibody treatments. However, conditioned media from Marfan VSMC causes an evident nuclear translocation of SMAD2, which was equally blocked by both treatments.
Supplemental Fig V. Comparative mRNA expression levels of MYOCD, SRF, PAI1 and CTGF in VSMC treated with TGF-β. Relative mRNA levels of these genes were analyzed by real-time RT-PCR in control VSMC with (T) or without (C) exogenously added TGF-β (T; 2 ng/ml) for 24 or 48 h. After the addition of the cytokine, the increase in MYOCD mRNA expression is much higher than in the other three genes. Results from two different control VSMC (N=3). Statistical significance: *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001 VSMC treated with TGF-β in comparison with control not-treated cells.
Supplemental Figure VI. Myocardin related transcription factor in human Marfan VSMC. (A) Relative mRNA levels of MRTF-A (MKL1) were analyzed by quantitative real-time RT-PCR. Marfan and control cells did not showed differences. Results from two control and three Marfan VSMC. N=4. Statistical significance: ###p ≤ 0.001 vs not-treated Marfan cells. (B) Representative images of three control and Marfan VSMC lines stained with antibodies to MRTF-A. Marfan cells showed a higher nuclear staining of MRTF-A than control cells. *p ≤ 0.05.
Supplemental Figure VII. Reduced migration capacity of cultured Marfan VSMC. Representative experiment of the migration assay monitored in real-time by XCELLigence system. Results show reduced migration capacity of Marfan VSMC compared with control cells. Bars represent the quantitative value of the slope for each cell line examined. Results obtained from two control and three Marfan VSMC. N=4. Statistical significance: *p ≤ 0.05.
SUPPLEMENTAL MATERIAL AND METHODS

VASCULAR SMOOTH MUSCLE CELL PHENOTYPIC CHANGES IN PATIENTS WITH MARFAN SYNDROME


From the University of Barcelona School of Medicine (Barcelona, Spain) (E.C.-M., T.M., C.S.-P., J.S., D.G., Y.M., V.H., E.S., J.J.U., D.N., I.F., G.E.); Institut d’Investigacions Biomèdiques August Pi i Sunyer (IDIBAPS) (Barcelona, Spain) (G.E.); Institut de Nanociència i Nanotecnologia (IN²UB) (Barcelona, Spain) (G.E.); Institut de Bioenginyeria de Catalunya (IBEC) (Barcelona, Spain) and CIBER de Enfermedades Respiratorias (CIBERES) (D.N.); Heart Institute (InCor), University of São Paulo School of Medicine (São Paulo, Brazil) (T.M.); Institut d’Investigació Biomèdica de Bellvitge (IDIBELL) (L’Hospitalet de Llobregat, Barcelona) (E.C.-M., J.L.-L., L.C., E.B., I.F.); Centro de Biología Molecular Severo Ochoa (CSIC-UAM, Madrid) (O.B., F.R.-P.); Hospital de Bellvitge-IDIBELL (L’Hospitalet de Llobregat, Barcelona) (C.G.-C., E.C., D.T.); Hospital Clinic i Provincial (Barcelona) (M.C.); Hospital 12 de Octubre (Madrid) (A.F.); Johns Hopkins University and Howard Hughes Medical Institut (Baltimore, USA) (H.D.D.)

