1 *Running title:* **Generation of the first hiPSC-derived vascular model of**
2 **Marfan syndrome**

3 **Title:** An iPSC-derived vascular model of Marfan syndrome identifies key
4 **mediators of smooth muscle cell death**

5 **Author list:** Alessandra Granata*, Felipe Serrano, William George Bernard,
6 Madeline McNamara, Lucinda Low, Priya Sastry, and Sanjay Sinha

7 **Affiliations:** Wellcome Trust-MRC Cambridge Stem Cell Institute, Anne
8 McLaren Laboratory, University of Cambridge, Cambridge, UK.

9 Division of Cardiovascular Medicine, University of Cambridge, UK

10 * Division of Neurology, Cambridge Biomedical Campus, University of
11 Cambridge, Cambridge, UK.

12 Corresponding author: Sanjay Sinha, ss661@cam.ac.uk, tel +44 1223

13 747479

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Abstract

Marfan syndrome (MFS) is a heritable connective tissue disorder caused by mutations in fibrillin-1, an extracellular matrix protein. To investigate the pathogenesis of aortic aneurysms in MFS, we have generated the first vascular model derived from human induced pluripotent stem cells (hiPSC). Our MFS-hiPSC derived smooth muscle cells (SMC) accurately recapitulated the pathology seen in Marfan aortas, including defects in fibrillin-1 accumulation, extra-cellular matrix degradation, TGF- β signaling, contraction, and apoptosis; abnormalities are corrected by CRISPR-editing of the fibrillin-1 mutation. TGF- β inhibition rescued abnormalities in fibrillin-1 accumulation and matrix metalloproteinase expression. However, only the non-canonical p38 pathway regulated SMC apoptosis, a pathological mechanism also governed by KLF4. This model has allowed us to dissect the molecular mechanisms of MFS, to identify novel targets for treatment, such as p38 and KLF4, and has provided an innovative human platform for the testing of new drugs.

Introduction

Marfan syndrome (MFS) is a heritable autosomal dominant multi-system disorder of connective tissue affecting 1 in 5,000 individuals^{1–4}. Premature death is due to the development of thoracic aortic aneurysm (TAA); a potentially devastating process that can progress to aortic dissection or rupture without prior symptoms⁴. There are currently no effective medical treatments and only surgical replacement of the aortic root increases life expectancy in patients with MFS⁵.

MFS is caused by mutations in the fibrillin-1 (*FBN1*) gene, which encodes a major constituent of microfibrils found in the extracellular matrix (ECM)^{6,7}. Fibrillin-1 interacts with and controls the bioavailability of TGF- β , a potent cytokine that regulates proliferation, differentiation, ECM modeling and apoptosis^{8–10}. Studies in a mouse model for MFS indicated that enhanced activation of the non-canonical (ERK-mediated) TGF- β pathway is the principal driver of TAA progression^{11,12}. In particular, these studies showed that losartan, an angiotensin II receptor type 1 (AGTR1) blocker, reduced AGTR1-induced TGF- β activity and mitigated TAA in MFS mice.

However, the precise functional role of TGF- β in aneurysm development remains controversial^{13,14}. Indeed, recent studies in mice have exposed the complexity of TGF- β signaling in MFS, by showing that both TGF- β canonical and non-canonical pathways contribute to aneurysm growth and that TGF- β exerts both protective and detrimental effects¹⁵. The complex pleiotropic role of TGF- β in aortic remodeling may also account in part for the finding that in a recent clinical trial, losartan showed no benefit over the β -blocker atenolol in regulating the rate of aortic dilatation¹⁶.

Currently, most of our knowledge of MFS pathogenesis has been gained from studies in animal models, including *Fbn1*^{mgR/mgR} and *Fbn1*^{C1039G/+} mice^{11,17–19}. However, the emergence of human induced pluripotent stem cells (hiPSC) for disease modeling²⁰ provides an opportunity to use a new approach to study both the early development and disease pathology of MFS on a susceptible human genetic background.

Here we describe the generation of the first vascular human model of MFS. Patient-derived hiPSC lines are differentiated into embryonic lineage-specific smooth muscle cells (SMC)²¹ that reliably phenocopy the aortic pathology in patients with MFS. Importantly, our study demonstrates that at an early stage TGF- β appears to contribute to the developing MFS phenotype but at later stages, blockade of TGF- β signaling by using losartan is ineffective in averting SMC death. In contrast, using our human MFS-SMC model, we highlight a previously undocumented role for p38-MAP kinase in regulating SMC apoptosis and proliferation, and identify Kruppel-like factor 4 (KLF4) as a new potential contributing factor to MF pathology.

Results

MFS-specific hiPSCs and differentiation into SMC lineages

To generate hiPSCs, human dermal fibroblasts from two MFS patients carrying known pathogenic mutations in the *FBN1* gene and severe aortic disease were used (*Supplementary Table 1*). The first line, MFC^{C1242Y}, carries a missense mutation in exon 30 (3725G>A)²² representing the commonest type of Marfan-causing mutation, namely a cysteine substitution in an EGF domain²³. The second line, MFG^{G880S}, harbors a missense mutation in exon 21 (2638G>A) leading to a glycine-to-serine substitution²⁴. Genomic DNA sequencing confirmed the specific *FBN1* mutations (**Fig. 1a** and *Supplementary Fig. 1a*). We established two sub-clonal hiPSC lines for each MFS line. Three wild-type hiPSC lines from healthy individuals were used as controls (*Supplementary Table 1*) and all the results represent the average of these three lines (WT).

We confirmed that hiPSC lines expressed the human pluripotency-associated gene products and that they were karyotypically normal (**Fig. 1b** and *Supplementary Fig. 1b-d*). MFS hiPSC pluripotency was assessed by differentiation into all three embryonic germ layers (*Supplementary Fig. 1e,f*) and by teratoma formation in vivo (*Supplementary Fig. 1g*).

hiPSC were then differentiated into the three embryonic origin-specific SMC lineages as previously described (**Fig. 1c**, *Supplementary Fig. 2a,b*)^{21,25}. The SMCs were then passaged and matured in 10% serum-containing media for periods ranging from one to three months (S30). Neuroectoderm was generated as described previously²⁶ and then trypsinized to single cells and passaged up to 12 times to generate neural crest-like cells (NC; *Serrano F*,

Bernard WG, Granata A, Iyer D, Kim M, Gambardella L, and Sinha S; in submission). Similar to the mesoderm intermediates, NC were differentiated to SMC and allowed to mature in serum-containing medium (Supplementary Fig. 2). The intermediate populations were characterized for expression of specific markers respectively of lateral mesoderm, paraxial mesoderm and NC cells at both mRNA and protein level (Supplementary Fig. 2a-b). In response to TGF- β 1 and PDGF-BB treatment, the expression of these intermediate markers was downregulated while the levels of specific SMC markers, including *CNN1* and *MHY11* increased (Supplementary Fig. 2a).

Abnormal fibrillin-1 deposition and increased TGF- β

After a month in serum-containing media, extracellular fibrillin-1 deposition appeared irregular and less abundant in MF^{C1242Y} LM-, PM- and NC-derived SMC compared to equivalent WT SMC subtypes by immunofluorescence (Fig. 1d). Abnormal organization of fibrillin-rich microfibrils in MF^{C1242Y} ECM was confirmed by transmission electron microscopy (Fig. 1e and Supplementary Fig. 3). Fibrillin-1 immuno-staining showed that the phenotype was more severe in MF^{C1242Y} NC-SMC in comparison with SMC of LM- and PM- origins (Fig. 1f). A similar phenotype was observed for MF^{G880S} NC-SMC (Supplementary Fig. 4a,b). By showing a stronger phenotype, SMC of NC origin appeared to be more affected by the mutant fibrillin-1. This is largely consistent with the clinical distribution of aneurysms, which occur preferentially in the aortic root, ascending aorta and arch, regions populated predominantly by NC-derived SMC. Importantly, neither MF mutation, both of which result in an amino acid substitution, affected fibrillin-1 synthesis

(*Supplementary Fig. 4c-d*).