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Reagents and antibodies
The following polyclonal antibodies were used: anti-actin antibody from Cytoskeleton (Denver, CO, USA); anti-SMA from Merck Millipore (Billerica, MA); anti-SM22α from Abcam (Cambridge, UK), anti-Calponin-1 from Sigma-Aldrich (St Louis, MO, USA); anti-phospho-paxillin [Y118] from Invitrogen (Carlsbad, CA, USA); anti-Focal Adhesion Kinase (FAK) from Abcam; anti-phospho-FAK (Tyr397) from Cell Signaling; anti-phospho-SMAD2 and anti-phospho-SMAD3 from Cell Signaling (Boston, MA, USA); anti-collagen I from Millipore (catalogue number AB758); anti-MRTF-A from Santa Cruz Biotechnology (catalogue number sc-47282). The following monoclonal antibodies were used: anti-Rhoa-GTP from New East Biosciences (Malvern, PA, USA); anti-α-tubulin, anti-actin and anti-vinculin from Sigma-Aldrich; anti-paxillin and anti-Rhoa from BD Biosciences (San Jose, CA, USA); anti-Smoothelin (R4A) from Santa Cruz (Dallas, TX, USA); anti-SMAD2 and SMAD3 from Cell Signaling. Secondary polyclonal goat anti-rabbit, goat anti-mouse, donkey anti-goat IgG horseradish peroxidase antibodies were from Promega (Eugene, OR, USA). Anti-mouse and anti-rabbit Alexa 488- and Alexa 488-conjugated secondary antibodies were from Invitrogen. Tetramethyl Rhodamine Iso-Thiocyanate (TRITC)-phalloidin and Elastin Stain Kit (HT25A-1KT) were purchased from Sigma-Aldrich. Anti-TGF-β1 antibody [2Ar2] (Abcam, Cambridge, UK; Ref: ab64715). Human TGF-β1 Quantikine ELISA Kit was from R&D Systems (DB100B).

Tissue collection, ethics statements and subjects
Normal ascending aortic tissue was collected from heart donors (n = 6) through the organ donation organization at the Hospital Clinic i Provincial (Barcelona, Spain) and Hospital De Bellvitge de l’Hospital de Llobregat (Barcelona, Spain). The age and gender of heart donors were unknown because Spanish law protects personal information about organ donors. Ascending aortic aneurysm samples were collected from MFS patients (n = 9; four males and five females with ages ranging from 23 to 59 years) undergoing aortic aneurysm repair surgery. All the patients in whom aortas were resected fulfilled Marfan diagnostic criteria according Ghent nosology1, but no genetic information of putative FBN1 mutations was available. In all patients, we obtained a 3 x 3 cm sample from two areas: the dilated zone, corresponding to the sinuses of Valsalva, and the non-dilated aorta corresponding to the distal ascending aorta. The aortas were maintained in cold saline solution before delivery to the laboratory. Human tissues were collected with the required approvals from the Institutional Clinical Review Board from clinical centers both in the USA (Johns Hopkins Hospital) and Spain (Hospital Clinic i Provincial and Hospital de Bellvitge in Barcelona, and The Marfan Unit from Hospital 12 de Octubre in Madrid) and patients’ written consent conformed to the ethical guidelines of the 1975 Declaration of Helsinki. All aortic tissues described in the manuscript are those obtained from Spanish heart donors and Marfan patients.

Human Vascular smooth muscle cells culture
Human VSMC were isolated from healthy and Marfan aortas using the primary culture explant methodology2. Ascending aortic tissue was first cleaned of fatty tissue and separated from intima and adventitia layers, leaving tunica media alone. Subsequently, tunica media was cut into 1–2 mm cubes, which were transferred to 100 mm culture plates. After their adhesion at 37°C for 45 min in the incubator, aortic media small cubes were gently covered with 4 mL of 231 culture medium (Gibco) supplemented with 25 mL of Smooth Muscle Growth Supplement (SMGS) from Gibco, 100 mg/mL streptomycin and 100 U/mL penicillin, and plasmocin. Cell cultures were maintained at 37°C in a humidified 5% CO2 atmosphere. Explants were left undisturbed for 4 days to prevent detachment, and half the medium was replaced every 4 days. VSMC migrate out from the explants within 1–2 weeks. Then, after removing the explants from the flask surface, the cells were trypsinized, used as P1 stage cells and routinely
subcultured. Primary cultures have limited expansion and were used for experiments between passages P3 to P10. Cultured control VSMC used in this study were only those that successfully derived from explants of small aortic media pieces available from ascending aorta from heart donors.

**Western blotting analysis**

Aortic samples were homogenized with a pestle in a liquid nitrogen-cooled mortar. The disaggregated tissue was incubated with lysis buffer (30 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 2% SDS, 5 mM EGTA, 0.5% NP40, and proteases and phosphatases inhibitors) for 1 h at 4°C with continuous shaking. Samples were subsequently centrifuged at 1,500 x g for 10 min at 4°C.