In MFS, abnormal fibrillin-1 may lead to excessive release of TGF- β from the ECM⁹. Accordingly, we measured TGF- β 1 in the WT and MFC^{C1242Y} SMC supernatants by ELISA. There were increased TGF- β 1 levels in all MFC^{C1242Y} SMC populations compared with the WT and this increase was greatest in the supernatant of NC-derived MFC^{C1242Y} SMC (**Fig. 1g**). Equally, MFG^{G880S} NC-SMC also showed higher TGF- β 1 levels (*Supplementary Fig. 4e*).

Consistent with these observations, TGF- β 1 protein, extracted from SMC and extracellular matrix, was increased in MFC^{C1242Y} and MFG^{G880S} NC-SMC compared with WT and the two other lineages (**Fig. 1h**). TGF- β 1 mRNA levels did not significantly differ in WT and MF SMC lineages, suggesting that increased protein levels were due to increased accumulation in the ECM (*Supplementary Fig. 4f*). We observed increased mRNA levels of plasminogen activator inhibitor-1 (PAI-1), a known target of TGF- β 1 signaling²⁷, and of a range of matrix metalloproteinases (MMPs) in MFC^{C1242Y} SMC, especially in the NC subtype (**Fig. 1i,j**). Data supporting higher TGF- β 1 activity in MFC^{C1242Y} and MFG^{G880S} NC-SMC were obtained by measuring the luciferase signal in Mink Lung Epithelial Cells stably transfected with human PAI-1 luciferase reporter and incubated with NC-SMC conditioned media (*Supplementary Fig. 4g*).

MFC^{C1242Y} NC-SMC recapitulate the MFS aortic phenotype

Our MFS hiPSC-derived SMC model allows us to observe changes in the NC-SMC phenotype from an early stage of development (PTD12) to a more mature stage (S30), which may reflect the disease progression occurring in

MFS patients²⁸.

We observed morphological differences between WT and MFC^{C1242Y} NC-SMC following prolonged culture in serum-containing media (S30), with WT NC-SMC appearing spindle shaped while MFC^{C1242Y} NC-SMC were larger and more stellate in appearance (**Fig. 2a**). mRNA analysis showed increased SM markers in MFC^{C1242Y} NC-SMC at S30 versus WT NC-SMC (**Fig. 2a,b**).

Furthermore, the proliferative capacity of both MFC^{C1242Y} NC-SMC clones decreased dramatically during maturation, compared with WT (**Fig. 2c,d**).

Similar morphology and behavior were observed in MFG^{G880S} NC-SMC (*Supplementary Fig. 5a,b*). Moreover, MFC^{C1242Y} NC-SMC displayed higher poly-caspase activity at an early stage (PTD12); while at a more mature stage, MFS NC-SMC showed a higher incidence of cell death in both mutant lines compared to WT NC-SMC, consistent with increased SMC loss in MFS aortic dilatation (**Fig. 2e**; *Supplementary Fig. 5c*).

MFC^{C1242Y} NC-SMC demonstrated reduced contractility in response to stimulation with the cholinergic agent, carbachol (**Fig. 2f,h**). The intracellular calcium responses of WT and both lines of MFS NC-SMC were also remarkably different, with WT NC-SMC generating robust cyclic calcium waves, while MFS NC-SMC cells showed equivalent initial calcium release upon carbachol treatment as the WT but failed to generate propagating calcium waves and returned abruptly to basal levels (**Fig. 2g,i**; *Supplementary Fig. 5d and Supplementary material movie1 and 2*). Similar contraction and Ca²⁺ flux abnormalities were previously seen in the C1039G mouse model^{29,30}. Together these observations indicate that MFS NC-SMC show functional changes typical of those observed in the aortas of Marfan patients

TGF- β signaling and cyclic stretching in MFC^{1242Y} model

The MFS mouse model has implicated both TGF- β canonical and non-canonical (ERK-mediated) signaling pathways in contributing to the progress of the Marfan phenotype¹¹. We used our human *in vitro* model to dissect TGF- β pathways at both early and mature stages. We observed that phosphorylated levels of a TGF- β canonical signaling component, such as P-SMAD2, and non-canonical components, including P-ERK1/2 and P-p38, were increased in MFC^{1242Y} and MFG^{880S} NC-SMC at PTD12 stage compared to WT, while no differences were detected between WT and MF fibroblast lines (**Fig. 3a,b** and *Supplementary Fig. 6a*). At stage S30, the increase in ERK1/2 phosphorylation levels was no longer detectable while P-p38 and P-SMAD2 levels remained elevated (**Fig. 3b**). These findings are consistent with recent observations of developmental stage-dependent differences in signaling in the MF mouse¹⁵.

When we looked at the transcriptional levels of MMPs, downstream targets of TGF- β signaling, we observed that while fibroblast lines did not show significant differences (*Supplementary Figure 6b*), MMPs expression levels were increased in both MF NC-SMC mutants at PTD12 (*Supplementary Fig. 6c*) and at mature stages (S30; **Fig. 3c**). The tissue specific inhibitors of MMPs (TIMPs), showed a biphasic expression pattern with increased expression at an early stage (*Supplementary Fig. 6d*), but with lower TIMP expression levels at S30 than in WT (**Fig. 3d**) suggesting increasing proteolysis with prolonged culture. To confirm increased MMP activity in the

MF NC-SMC at S30, we also performed a FITC-gelatin degradation experiment, which showed extensive proteolytic activity in MF NC-SMC compared to the WT (**Fig. 3e,f**, *Supplementary Fig. 6e*).

SMC cyclic stretch exacerbates the MF phenotype

WT and MF SMC were mechanically stretched for 24h using 10% cyclic stretching at 1Hz to mimic aortic hemodynamic forces. After 24h, both WT and MF^{C1242Y} NC-SMC were aligned parallel to the direction of the stretch (**Fig. 4a**). SM markers showed increased expression in MF^{C1242Y} NC-SMC (**Fig. 4b**). Also, *MMP9* and *MMP10* were significantly increased in MF^{C1242Y} NC-SMC in response to stretching (**Fig 4c**). Furthermore, we observed that Collagen type I (COL-1) expression and deposition were higher in MF NC-SMC compared to WT and further increased upon stretching (**Fig. 4d-e**), which may lead to greater stiffness of the ECM³³. Moreover, we observed increased binding of phalloidin-FITC and immunostaining for Vinculin in MF^{C1242Y} SMC in static conditions compared to WT, which were both further incremented after stretching, suggesting that MF^{C1242Y} SMC have higher density of stress-fibres (Phalloidin) and focal adhesions (Vinculin) than WT SMC (**Fig. 4f-h**). Remarkably, we also observed higher expression of p38 in MF^{C1242Y} SMC in response to cyclic stretch at both mRNA and protein levels (**Fig. 4i,j**).

Effect of general TGF- β inhibition on the MF phenotype

To test whether TGF- β signaling may have different roles at different stages of NC-SMC development, both WT and MF^{C1242Y} NC-SMC cells were treated

with anti-TGF- β neutralizing antibody and Losartan, a specific AGTR1 blocker (*Supplementary Fig. 7a*). In mature NC-SMC cells (S30), both treatments promoted fibrillin-1 accumulation in MFC^{C1242Y} NC-SMC (**Fig. 5a,b**), associated with reduced canonical TGF- β activity, as measured by transfecting a 4xSmad binding element (SBE4) promoter-luciferase reporter in both WT and MFC^{C1242Y} NC-SMC (**Fig. 5c**). Moreover, anti-TGF- β blocking antibody and Losartan treatments were both able to downregulate efficiently the expression levels of MMPs and also to upregulate *TIMP3* both in mature cells (S30; **Fig. 5d**) and at early stages (PTD12, *Supplementary Fig. 7b*). We therefore propose that excessive TGF- β activity, through effects on MMP and TIMP expression, is responsible at least in part for the reduced amount of fibrillin-1 in MFS NC-SMC.

Notably, Losartan was more effective in reducing ECM degradation than Doxycycline (DOX), a nonspecific inhibitor of MMPs, suggesting losartan may be acting through additional mechanisms beyond simple MMP inhibition (*Supplementary Fig. 7c,d*). Extracellular fibrillin-1 only partly increased following DOX treatment, suggesting that the changes seen are consequence of abnormalities in both degradation and deposition and that mutant fibrillin-1 may be more prone to proteolytic degradation than WT (*Supplementary Fig. 7e,f*). High level of TGF- β 1 is likely to be caused by ECM breakdown, as it decreases upon DOX treatment (*Supplementary Fig. 7g*).