Cell extracts were prepared by aspirating cell culture medium, washing in ice-cold PBS, adding cold lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, pH 7.4) containing protease (aprotinin, leupeptin, and phenylmethylsulfonyl fluoride and pepstatin A) and phosphatase (sodium orthovanadate inhibitors). Cells were harvested with a scraper, and samples were incubated for 10 min on ice, then sonicated (two rounds of 5 s at 80%) and finally centrifuged (1000 x g for 10 min at 4°C).

To extract cytoskeleton proteins, cells were washed in ice-cold PBS and incubated with ice-cold cytoskeleton buffer (10 mM PIPES, 100 mM NaCl, 300 mM Sucrose, 3 mM MgCl₂, 1 mM EGTA, plus the above mentioned protease and phosphatase inhibitors) for 30 min at 4°C. Subsequently, cells were scraped and homogenates were centrifuged at 4,500 x g, for 15 minutes at 4°C; pellets were resuspended in ice-cold PBS, and centrifuged again at 18,000 x g, for 10 min at 4°C. Finally, the clarified pellets were resuspended in lysis buffer (25 mM HEPES, 2.5 mM EDTA, 0.1% Triton TX-100, plus protease and phosphatase inhibitors) and gently sonicated (three rounds of 10 s at 40%).

For SMAD proteins analysis cells were incubated with a lysis buffer containing 30 mM Tris-HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10% glycerol, leupeptin, sodium orthovanadate and PMSF for 1 h at 4°C, and centrifuged at 13,000 rpm for 10 min at 4°C. Subsequently, pellets were resuspended in ice-cold PBS, and centrifuged again at 18,000 x g, for 10 min at 4°C. Protein was determined with the DC Protein Assay from BioRad (Hercules, CA, USA).

Equal amounts of lysates were loaded with loading buffer 5x (containing 10% β-mercaptoethanol), subjected to 10% (v/v) sodium dodecylsulfate (SDS)-polyacrylamide gel electrophoresis and transferred (90 min, 100 mV) to nitrocellulose or PVDF membranes. Blots were blocked in 5% BSA or 5% low fat milk/Tris Buffered saline (TBS) with Tween buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) for 1 h, and immunoblotted to test the various antigens listed above. Primary antibodies were diluted in phosphate-buffered saline (PBS) containing sodium azide, and blots were incubated overnight at 4°C. The following day, blots were rinsed and incubated with secondary horseradish peroxidase -tagged IgG antibodies for 1 h in 5% BSA or low fat milk/TBS with Tween buffer. After several rinses, blots were developed for 1 min at room temperature with Western Blotting Luminol Reagent (Santa Cruz, Delaware, CA, USA). Bands were visualized by exposure of the membrane to Hyperfilm (Amersham Pharmacia Biotech, Uppssala, Sweden). Band intensities were measured by densitometry scanning of the film using Image J software. Immunoblots shown are representative of at least three independent experiments.

Working dilutions of (1) polyclonal antibodies: anti-actin antibody, 1/5000; anti-SMA; anti-SM22α, 1/3000; anti-calponin-1, 1/1000; anti-phospho-paxillin [Y118], 1/100; anti-Focal Adhesion Kinase (FAK), 1/1000; anti-phospho-FAK (Tyr397), 1/1000; anti-phospho-SMAD2 and anti-phospho-SMAD3, both at 1/1000; (2) monoclonal antibodies: anti-Rhoa-GTP, 1/1000; anti-α-tubulin, 1/5000; anti-paxillin, 1/4000; and anti-Rhoa, 1/2000; anti-smoothelin (R4A), 1/1000; anti-vinculin, 1/400. Secondary polyclonal goat
anti-rabbit, goat anti-mouse, donkey anti-goat IgG horseradish peroxidase antibodies, 1/3000.