Despite beneficial effects on fibrillin-1 deposition and matrix proteolysis, anti-TGF- β blocking antibody treatment failed to rescue the impaired proliferation of MFC^{C1242Y} NC-SMC, while Losartan provided partial rescue (**Fig. 5e**).

Furthermore, the increased apoptosis seen in MFC^{C1242Y} NC-SMC was

unaffected upon Losartan treatment as shown by AnnexinV analysis (**Fig. 5f**).

Likewise, a poly-caspase assay also showed no benefit with losartan on

MF^{C1242Y} NC-SMC cell death (*Supplementary Fig. 8a*).

Taken together, these results suggest that ECM turnover in MFS is regulated through pathways that are distinct from those regulating SMC proliferation and death.

Emerging roles of p38, KLF4 and β 1 integrin in MFS model

To better understand the contribution of different TGF- β signaling pathways in the development of the MFS phenotype, NC-SMC were treated with inhibitors of either the canonical pathway (SB431542), ERK1/2 phosphorylation (PD98059) or p38-MAP kinase (SB203580) (**Fig. 6** and *Supplementary Fig.*

9a,b). Analysis of fibrillin-1 deposition by immunostaining showed that inhibition of p38 phosphorylation was surprisingly effective in rescuing the phenotype, comparable to Losartan in both MF^{C1242Y} and MF^{G880S} (**Fig. 6a,b** and *Supplementary Fig. 10a,b*), while inhibition of canonical Smad signaling or ERK1/2 by SB431542 or PD98059 respectively had no significant effects (**Fig. 6a,b** and *Supplementary Fig. 10a,b*). Comparable results were found for effects on cell proliferation with significant rescue of suppressed proliferation only by the p38-MAP kinase inhibitor, SB203580 and Losartan (**Fig. 6c**).

To investigate further the proliferative mechanisms affected in MF NC-SMC, we looked at potential regulators, including KLF4 (Krüppel-like factor 4) and observed a dramatic increase in KLF4 mRNA levels in MF lines compared to WT NC-SMC (**Fig. 6d** and *Supplementary Fig. 11a*). Both inhibition of p38 upon SB203580 treatment and efficient knock-down of KLF4 using siRNA

(siRKLF4) were able to increase mRNA levels of the proliferation marker, *CCND1* (cyclinD1), which were significantly lower in MF^{C1242Y} NC-SMC compared to WT (**Fig. 6e**). Moreover, both *TP53* (tumor suppressor protein p53) and *CDKN1A* (*p21*; Cyclin-Dependent Kinase Inhibitor 1A), which promote cell cycle arrest, were upregulated in MF cells and reduced in response to SB203580 and SiKLF4 treatments (**Fig. 6f,g**). Therefore, silencing KLF4 had also a positive effect on MF^{C1242Y} and MF^{G880S} NC-SMC proliferation (*Supplementary Fig. 11b,c*). Moreover, silencing of KLF4 increased the deposition of fibrillin-1 in the matrix compared to the control scramble siRNA (siScr; **Fig. 6h,i**).

Similarly to KLF4 siRNA approach, a specific inhibitor of the MEK5/ERK5 pathway, BIX02189, exclusively reduced KLF4 mRNA and protein levels in MF^{C1242Y} NC-SMC (**Fig. 6j** and *Supplementary Fig. 11d*). Conversely, SB203580 and Losartan, did not affect total KLF4 mRNA and protein levels, suggesting that p38 and KLF4 contribute independently to the same signaling pathway that controls SMC proliferation (**Fig. 6j** and *Supplementary Fig. 11d*). Consequently, inhibiting the MEK5/ERK5 pathway by BIX02189 restored normal proliferation rate and mRNA levels for *TP53*, *CCND1* and *CDKN1A* in MF^{C1242Y} NC-SMCs comparable to WT (**Fig. 6c** and *Supplementary Fig. 11e*).

Notably, both SB203580 and KLF4 siRNA reduced the apoptotic activity of both MF mutant lines (**Fig. 7a** and *Supplementary Fig. 10d*). This may indicate that both p38 and KLF4 contribute to the same regulatory apoptotic mechanism, which is exclusive of SMC, since no difference in cell death was seen between WT and MF fibroblasts (*Supplementary Figure 10c*). Whereas, MF^{C1242Y} NC-SMC apoptotic levels were found unchanged or increased upon

Losartan and PD98059 or U0126 (selective inhibitor of MEK1/2) treatments respectively, suggesting that the ERK1/2-mediated pathway has a protective role for cell death in this model (**Fig. 7a** and *Supplementary Fig. 12a*). Moreover, MF NC-SMC apoptosis was substantially reduced in cells with plasminogen activation, implying that pericellular proteolysis is partially responsible for MF SMC death³⁴ (*Supplemental Fig. 12b*). Finally, a new insight about the MF cell death mechanisms comes from the stretch studies. Upon cyclic stretching, MF^{C1242Y} NC-SMC expressed higher levels of *KLF4*, *CDKN1A* and *TP53*, which may contribute to MF apoptosis (**Fig. 7b**). In addition, $\beta 1$ integrin, an essential adhesion molecule, increased in response to cyclic stretch at both mRNA and protein levels (**Fig. 7c,d**). A blocking anti- $\beta 1$ integrin antibody specifically downregulated p38 phospho-protein levels and consequently *TP53* expression (**Fig. 7e,f**). The hypothesis that $\beta 1$ integrin may play a role in promoting apoptosis, potentially mediated by p38 in MF NC-SMC is consistent with the AnnexinV study that showed decreased apoptotic levels in MF NC-SMC upon anti- $\beta 1$ integrin blockade (**Fig. 7g**). And since $\beta 1$ integrin was more expressed in NC-SMC compared to the other lineages, these findings may be a potential explanation of why NC-SMC are more vulnerable to fibrillin-1 mutation (*Supplementary Fig. 13a,b*).

Correction of C1242Y mutation by CRISPR/Cas9 *FBN1* editing

Notably, the upregulation of both P-p38 and *KLF4* observed in our hiPSC-derived MF^{C1242Y} NC-SMC model was also found in aortic sections of two patients affected by Marfan syndrome, compared to healthy individuals, validating our *in vitro* findings (**Fig. 8a-d**).

To verify that the MF^{C1242Y} hiPSC-derived SMC phenotype is caused solely by the C1242Y mutation, we generated a CRISPR/Cas9 isogenic MF hiPSC line where the nucleotides in the mutant allele were replaced with WT nucleotides, which we refer to as CRISPR MF corrected (*Supplementary Fig. 14a,b*). In parallel, we generated as control an isogenic CRISPR/Cas9 MF hiPSC line where the WT nucleotides were inserted in the WT allele, therefore retaining the C1242Y mutation (CRISPR MF mutant; *Supplementary Fig. 14b-d*). CRISPR MF corrected showed a WT fibrillin-1 phenotype compared to MF^{C1242Y} and CRISPR MF mutant (**Fig. 8e,f**). In CRISPR MF corrected NC-SMC, the phosphorylated levels of TGF- β pathway effectors were lowered to levels comparable to WT (**Fig. 8g**). In addition, TGF- β levels in the supernatant, the mRNA levels of MMP10, and the extent of matrix degradation of CRISPR MF corrected NC-SMC were significantly reduced (**Fig. 8h** and *Supplementary Fig. 15a-f*). In conclusion, our hiPSC-derived SMC have proven to be a robust model for MFS and a unique tool for the identification of new targets, including p38 and KLF4, which may represent novel therapeutic opportunities for MFS treatment (**Fig. 8i**).

Discussion

This study describes the first Marfan Syndrome (MFS) patient-derived hiPSC model that faithfully mimics the human vascular phenotype, which is the main cause of premature death in MFS patients⁷. Recently, Quarto and colleagues described the MFS skeletal phenotype using a human iPSC model³⁵. However, our model recapitulates the key aspects of MFS vascular pathology and highlights the complexity of the downstream signaling pathways identifying a key role for p38 and KLF4 in disease development.

A crucial step in these studies was to generate vascular SMC of specific embryonic origins to model different aortic regions as aneurysms in MFS are found principally in the aortic root, ascending aorta and arch, regions invested by NC-SMC^{36,37}. Interestingly, our model indicated that SMC of neural crest origin, which deposit higher levels of extracellular fibrillin-1 (Fig. 2a,b) and appear to be more proliferative when compared to the other two lineages, are most severely affected by the disease. Thus, NC-SMC appear to possess intrinsic properties, for instance differential expression of β 1 integrin, that make them more susceptible to *FBN1* mutations, reinforcing the importance of using lineage-specific systems for vascular disease modeling.

Abnormal TGF- β activation and signaling have been documented in MFS mouse models^{12,19,38} and has been proposed as a common factor in MFS-related disorders, such as Loeys-Dietz syndrome^{39–41}. As TGF- β bioavailability seems to be affected by inhibition of MMP activity via Doxycycline, we suggest that increased proteolysis is responsible for high TGF- β levels detected in our MF model⁴². Importantly, blocking TGF- β , whether by using TGF- β neutralizing antibody or the AGTR1 blocker,

Losartan, attenuated or prevented aortic root dilatation in mice⁴³. However, recent randomized clinical trials in patients with MFS have been disappointing with losartan showing no benefit over either placebo or beta-blockade^{16,44,45}. The rationale for using Losartan as a TGF- β blocker is that Angiotensin II can regulate TGF- β 1 mRNA and protein expression and potentially TGF- β activation^{46,47} and cross-talk with TGF- β signaling by promoting the phosphorylation of MAP kinases⁴⁸. Interestingly, in C1039G MFS mice, TGF- β -mediated ERK1/2 activation was driving aneurysm formation¹⁹. However, other studies have shown that TGF- β neutralization either exacerbated or mitigated TAA progression depending on whether the treatment was initiated before or after aneurysm formation^{15,49}. Indeed, dual treatment with losartan and late TGF- β blockade entirely blocked aortic aneurysm development in the mgR mouse accompanied by a dramatic increase in phosphorylated ERK1/2¹⁵. These findings suggest that the link between ERK1/2 phosphorylation and disease development is not simple. Interestingly, p38 activation has been documented in *Fbn1*-null mice (mgN) with a more severe aortic phenotype⁵⁰ and systemic p38 blockade has been found to normalize P-smad2 levels in the mgN mouse. Correspondingly, we demonstrated that increased TGF- β activity in MFS SMC was associated with increased phosphorylation of endogenous SMAD2, ERK1/2 and p38 at an early stage as reported in MFS mouse models^{19,50}, while at a more mature stage, ERK1/2 phosphorylation reduced to WT levels, which may indicate the need for distinct therapies at different disease time-points, as also suggested by Ramirez and colleagues using the mgR mouse model¹⁵.

393 In our model, as consequence of reducing TGF- β activity, Losartan was
394 therefore able to lower MMP expression and reduce ECM degradation when
395 cells were treated both at PTD12 and S30 stages. However, Losartan had
396 minimal or no effect in reducing the apoptotic activity in MF SMC (Fig. 5f).
397 By dissecting TGF- β pathways using both canonical and non-canonical
398 pathway inhibitors, we observed that blocking ERK1/2 aggravated the
399 phenotype by reducing MF-SMC proliferation and increasing apoptotic rates.
400 Conversely, p38 inhibition by SB203580, had previously unreported beneficial
401 effects on MF^{C1242Y} SMC proliferative ability and viability. The improved SMC
402 viability was associated with upregulation of proliferation makers and
403 downregulation of *TP53* and *P21*, known to inhibit the cell cycle with a role in
404 apoptosis^{51,52}. Taken together, these data suggest that in this human system,
405 there are elements of TGF- β non-canonical signaling that depending on the
406 disease stage, may have a detrimental impact, such as p38, while others,
407 such as ERK1/2, may have a protective role in MFS pathogenesis¹⁵.
408 Consequently, the broad upstream inhibition of TGF- β signaling may not fully
409 reverse the MF phenotype and targeting of regulatory pathways such as p38
410 and the identification of novel targets, such as KLF4^{53,54}, may be essential. In
411 response to cyclic stretch, we observed a more severe phenotype in MF
412 SMC, including upregulation of MMPs and p38, increased collagen I
413 deposition and increases in stress fibers and focal adhesions, resembling the
414 elevated cellular and ECM stiffness seen in SMC from Marfan patients³³.
415 These observations indicate a complexity of signaling, which may reflect
416 additional non-TGF- β mediated mechanotransduction caused by defective
417 fibrillin-1, as a major contributor to the phenotype^{55,56} and uncover β 1 integrin

as a potential mediator of this process.

Our findings suggest that $\beta 1$ integrin, which is known to interact with fibrillin-1⁵⁷, appears to have an important role in regulation of MF SMC death, potentially through p38 as shown previously⁵⁸ and may explain the susceptibility of the ascending and aortic arch to aneurysm development, since it is highly expressed in NC-derived SMC.

Ultimately, the results obtained with our hiPSC-SMC *in vitro* model, including high levels of both KLF4 and phospho-p38 were validated in patient samples, suggesting that the *in vitro* system accurately models human disease.

In conclusion, our model represents an innovative tool to dissect molecular and mechanosensing mechanisms of MFS and identify novel targets for potential new treatments, as well as a resourceful platform for testing new drugs. Finally, our human lineage-specific SMC model has opened the door for the use of hiPSC harboring different *FBN1* mutations to understand the mechanisms underlying genotype-phenotype variability in MFS, which will allow the development of preventative strategies and precision medicine for individual patients.

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Author contributions-

Alessandra Granata conception, design, acquisition, analysis and

interpretation of iPSC-SMC data and to the drafting of the article;

Felipe Serrano contributed to the conception and design and the analysis of

the CRISPR/Cas9 iPSC clones, and to the neural crest protocol.

William George Bernard contributed to the Neural Crest protocol for deriving

NC-SMC.

Madeline McNamara carried out the qPCR analysis of fibroblasts and iPSC-

derived SMC lines and the stretch studies.

Lucinda Low carried out the teratoma assay;

Priya Sastry contributed by providing human aortic tissue;

Sanjay Sinha contributed conceptually to the design of the experiments,

obtained funding and supervised all studies. All authors contributed to revision

of the article.

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622

Figure 1: Generation of hiPSC and lineage-specific SMC from Marfan

MF^{C1242Y} fibroblasts show greatest perturbation of fibrillin-1 deposition,

TGF- β levels and MMP expression in MF^{C1242Y} NC-SMC

a) DNA sequencing analysis of MF hiPSC showing 3725G>A heterozygous

mutation in *FBN1* in exon 30. **b)** Immunofluorescence staining of MF^{C1242Y}

hiPSC colonies for the pluripotency markers, OCT3/4, SOX2, SSEA4 and

TRA-1-60. Scale bar = 100 μ m. **c)** Schematic representation of hiPSC

differentiation to three different smooth muscle cell (SMC) origins: lateral

mesoderm (LM), paraxial mesoderm (PM) and neuroectoderm (NE)/neural

crest (NC). **d)** Immunostaining analyses of extracellular fibrillin-1 in MF^{C1242Y}

and WT SMC of LM, PM and NC embryonic origins after 30 days of culturing

in serum-containing medium (S30) (scale bar=100 μ m). **e)** Transmission

electron microscopy (TEM) of fibrillin-rich microfibrils (arrows) of WT and

MF^{C1242Y} NC-SMC (scale bar = 500nm). **f)** Quantification of fibrillin-1 staining

relative to cell number in MF^{C1242Y} and WT SMC of all three embryonic

lineages. **g)** Levels of TGF- β 1 measured by ELISA in the supernatant of

MF^{C1242Y} SMC and WT SMC. **h)** Cropped blot of total TGF- β 1 in WT, MF^{C1242Y}

and MF^{G880S} SMC lysates. **i)** RT-qPCR of plasminogen activator inhibitor-1

(PAI-1) expression in WT and MF^{C1242Y} LM-, PM- and NC-SMCs. **j)** *MMP1*

and *MMP9* mRNA levels detected in WT and MF^{C1242Y} SMC lineages. The

relative mRNA level was normalized to its *GAPDH/PBGD* content. The results

are presented as means $\pm$ SD of three independent experiments. The

asterisks indicate statistically significant differences; * $p < 0.05$; ** $p < 0.01$. ns

= non significant.