**Immunohistochemistry and immunofluorescence**

For immunohistochemical analysis, aortic specimens were fixed in buffered formalin for at least 24 h, processed, and embedded in paraffin. Expression and localization of antigens of interest were analyzed using immunohistochemistry on 5-µm-thick paraffin sections of aortas. Primary antibodies were pSMAD2 (1/500), SMA (1/3000), SM22α (1/3000), calponin-1 (1/1000), smoothelin (1/1000), MRTF-A (1/250), and collagen I (1/1000). Consecutive paraffin sections of the same aortic tissue sample were stained for the different antibodies, which were previously subjected to titration to give the best signal/noise ratio. Tissue was incubated overnight in a humidified chamber at 4°C with a primary antibody. Anti-mouse and anti-rabbit Alexa 546- and Alexa 488-conjugated secondary antibodies were from Invitrogen and used at 1/50, 1/250 and 1/500, respectively. A secondary antibody (Dako LSAB + System HRP) was applied for 1 h, followed by Liquid DAB+Substrate Chomogen System (Dako) for 1-7 min. Sections were counterstained with hematoxylin to visualize the cell nucleus, dehydrated in ethanol and xylene, and mounted permanently in DPX (BDH). Negative controls were processed in the same manner but without the primary antibody. Tetramethyl Rhodamine Iso- Thiocyanate (TRITC)-phalloidin was used at 0.02 mg/mL. For quantitative analysis, three or four areas of each paraffin section were quantified and the mean was calculated for each sample. Results are expressed as the percentage of immunostained area in relation to total image area. For pSMAD2, stained nuclei were represented as the percentage respect to the total nuclei. All analyses were made using Image J software from pictures taken under oil immersion x40 objective magnification. The results are plotted as mean of all samples ± SEM.

For immunofluorescence, VSMC grown on coverslips were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature, rinsed in PBS (3 x 5 min) and incubated in PBS containing 50 mM ammonium chloride. Cells were then permeabilized for 10 min with PBS containing 0.1% saponin and 1% BSA. Primary antibodies were incubated for 1 h at room temperature or at 4°C overnight and secondary antibodies for 45 min in PBS containing 1% BSA. For MRTF-A, fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min, and blocked with 1% FBS in PBS for 30 min. In this case, cells were also co-stained with DAPI (300 nM) for 5 min. For MRTF-A quantitative analysis, the fluorescence intensity of the nucleus was measured and related to the total fluorescence intensity of each cell examined.

Actin cytoskeleton was visualized by incubating cells for 30 min with TRITC- or FITC-labelled phalloidin. Coverslips were mounted onto microscope slides using Mowiol. Cells were observed on an Olympus BX60 epifluorescence microscope and recorded with a cooled Orca-ER CCD camera (Hamamatsu Photonics, Japan). The images were processed using Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA) and Image J software. Microscopy and imaging were performed with an Olympus BX60 epifluorescence microscope with a cooled ORCA-ER CCD camera (Hamamatsu Photonics, Japan).

To analyze the collagen network, VSMC were grown to confluence on coverslips and supplemented with ascorbic acid (50 µg/mL) for 3 days to start collagen ECM production. After this period, we followed the same fixation and immunostaining protocol described above.

**Filamentous-/Globular-actin ratio**

VSMC were homogenized in lysis buffer (50 mM PIPES, pH 6.9, 50 mM KCl, 5 mM MgCl₂, 5% (v/v) glycerol, 0.1% Nonidet P40, 0.1% Triton X-100, 0.1% Tween 200, 1% 2-mercaptoethanol and 0.001% antifoam C) containing 250 nM of TRITC-phalloidin. Lysates were centrifuged at 100,000 x g for 1 h at room temperature. Pellet contained
the F-actin fraction and the supernatant contained the G-actin fraction. Samples were analyzed by immunoblotting for actin.