Figure 2: MF^{C1242Y} hiPSC-derived SMC exhibit functional abnormalities

consistent with the human disease phenotype.

a) WT and MF^{C1242Y} NC-SMC immunofluorescence staining for CNN1. **b)**

Expression analysis of SM markers, *CNN1*, *ACTA2*, *TAGLN* and *MYOCD* at

hiPSC stage, early (PTD12) and mature stage (S30) of WT and MF^{C1242Y} NC-

SMC. Proliferation of MF^{C1242Y} and WT NC-SMC at intermediate NC stage, at

PTD12 and at S30 by **c)** MMT assay and by **d)** BrdU staining (at S30; scale

bar=50μm). Ratio of BrdU-positive cells is expressed relative to total number

of cells (nuclei-DAPI staining). **e)** Apoptotic pathway activation measured by

FAM poly-caspase flow cytometry in MF^{C1242Y} NC-SMC and WT NC-SMC at

early stage PTD12 and at S30. Cell viability assessed by PI staining.

Quantification is shown in *Supplemental Figure 5c*. **f)** Surface area of WT and

MF^{C1242Y} NC-SMC before and 3 minutes after carbachol stimulation to

measure contractile ability (basal, blue line; contracted, red line). **g)** Ca²⁺ flux

measured by Fluo-4AM loading and intensity of WT and MF^{C1242Y} NC-SMC at

basal (0s), stimulation with carbachol (4s), after 30s stimulation and total

loading after triton (tx) treatment. **h)** Quantification of contractility by

measuring the change in cell surface areas in MF^{C1242Y} NC-SMC compared to

WT NC-SMC (average n of cells=15). **i)** Single cell fluorescence tracing of WT

and MF^{C1242Y} SMC before and after carbachol stimulation (4s), relative to

basal level and total Fluo-4AM loading.

The results are representative of three independent experiments (means ±

SD). The asterisk indicates statistically significant difference (* p< 0.05).

Figure 3: Increased TGF-β signaling correlates with MMP expression and

matrix degradation in MF^{C1242Y} NC-SMC. a) Cropped western blot analysis

of total and phosphorylated levels of TGF- β canonical (SMAD2) and non-canonical (ERK1/2 and p38) pathways in WT and MFC^{C1242Y} NC-SMC at PTD12 and S30. The result is representative of three independent experiments. **b)** Quantification of the phosphorylated levels of SMAD2, ERK1/2 and p38 relative to the total protein levels and to β -actin. **c,d)** RT-qPCR expression profile of a selection of MMPs and TIMPs in MFC^{C1242Y} and WT NC-SMCs at S30 stage. The results for each sample were normalized to its *GAPDH* and *PBGD* content. **e)** FITC-gelatin degradation assay performed with WT and MFC^{C1242Y} S30 NC-SMC over 48h. Cells were then fixed and stained for the SMC marker, CNN1 (red). **f)** Quantification of the FITC-degraded areas for S30 MFC^{C1242Y} NC-SMCs compared with WT. The results are presented as means $\pm$ SD of three independent experiments. The asterisks indicate statistically significant differences; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. ns = non significant.

Figure 4: Effect of mechanical cyclic stretching on MFC^{C1242Y} SMC phenotype. **a)** Morphology of NC-SMC before and after cyclic stretching (Flexcell FX-5000, 10% elongation); double arrows indicate stretch directionality. RT-qPCR mRNA profile of SM markers (*CNN1*, *ACTA2* and *TAGLN*, **b**), *MMP9* and *MMP10* (**c**) and Collagen type I (*COL-1*; **d**) in WT and MFC^{C1242Y} NC-SMC before and after 24h stretching. Immunofluorescence analysis of WT and MFC^{C1242Y} SMC before and after stretching for Collagen I (**e**), Phalloidin-FITC (**f**) and Vinculin (**g**). **h)** Quantification of Collagen type I, Phalloidin and Vinculin staining intensity relative to cell numbers in WT and MFC^{C1242Y} SMC in un-stretched and stretched conditions. **i)** mRNA expression

levels of p38 in WT and MFC^{C1242Y} SMC before and after stretching. The asterisks indicate statistically significant differences; * p< 0.05; ** p< 0.01. **j)** Cropped western blot of total and phospho-protein levels for p38 in unstretched and stretched NC-SMC. Results are presented as means $\pm$ SD of two independent experiments.

Figure 5: Inhibition of TGF- β - and AGTR1-mediated signaling rescues the loss of fibrillin-1 but does not rescue MFC^{C1242Y} SMC proliferation and cell death. a) Immunostaining for extracellular fibrillin-1 in S30 WT and MFC^{C1242Y} NC-SMCs following treatment with TGF- β neutralizing antibody (α -TGF- β Ab) or the AGTR1 inhibitor, Losartan. **b)** Quantification of fibrillin-1 immunostaining expressed relative to cell numbers in control and treated samples. **c)** TGF- β signaling activity was assessed by transiently transfecting control and treated WT and MFC^{C1242Y} NC-SMC with 4 Smad binding element (SBE4)-Luciferase reporter vector and Luciferase luminescent signal was measured after 12h. Luciferase activity expressed relative to protein concentrations. **d)** mRNA was extracted from control and samples treated with TGF- β neutralizing antibody and Losartan. Levels of *MMP1*, *MMP9*, *MMP10* and *TIMP3* were detected by RT-qPCR in WT and MFC^{C1242Y} NC-SMCs and **e)** cell proliferation was measured using an MMT assay after 48h of treatment. **f)** Flow cytometric staining for AnnexinV shows apoptotic levels in both MFC^{C1242Y} SMC control (red) and Losartan treated (purple) compared to WT control (blue) and treated (green). Results are presented as means $\pm$ SD of three independent experiments. The asterisks indicate statistically significant differences; * p< 0.05; ** p< 0.01. ns=

non significant.

Figure 6: Inhibition of p38 phosphorylation or AGTR1 and knock-down of KLF4 rescue fibrillin-1 deposition and SMC proliferation defects. a) Immunofluorescence shows fibrillin-1 staining in S30 NC-SMC after 7 days treatment with a range of inhibitors: CTL= control; SB431542 (ALK5 inhibitor); SB203580 (p38 inhibitor); PD98059 (ERK1/2 inhibitor) and Losartan (ATR1 inhibitor). **b)** Quantification of fibrillin-1 staining relative to cell number (nuclei) using ImageJ. **c)** MMT assay to measure NC-SMC proliferation after 48h treatment with SB203580, BIX02189 (MK5/ERK5 inhibitor) and Losartan. **d)** *KLF4* mRNA levels in MFC^{C1242Y} NC-SMCs and WT NC-SMC in response to *KLF4* siRNA (siRKLF4) and scrambled siRNA (siScr) transfections. **e)** *CCND1* (CyclinD1) expression **f)** *TP53* (p53 protein) expression and **g)** *CDKN1A* (P21) mRNA expression levels in MFC^{C1242Y} NC-SMC and WT in response to SB203580 and siRKLF4. **h,i)** Immunostaining analysis and quantification of WT and MFC^{C1242Y} NC-SMC showing fibrillin-1 deposition in response to *KLF4* knockdown (siRKLF4) compared to scrambled siRNA (siScr). **j)** Immunoblot for total *KLF4* in WT and MFC^{C1242Y} NC-SMC samples transfected with siRKLF4 or treated with SB203580 (p38 inhibitor) or BIX02189 (MEK5/ERK5 inhibitor).

The results are presented as means $\pm$ SD of three independent experiments. The asterisks indicate statistically significant differences; * $p < 0.05$; ** $p < 0.01$.

Figure 7: MFS SMC death is regulated by p38, KLF4 and β 1 integrin. a)

Flow cytometric staining for AnnexinV to determine apoptosis levels of WT

and MF^{C1242Y} NC-SMC following treatment with SB203580, Losartan, PD98059 (ERK1/2 inhibition) and by transfection with KLF4 siRNA. mRNA expression levels for *KLF4*, *CDKN1A* and *TP53* **b)** and β 1 integrin **(c)** in un-stretched and stretched conditions in WT and MF NC-SMC. **d)** Flow cytometric analysis of β 1 integrin-APC levels in NC-SMC before and after stretching. **e)** Cropped western blot for SMAD2, ERK1/2 and p38 phosphoproteins in control (CTL, IgG), stretched conditions and after treatment with anti- β 1 integrin blocking antibody (anti- β 1 Ab) in WT and MF^{C1242Y} NC-SMC. **f)** RT-qPCR profile of *TP53* expression in control (CTL) and anti- β 1 Ab treated WT and MF NC-SMC. **g)** Comparison of apoptotic levels by AnnexinV flow cytometry in control (CTL) and upon treatment with anti- β 1 integrin antibody of WT and MF^{C1242Y} NC-SMC in resting and stretched conditions.