Atomic Force Microscope (AFM) measurements of stiffness
Human vascular smooth muscle cells explanted from ascending aortas from heart donors and from a MFS patients were seeded sparse on 35 mm culture dishes with growth medium and led to adhere on the plastic surface for 48 h. To estimate the VSMC stiffness each cell (n= 5) was measured on three different perinuclear points of the plasma membrane (illustrated with colored dots in upper drawing of Fig. 6D). To estimate the stiffness of the secreted ECM, cells were seeded on 35 mm culture dishes and cultured with growth medium containing ascorbic acid (50 μg/mL) for 8 days post-confluence. At this time of culture, a mature ECM is already formed (see Fig. 5D). The growth medium containing freshly prepared ascorbic acid was changed every 48 h. AFM measurements were performed in five different points separated each by 5 μm onto the biological material located on cell-to-cell junctions (illustrated with colored dots in lower drawing of Fig. 6D), where most likely ECM is measured. The VSMC and ECM stiffness was probed with a custom-built atomic force microscope attached to an optical inverted microscope (TE2000, Nikon, Japan) following the procedure described previously. Measurements were carried out at room temperature by using a V-shape Au-coated cantilever of nominal spring constant (k) of 0.01 N/m with a microsphere (4.5 μm in diameter) attached at its end (Novascan, IA). The cantilever was displaced in 3-D with nanometric resolution with piezoactuators coupled to strain gauge sensors (Physik Instrumente, Germany) to measure cantilever displacement (z). The deflection of the cantilever (d) was measured with the optical lever method. The sensitivity of the optical system was calibrated by recording a deflection-displacement (d-z) curve in a bare region of the glass slide. A fully linear calibration curve with a sharp contact point was taken as indicative of a clean and undamaged tip. The force applied by the cantilever was computed as F = k·d. At each measurement point, five force-displacement curves (F-z) were recorded by vertically oscillating the cantilever with triangular displacement at 1 Hz and peak-to-peak amplitude of 4 μm (cantilever speed of 8 μm/s) to reach a maximum indentation of ~1 μm. The indentation (δ) of the sample was computed as δ = (z-zc)-(d-do), where zc is the position of the contact point, and do the offset of the cantilever deflection. The effective Young’s modulus (E) was computed by fitting the elastic Hertz contact model of a sphere to the force-indentation data using the spherical Hertz model:

\[
F = \frac{4kR^{1/2}}{3(1-\mu^2)}\delta^{3/2}
\]

where \(\mu\) is the Poisson’s ratio assumed to be 0.5. The spherical Hertz model can be expressed in terms of cantilever displacement and deflection as

\[
d = do \frac{4kR^{1/2}}{3(1-\mu^2)}[z - zc - (d - do)]^{3/2}
\]

The Young’s modulus together with zc and do were computed by least squares fitting of Eq. 2 to the approaching phase of the d-z curve using custom built software (Matlab, The MathWorks, MA). The fitting was performed with a maximum indentation of ~0.5 μm to avoid inaccuracy of zc determination at shallow indentations and the effect of the rigid substrate at deep indentations. For a given measurement point, E was computed as the average of the values obtained in the five d-z curves. The stiffness of each cell was taken as the average of the E values computed in the three measured points. The stiffness of the ECM at each cell-cell junction was characterized as the average of the E values computed in the five measured points.
TGF-β content
Cultured VSMC from control and Marfan patients were seeded at a confluent cell density (200,000 cells in a p60 culture plate) and let them grow for seven days without to change the culture medium. Thereafter, the respective extracellular medium was collected and the total cytokine present was quantitated by ELISA kit (R&D Systems, Minneapolis, MN). To analyze the bioactivity of the secreted TGF-β, the collected medium was given in parallel to human hepatocellular carcinoma Hep3B cells. Phospho-Smad2 protein levels in Hep3B cells after being incubated for 2 h with diluted series of the control and Marfan VSMC conditioned medium were examined by western blotting.

Gene expression analysis
RNAeasy Mini Kit (Qiagen, Valencia, CA, USA) was used for total RNA isolation. Reverse transcription (RT) was carried out using the High Capacity Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA), and 1µg of total RNA from each sample for complementary DNA synthesis. PCR products in semiquantitative reactions were obtained after 30–35 cycles of amplification at annealing temperatures of 57-62 ºC, and analyzed by 1.5% agarose gel electrophoresis. Expression of 18S rRNA was analyzed as a loading control, as indicated. For Real-Time PCR, expression levels were determined in duplicate in a LightCycler® 480 Real-Time PCR System, using the LightCycler® 480 SYBR Green I Master (Roche Applied Science) for human primers. Real-time PCR and semiquantitative PCR reactions were performed using the primers related in the following supplemental Tables I and II, respectively. Expression of RPL32 gene used was used as house-keeping control.

Supplemental Table I: Real-time PCR primer sets used

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5'-3')</th>
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<tbody>
<tr>
<td></td>
<td>Sense</td>
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<tr>
<td>RPL32</td>
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<tr>
<td>TGFBR1</td>
<td>ACATGATTCAGCCACAGATACC</td>
</tr>
<tr>
<td>TGFBR2</td>
<td>CTGTGGATGACCTGGTAAAC</td>
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<tr>
<td>TGFBR2</td>
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<tr>
<td>TGFBR3</td>
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<tr>
<td>SMAD2</td>
<td>CATTTCCAGAGCACCAGAG</td>
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<tr>
<td>SMAD3</td>
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<tr>
<td>ACTA2</td>
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<td>SMTN</td>
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<tr>
<td>CNN1</td>
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<td>TAGLN</td>
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<tr>
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<tr>
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<tr>
<td>CTGF</td>
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</table>
Supplemental Table II: Semiquantitative PCR primer sets used

<table>
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<th>Gene</th>
<th>Primer sequences (5'-3')</th>
<th>Primer sequences (5'-3')</th>
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<td>Antisense</td>
<td>Sense</td>
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<tr>
<td>ZFYVE9</td>
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<td>TGTCCATTAGCAGAGTAGCC</td>
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<tr>
<td>ENG</td>
<td>GCTGCACTCTGGTACATCTAC</td>
<td>ACTGGGTGAGGTTCTGTG</td>
</tr>
</tbody>
</table>

Cell migration assay

Cell motility was examined by real-time migration assay through the xCELLigence System (Roche, Mannheim, Germany). 4x10^4 cells/well were seeded onto the top chamber of a CIM plate, which features microelectronic sensors integrated on the underside of the microporous membrane of a Boyden like chamber. CIM plates were placed onto the Real-Time Cell Analyzer (RTCA) station. Cell migration was continuously monitored throughout the experiments by measuring changes in the electrical impedance at the electrode/cell interface, as a population of cells migrated from the top to the bottom chamber. Continuous values were represented as Cell Index (CI), a dimensionless parameter, which reflects a relative change in measured electrical impedance, and quantified as a slope (h^-1) of the first 5 hours.

Determination of collagen into cell culture medium

VSMC from control and Marfan patients were grown to confluence in 10 cm² wells. 1 mL of the culture media was collected after 3 days of culture in the presence of 50 µg/mL of ascorbic acid. The total amount of collagen (types I to V) in the culture medium was determined using the Sircol® collagen assay kit (Biocolor Ltd., Northern Ireland, UK), a colorimetric assay based on the specific binding of Sirius Red in picric acid dye reagent to collagen. Briefly, first collagen in the culture medium was concentrated in Tris-HCl buffer at pH 7.6 containing polyethylene glycol. Then collagen was incubated with the dye reagent to form a collagen-dye complex that was precipitated, the reagent combined to collagen was solubilized, and the absorbance of the solution was measured at 555 nm wavelength. Collagen concentration in the sample was estimated in a collagen standard curve.

Statistics

Quantitative real-time RT-PCR and immunoblotting densitometry data were expressed as mean ± standard deviation (SD) and analyzed for statistical significance using GraphPad prim software. Student’s t test was used when two groups -control and Marfan cells- were compared (as shown in Figs. 4C, 5D and 5E, 6C, 6D and 6F, and Supplemental Figs. II A, V and VII). Two-way analysis of variance (ANOVA) was used when two groups -control and Marfan cells- and two experimental conditions -untreated and LY-treated cells- were compared (as shown in Figs. 4A, 5A, 5B and 5C, and Supplemental Fig. VI A). Immunohistochemical and immunofluorescence data were respectively analyzed for statistical analysis by one-way ANOVA, Tukey’s multiple comparison test. The number of different control and Marfan cells used in the study is stated in figure legends. “N” means the number of independent experiments done
using the different control and Marfan cells examined. p values equal to or less (≤) than 0.05 (*; #; $), 0.01 (**; ##; $$) or 0.001 (***; ###; $$$) were considered significant.

SUPPLEMENTAL REFERENCES