The results are presented as means $\pm$ SD of three independent experiments. The asterisks indicate statistically significant differences; * $p < 0.05$; ** $p < 0.01$.

Figure 8: Correction of MF mutation in hiPSC by CRISPR/Cas9 editing rescues fibrillin-1 phenotype. a) Immunostaining of human ascending aorta sections of healthy patients and MF patients for P-p38 (green), ACTA2 (magenta) and DAPI (blue). **b)** Quantification of P-p38 staining relative to number of ACTA2 positive SMC cells in healthy and MF aortic sections (n=2). **c)** Immunostaining for KLF4 (green), ACTA2 (magenta) and DAPI (blue) in aortas of healthy and MF patients. **d)** KLF4 staining quantification relative to the number of ACTA2 positive cells in healthy and MF aortic sections (n=2). **e)** Immunostaining for fibrillin-1 in S30 NC-SMC derived from MF^{C1242Y} hiPSC, CRISPR MF mutant (MF^{C1242Y} hiPSC CRISPR-edited with WT sequence into

the WT allele), CRISPR MF corrected (MF^{C1242Y} hiPSC CRISPR-edited with WT sequence into the mutant allele to correct the C1242Y mutation) and WT hiPSC. **f)** Quantification of fibrillin-1 staining levels in the four groups depicted in **e** normalized for cell numbers. The asterisks indicate statistically significant differences; * p< 0.05; ** p< 0.01. **g)** Cropped blots of phosphorylated and total SMAD2, ERK1/2 and p38 with β -actin loading controls in S30 NC-SMC derived from MF^{C1242Y} hiPSC, CRISPR MF mutant, CRISPR MF corrected and WT hiPSC. The result is representative of two independent experiments. **h)** AnnexinV assay to determine apoptotic rate in CRISPR MF corrected compared to CRISPR MF mutant. The results are presented as means $\pm$ SD of two independent experiments. **i)** Schematic of the proposed mechanism for regulating SMC loss and matrix breakdown in MFS.

Supplementary Material and Methods

hiPSC Derivation and Culture Conditions

MF^{C1242Y} fibroblasts were purchased from Coriell cell bank (GM21943).

MF^{G880S} fibroblasts were obtained from Addenbrooke's hospital.

The generation of patient-derived hiPSC was approved by NRES Committee, Cambridgeshire (ethic code; 11/EE/0053) and informed consent was obtained from all the patients. Fibroblasts were cultured in DMEM (Sigma) with 10% FBS (Sigma), 50 U ml⁻¹ penicillin (P) and 50 µg ml⁻¹ streptomycin (S). To generate hiPSC, MF fibroblasts harboring *FBN1* mutations were transfected using commercially available monocistronic iPSC reprogramming kit from Vectalys, consisting of four vectors encoding: *OCT4*, *SOX2*, *KLF4*, *v-MYC*; retroviral transduction was performed on 100 000 cells per one well of 6-well plate in mitotically inactive mouse embryonic fibroblasts (MEF) media without P/S per patient line. Five days post-transduction, the cells were resuspended with trypsin, and 1 × 10⁵ cells were and seeded onto 10-cm dishes pre-plated with irradiated MEF feeders (CF-1 MEF IRR). Colonies appeared between day 12 and 32 after transfection. Colonies with hESC-like morphology were manually picked and transferred to 12-well plates pre-plated with inactivated MEF feeders containing 10µM p160ROCK (Rho-Associated coiled-coil containing Protein Kinase 1) inhibitor (Y-27632; Tocris). Two independent colonies were picked and expanded from each of the MF mutant lines. All hiPSC were maintained on irradiated mouse feeders in typical DMEM/F12 medium, supplemented with 20% Knockout Serum Replacement (Gibco), 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM β-mercaptoethanol, and 4 ng/ml FGF-2 (R&D System). Medium was changed

every day. Cells were routinely passaged using 1 mg/ml type IV collagenase (Invitrogen).

Human WT hiPSC were obtained from the Cambridge Biomedical Research Centre iPS Core Facility. Marfan patient specific hiPS and control hiPS cells are referred as MF hiPSC and WT hiPSC respectively.

All hiPSC lines were validated by Cambridge Biomedical Research Centre iPS Core Facility for expression of endogenous pluripotency markers versus transgenes by qPCR and by *in vitro* differentiation into the three germ layers.

hiPSC lines were routinely tested for presence of mycoplasma contamination by Mycoplasma Experience LTD.

CRISPR-mediated FBN1 gene editing

i. Construction and cloning strategy of CRISPR guide RNA/CAS9 plasmid

The Cas9 and single guide RNA (sgRNA) plasmid pSpCas9(BB)-2A-Puro (PX459) was obtained from Addgene (plasmid 48141). The sgRNAs targeting *FBN1* gene were designed according to the rule of 5'-GN₂₀NGG-3' (*Supplemental Table 2*). sgRNAs were synthesized and ligated to the PX459 plasmid that was digested with BbsI (New England Biolabs) to obtain an expression vector of Cas9 and *FBN1* sgRNA. CRISPR2 guide RNA (*Supplementary Table 2*) was selected for targeting using Transgenomic™ Surveyor™ Mutation Detection Kit (Thermo Fisher).

A fragment of genomic DNA from *FBN1* containing 1063 BP upstream and 970 BP downstream flanking Exon 30 was amplified by PCR with specific primers (*Supplemental Table 2*). This fragment was cloned into a pUC18plasmid using PstI and SacI restriction sites. To facilitate the

screening, a silent mutation, which removes the putative ClaI restriction site in Exon 30, was created by directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit, Qiagen). The piggyBac PGK-PuroR-pA cassette (a kind gift from Dr Pentao Liu) was inserted into the donor plasmid at 40bp into the intron upstream of Exon 30. This intronic location was checked with Human Splicing Finder software to avoid exon skipping⁵⁹. KpnI and BclI restriction sites were then created by direct mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit, Quiagen) to generate the final donor plasmid with a selectable marker in the 5' intronic region, the wildtype exon 30 and two intronic homology arms for HDR (*Supplemental Figure 10A*).

ii. Gene targeting in Marfans hiPSC

For gene targeting, 2.5×10^6 MF hiPSCs were electroporated with 1 ug each of the donor plasmid and Cas9 sgRNA plasmid (Addgene) in 82ul Lonza Stem Cell Solution + 18 μ L *supplement 1* (Lonza) using CA137 program of 4D-nucleofector system (Amaxa). Transfected cells were plated onto DR4 strain feeders (Jackson Laboratory) and cultured in CDM BSA+10% KSR supplemented with 4 ng/ml FGF-2 and 10uM of Y-27632. 36 hours after transfection, puromycin selection (1 μ g/mL) was applied and the surviving colonies were picked and expanded for PCR screening verification. Primers used are listed in *Supplementary Table 2*.

hiPSC differentiation protocols

i. 3 germ layers differentiation

Differentiation assays were performed as previously described²⁵. For differentiation into the embryonic germ layers, hiPSC were cultured in

860 chemically defined CDM medium with 10 ng/mL Activin A (R&D system) and
861 12 ng/mL FGF2 (R&D system) on 0.1% gelatin coated plates as described
862 previously⁶⁰. CDM-BSA comprised Iscove's modified Dulbecco's medium
863 (Gibco) plus Ham's F12 NUT-MIX (Gibco) medium in a 1:1 ratio,
864 supplemented with Glutamax-I, chemically defined lipid concentrate (Life
865 Technologies), transferrin (15 µg/ml, Roche Diagnostics), insulin (7 µg/ml,
866 Roche Diagnostics) and monothioglycerol (450 µM, Sigma). hiPSC were
867 induced to differentiate into endoderm using CDM with with polyvinyl alcohol
868 (PVA, 1 mg/ml, Sigma) with 100 ng/mL Activin, 10 ng/mL bone morphogenetic
869 protein 4 (BMP4), 20 ng/mL FGF2, 10 µM phosphoinositide 3-kinase (PI3K)
870 inhibitor Ly (LY294002) and 3 µM glycogen synthase kinase3 inhibitor
871 CHIR99021⁶¹. To obtain mesoderm precursors, hiPSC were grown for the 3
872 following days in CDM-PVA in 20 ng/mL FGF2, 10 ng/mL BMP4 and 10 µM
873 LY294002. Ectoderm was obtained using 1 µM Retinoic Acid (RA) and 25
874 ng/ml BMP4 (R&D Systems).

875 The efficiency of differentiation into the three germ layers was tested for
876 expression of specific markers (*SOX17*, *BRACHYURY* and *PAX6*;
877 *Supplementary Table 3*) by RT-PCR and immunostaining (*SOX17*,
878 *BRACHYURY* and *SOX1*; *Supplementary Table 4*).

879 *ii. SMC lineages differentiation*

880 Lateral mesoderm and paraxial mesoderm intermediate populations were
881 generated according the protocol previously described²¹. The neural crest
882 population was derived using a modified version of the previously described
883 protocol to generate neuroectoderm²⁵. For mesoderm subtype differentiation,
884 we differentiated the hiPSC to 36-h early mesoderm (FlyB) with a combination

of FGF2 (20 ng/ml), LY294002 (10 μ M) and BMP4 (10 ng/ml). Subsequent mesoderm subtype specification was further obtained with FGF (20 ng/ml) and BMP4 (50 ng/ml) for lateral mesoderm (LM) and FGF (20 ng/ml) and Ly (10 μ M) for paraxial mesoderm (PM) for a further 4 days. To produce neural crest (NC), we modified the previously described protocol for neuroectoderm differentiation obtained by using fibroblast growth factor 2 (FGF2, 12 ng/ml) and the activin/nodal inhibitor SB431542 (10 μ M) and passaged the cells first at day 4 and then repeatedly in FGF2 and SB431542 (; *Serrano F, Bernard WG, Granata A, Iyer D, Kim M; in submission*). After obtaining the intermediate populations, cells were trypsinized and cultured in SMC differentiation medium CDM-PVA containing PDGF-BB (10 ng/ml, Peprotech) and TGF- β 1 (2 ng/ml, Peprotech) for at least 12 days (PT). For long-term cultures, SMCs were subsequently grown in MEM medium (Sigma M5650) containing 10% fetal bovine serum (Sigma F7524) up to 10 passages. Each intermediate was tested for the expression of specific genes (NKX2.5 for LM, TBX15 or MEOM1 for PM and p75 for NC) by RT-PCR and immunostaining.

TGF- β 1 ELISA

hiPSC-SMC were cultured in serum-free medium for 24h. Levels of TGF- β 1 in culture supernatant were estimated by using DuoSet Sandwich ELISA according to the manufacturer's instructions (DY240-05; R&D System). The samples were treated with the activation reagent (1N HCl) for 10 min at room temperature followed by addition of the neutralization reagent (1.2 N NaOH/0.5 M HEPES). Treated samples were transferred to ELISA plates coated with the capture antibody overnight at 4°C and incubated for 2h at RT. After washing, samples were incubated with the detection antibody for further

910 2h at RT. After washing, samples were incubated with Streptavidin-HRP for
911 20 min at RT followed by the substrate solution for further 20 min at RT. The
912 optical density of each well was measured using a microplate reader set to
913 450 nm (Synergy). Recombinant human TGF- β 1 was used as a standard.

914 *Apoptosis assays*

915 *i. Caspases activity*

916 WT and MF SMC cells were harvested at the end of the differentiation
917 protocol (PT day12; PTD12) and at mature stage (after one month culture in
918 serum-containing media; S30). Cells were incubated with Cytofix/Cytoperm
919 Fixation solution (BD Biosciences) for 20 min at 4°C, then washed with Perm
920 Wash Buffer/PBS (BD Biosciences). Cells were treated for Caspases activity
921 using the Vybrant® FAM Poly Caspases Assay kit (V35117; Invitrogen). The
922 staining was done according to manufacture instructors. SMC cells were
923 trypsinised and the resuspension was incubated with FAM-488 for 1h at 37
924 degrees. After a couple of washes, cells were incubated for further 10 minutes
925 with PI (10 μ g/ml) to visualize death cells.

926 *ii. AnnexinV assay*

927 For AnnexinV staining, 1X10⁶ cells/ml were harvested and resuspended in 1X
928 annexin-binding buffer and incubated with 5 μ l of AnnexinV-488 (Alexa Fluor®
929 488 Annexin V/Dead Cell Apoptosis Kit; Life technologies) for 15 minutes at
930 RT. Cells were then resuspended in PBS and measured with a Beckman
931 Coulter Cyan_{ADP} cell analyzer. Flow cytometric data were analyzed with
932 FlowJo VX software.

933

934 *Cell Proliferation Assay*

935 *i. MMT assay*

936 Triplicate samples of 5×10^3 hiPSC-SMC were cultured in a 96-well microtiter
937 plate in MEM with or without serum for 72 h. Cell proliferation was measured
938 by the CellTiter® 96 Non-Radioactive Cell Proliferation Assay Kit (G4000;
939 Promega), according to the manufacturer's recommendations. After
940 incubation with the chromogenic solution for 1h at 37°C, the rate of formazan
941 dye formation was determined by measuring the absorbance at 570 nm. The
942 value of the proliferative cells was expressed relative to the basal value
943 (serum-starved non proliferative cells).

944 *ii. BrdU assay*

945 For BrdU labelling, hiPSC-SMC were treated with BrdU (10 μ M; BD
946 Bioscience) overnight before collection. Cells were fixed with PFA 4%
947 (Affymetrix) and blocked with 3 % BSA/0.5% Triton-X100 in PBS for 20 min at
948 RT. Cells were then treated with 1.5M HCl for 30 min at RT and stained with
949 an anti-BrdU antibody (1:50, Becton Dickinson) overnight at 4°C. The
950 following day, the cells were incubated with a secondary Alexa FITC-tagged
951 secondary antibody (1:500, Molecular Probes Invitrogen) and DAPI (10 μ g/ml,
952 Vector Laboratories) for 1h at RT. The numbers of BrdU-positive cells and
953 total cells were counted in a blinded manner and divided by the total number
954 of cells (DAPI stained nuclei).

955 *Calcium Fluo4-AM assay and contraction study*

956 SMCs were preloaded with the calcium-sensitive fluorophore Fluo-4 AM (2.5
957 μ M, Molecular Probes) in normal extracellular solution (NES; 140 mM NaCl, 5

mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose and 10 mM HEPES, pH 7.3) for 1 h at RT. Cells were washed for 15 min at RT. Intracellular calcium flux was monitored as time series with acquisition rates of 1 frame every 0.2ms over a period of 1 min using a Zeiss LSM 700 confocal microscope, before and after addition of carbachol (100 μ M, Sigma). The total Fluo4-AM uptake was measured by treating the cells with 0.1% Triton to permeabilise the cellular membranes. For both WT and MF SMCs, ten cells were randomly picked from a field of view and the fluorescent trace was analysed using ImageJ software.

FITC-gelatin degradation assay

Cells were plated into m-slide 8 glass bottom wells (Ibidi) previously coated with FITC-gelatin (1mg/ml; Life Technologies G13187), air dried, rehydrated with water for 15 min and fixed with glutaraldehyde for 30 min. Cells were grown on FITC-gelatin-coated wells for 48h and then fixed with 4% PFA, permeabilised with 0.5% Triton X-100, washed twice with PBS and blocked in 1% FBS blocking solution. Cells were then stained with a CNN1 specific antibody (1:2000; Sigma) overnight at 4°C followed by Alexa Fluor 588-tagged secondary antibody (1:500, Molecular Probes Invitrogen) and DAPI (10 μ g/ml, Vector Laboratories) for 1h at RT. Fluorescence intensity was analysed with ImageJ software and expressed as relative to cell numbers (DAPI staining). 5 images were taken for each well of each of the three independent. The results are presented as means $\pm$ SD of three independent experiments.

Immunofluorescence staining of hiPSC, intermediates and SMCs

981 Adherent cells were fixed with 4% PFA, permeabilised with 0.5% Triton-X100
982 (Sigma) in PBS and blocked with 5 % serum/PBS for 1h at RT.

983 The full list of primary antibodies used is provided in *Supplementary Table 4*.

984 hiPSC were incubated with primary antibodies: OCT3/4, SOX2, TRA-1-60 and
985 SSEA4 overnight at 4°C. Intermediate populations were stained with primary
986 antibody specific for LM (NKX2.5), PM (MEOX1) and NC (p75). SMC were
987 stained for SM markers, using primary antibodies for CNN1 (1:20,000,
988 Calponin1, Sigma) and ACTA2 (1:500, smooth muscle α -ACTIN, Sigma)
989 overnight at 4°C.

990 The 3 germ layers derived from hiPSC were stained using the Human Three
991 Germ Layer 3-Color Immunocytochemistry Kit (SC022; R&D System).

992 In WT and MF SMC, fibrillin 1 was detected in the extracellular matrix using a
993 specific monoclonal antibody (1:400, Millipore) and Collagen Type I using a
994 polyclonal antibody against subunit α 1 (1:200; R&D System). For cytoskeletal
995 and focal adhesion analysis, cells were stained with CytoPainter Phalloidin-
996 iFluor 488 (1:200; Abcam) and anti-Vinculin antibody (1:100; Sigma)
997 respectively. The cells were then stained with Alexa Fluor-tagged secondary
998 antibodies (1:500, Molecular Probes Invitrogen) and DAPI (10 μ g/ml, Vector
999 Laboratories) for 1h at RT.

1000 Images were taken with a Zeiss Axioplan microscope equipped with an
1001 Axiocam HRc digital camera. Fibrillin-1 staining was quantified using ImageJ
1002 software and expressed as relative to cell numbers (DAPI staining). 5 images
1003 were taken for each well of each of the three independent experiments done
1004 for each condition. The results are presented as means $\pm$ SD of three

1005 independent experiments.

1006 *Immunofluorescence staining of human tissues*

1007 Aortic tissues were obtained by Priya Sastry, Research Fellow at Papworth
1008 Hospital (project ID: 130372). The research was approved by NRES
1009 Committee East of England and informed consent was obtained from all the
1010 patients. The frozen tissues were sectioned and fixed with 4% PFA,
1011 permeabilised with 0.5% Triton-X100 in PBS and blocked with 5 %
1012 serum/PBS for 1h at RT. Section were incubated with anti-KLF4 (1:200;
1013 Abcam) or anti-phospho-p38 (1:200, R&D System) and ACTA2 (1:200)
1014 antibodies. Afterwards, sections were incubated with Alexa Fluor-tagged 488
1015 and 568 secondary antibodies for 1h at RT. Sections were then washed with
1016 PBS and mounted with VECTASHIELD-DAPI mounting medium (VECTOR
1017 Laboratories). The numbers of KLF4 or P-p38-positive cells were counted in a
1018 blinded manner and divided by the total number of ACTA2 positive-cells. The
1019 results are presented as means $\pm$ SD of two independent experiments.

1020 *Luciferase assays*

1021 *i. MLECs (Mink lung epithelial cells) PAI-1 reporter*

1022 MLECs stably transfected with an 800bp fragment of the 5' promoter end of
1023 the human PAI-1 gene, fused to the luciferase gene (4×10^4 cells) were plated
1024 into a 96-well plate with DMEM supplemented with 10% FBS and 200 μ g/ml
1025 G418. The day after, MLECs were treated with conditioned media from WT
1026 and MF SMC for 12h. Control cells were left untreated. After, MLECs were
1027 lysate with 20 μ l of Passive Lysis Buffer 1X (Luciferase system; Promega
1028 E1501). Luciferase assay reagent (100 μ l: Promega) was added to each well
1029 by injector and the relative luciferase unit (RLU) was read by 2030 Multilabel

1030 Reader (Perkin Elmer).

1031 ii. *SBE4 Reporter Luciferase assay*

1032 SMC were plated into a 24-well plate and transfected with SBE4-Luciferase
1033 reporter vector (Addgene plasmid #164965; 200 ng) using Lipofectamin2000
1034 (Life Technologies), following manufacturer instructions. On day 2 after the
1035 transfection, SMC were serum-starved. After 24 hours, SMC were treated with
1036 15 µg/ml of TGFβ or 5 µg/ml of anti-TGFβ blocking antibody or Losartan
1037 (1µM) for 12 hours. Control cells were kept in serum-free media. After, SMC
1038 were lysed with 60µl of Passive Lysis Buffer 1X and transferred in triplicates
1039 into a 96-well plate. Luciferase assay was performed and RLU was measured
1040 as previously described. The results are the mean ± SD of three independent
1041 experiments.

1042 *Cyclic Strain*

1043 SMC were plated on silicone elastomer-bottomed culture Collagen I,
1044 precoated plates and grown for 2 up to 7 days, and subjected to cyclic strain
1045 with a Cyclic Stress Unit (FX5000 Tension System, Flexcell International
1046 Corporation). Cyclic sine wave deformation (60 cycles/min) and 10%
1047 elongation were applied for 24h previous to cells harvesting for RNA analysis.

1048 *Static stretching*

1049 500µm-thick polydimethylsiloxane (PDMS) membranes were produced using
1050 spin-coating of silicon wafers (Christophe Verstreken at the Cambridge Stem
1051 Cell Institute). These were functionalised using plasma treatment and UV
1052 exposure of sulfo-sanpah (Thermo-Fisher), and incubated with collagen IV
1053 (Corning) overnight. NC-SMC were seeded onto the membranes at a density

1054 of 2×10^5 and left to attach for 4hrs at 37°C. The membranes were stretched
1055 20% uniaxially overnight using a custom-made device, while controls were left
1056 unstretched. Membranes were washed three times with PBS, fixed with PFA
1057 4% for 15 min, washed with PBS and permeabilised using a blocking buffer
1058 (0.5% Triton X-100) for 1hr at RT. Incubation with primary antibodies anti-
1059 vinculin antibody (1:100; Sigma) overnight at 4°C, was followed by
1060 incubations with Alexa Fluor-tagged 568 and CytoPainter Phalloidin-iFluor
1061 488 (1:200; Abcam) and DAPI for 1h at RT. Membranes were washed three
1062 times with PBS for 5min and were mounted using ProLong Gold (Thermo-
1063 Fisher).

1064 *TGF- β neutralizing antibody and inhibitors treatment*

1065 SMC cells at stage PT d12 or after one-month culture in serum-containing
1066 media (S30), were treated for a week with different inhibitors, which are listed
1067 in *Supplementary Table 5*. Samples were processed for fibrillin-1
1068 immunostaining analysis and RT-PCR analysis as described above.

1069 *KLF4 siRNA gene silencing*

1070 KLF4 knockdown was carried out using KLF4 Silencer Select from Ambion
1071 (siRKLF4; ID517793). A nonspecific siRNA (siScr; AllStars Negative control,
1072 Qiagen) was used as a negative control. hiPSC-derived SMCs were
1073 transfected with siRNA (20 nM for 1 well of 6-wells plate) using DharmaFECT
1074 transfection reagent (Thermo Scientific Dharmacon). The transfection was
1075 repeated after 2 days and the mRNA and protein analyses were done after
1076 24h and 48h respectively. The efficiency of *KLF4* knockdown by siRNA was
1077 approximately 90%.

1078

1079 *Statistical Analysis*

1080 The results in the figure legends are presented as the mean $\pm$ SD of three
1081 independent replicates. Statistical differences between the means were
1082 examined by Student's t test, two-sided. *P < 0.05 was considered statistically
1083 significant.

1084 Pre-specified effect size was not defined. Between 2 to 5 independent
1085 samples for experiments were used and estimates of normality were not
1086 necessary.

1087

1088 See Supplementary Notes for *Teratoma Formation, RNA Isolation, Reverse-*
1089 *Transcriptase Polymerase Chain Reaction and Quantitative PCR Analysis,*
1090 *Western blotting Analysis, Transmission Electron microscopy (TEM), Flow*
1091 *cytometric analysis and Antiplasmin viability assay.*

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