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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**
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15 **Abstract**

16 Marfan syndrome (MFS) is a heritable connective tissue disorder caused by
17 mutations in fibrillin-1, an extracellular matrix protein. To investigate the
18 pathogenesis of aortic aneurysms in MFS, we have generated the first
19 vascular model derived from human induced pluripotent stem cells (hiPSC).
20 Our MFS-hiPSC derived smooth muscle cells (SMC) accurately recapitulated
21 the pathology seen in Marfan aortas, including defects in fibrillin-1
22 accumulation, extra-cellular matrix degradation, TGF-β signaling, contraction,
23 and apoptosis; abnormalities are corrected by CRISPR-editing of the fibrillin-1
24 mutation. TGF-β inhibition rescued abnormalities in fibrillin-1 accumulation
25 and matrix metalloproteinase expression. However, only the non-canonical
26 p38 pathway regulated SMC apoptosis, a pathological mechanism also
27 governed by KLF4. This model has allowed us to dissect the molecular
28 mechanisms of MFS, to identify novel targets for treatment, such as p38 and
29 KLF4, and has provided an innovative human platform for the testing of new
30 drugs.
31

32 **Introduction**

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

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

49 However, the precise functional role of TGF-β in aneurysm development
50 remains controversial[13,14]. Indeed, recent studies in mice have exposed the
51 complexity of TGF-β signaling in MFS, by showing that both TGF-β canonical
52 and non-canonical pathways contribute to aneurysm growth and that TGF-
53 β exerts both protective and detrimental effects[15]. The complex pleiotropic role
54 of TGF-β in aortic remodeling may also account in part for the finding that in a
55 recent clinical trial, losartan showed no benefit over the β-blocker atenolol in
56 regulating the rate of aortic dilatation[16].

57 Currently, most of our knowledge of MFS pathogenesis has been gained from
58 studies in animal models, including Fbn1$^{mgR/mgR}$ and Fbn1$^{C1039G/+}$ mice[11,17–19].
59 However, the emergence of human induced pluripotent stem cells (hiPSC) for
60 disease modeling[20] provides an opportunity to use a new approach to study
61 both the early development and disease pathology of MFS on a susceptible
62 human genetic background.
63 Here we describe the generation of the first vascular human model of MFS.
64 Patient-derived hiPSC lines are differentiated into embryonic lineage-specific
65 smooth muscle cells (SMC)[21] that reliably phenocopy the aortic pathology in
66 patients with MFS. Importantly, our study demonstrates that at an early stage
67 TGF-β appears to contribute to the developing MFS phenotype but at later
68 stages, blockade of TGF-β signaling by using losartan is ineffective in averting
69 SMC death. In contrast, using our human MFS-SMC model, we highlight a
70 previously undocumented role for p38-MAP kinase in regulating SMC
71 apoptosis and proliferation, and identify Kruppel-like factor 4 (KLF4) as a new
72 potential contributing factor to MF pathology.
73

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, MF^{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 domain[23]. The second line, MF^{G880S}, harbors a missense mutation in exon 21 (2638G>A) leading to a glycine-to-serine substitution[24]. 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 previously[26] and then trypsinized to single cells and passaged up to 12 times to generate neural crest-like cells (NC; *Serrano F,*

99 *Bernard WG, Granata A, Iyer D, Kim M, Gambardella L, and Sinha S; in*
100 *submission*). Similar to the mesoderm intermediates, NC were differentiated
101 to SMC and allowed to mature in serum-containing medium (*Supplementary*
102 *Fig. 2*). The intermediate populations were characterized for expression of
103 specific markers respectively of lateral mesoderm, paraxial mesoderm and
104 NC cells at both mRNA and protein level (*Supplementary Fig. 2a-b*). In
105 response to TGF-β1 and PDGF-BB treatment, the expression of these
106 intermediate markers was downregulated while the levels of specific SMC
107 markers, including *CNN1* and *MHY11* increased (*Supplementary Fig. 2a*).
108

109 **Abnormal fibrillin-1 deposition and increased TGF-β**

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

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

125 In MFS, abnormal fibrillin-1 may lead to excessive release of TGF-β from the

126 ECM [9]. Accordingly, we measured TGF-β1 in the WT and MF^{C1242Y} SMC

127 supernatants by ELISA. There were increased TGF-β1 levels in all MF^{C1242Y}

128 SMC populations compared with the WT and this increase was greatest in the

129 supernatant of NC-derived MF^{C1242Y} SMC (**Fig. 1g**). Equally, MF^{G880S} NC-

130 SMC also showed higher TGF-β1 levels (*Supplementary Fig. 4e*).

131 Consistent with these observations, TGF-β1 protein, extracted from SMC and

132 extracellular matrix, was increased in MF^{C1242Y} and MF^{G880S} NC-SMC

133 compared with WT and the two other lineages (**Fig. 1h**). TGF-β1 mRNA levels

134 did not significantly differ in WT and MF SMC lineages, suggesting that

135 increased protein levels were due to increased accumulation in the ECM

136 (*Supplementary Fig. 4f*). We observed increased mRNA levels of

137 plasminogen activator inhibitor-1 (PAI-1), a known target of TGF-β1 signaling

138 [27], and of a range of matrix metalloproteinases (MMPs) in MF^{C1242Y} SMC,

139 especially in the NC subtype (**Fig. 1i,j**). Data supporting higher TGF-β1

140 activity in MF^{C1242Y} and MF^{G880S} NC-SMC were obtained by measuring the

141 luciferase signal in Mink Lung Epithelial Cells stably transfected with human

142 PAI-1 luciferase reporter and incubated with NC-SMC conditioned media

143 (*Supplementary Fig. 4g*).

144

145 **MF^{C1242Y} NC-SMC recapitulate the MFS aortic phenotype**

146 Our MFS hiPSC-derived SMC model allows us to observe changes in the NC-

147 SMC phenotype from an early stage of development (PTD12) to a more

148 mature stage (S30), which may reflect the disease progression occurring in

149 MFS patients[28].

150 We observed morphological differences between WT and MF^{C1242Y} NC-SMC
151 following prolonged culture in serum-containing media (S30), with WT NC-
152 SMC appearing spindle shaped while MF^{C1242Y} NC-SMC were larger and
153 more stellate in appearance (**Fig. 2a**). mRNA analysis showed increased SM
154 markers in MF^{C1242Y} NC-SMC at S30 versus WT NC-SMC (**Fig. 2a,b**).
155 Furthermore, the proliferative capacity of both MF^{C1242Y} NC-SMC clones
156 decreased dramatically during maturation, compared with WT (**Fig. 2c,d**).
157 Similar morphology and behavior were observed in MF^{G880S} NC-SMC
158 (*Supplementary Fig. 5a,b*). Moreover, MF^{C1242Y} NC-SMC displayed higher
159 poly-caspase activity at an early stage (PTD12); while at a more mature
160 stage, MFS NC-SMC showed a higher incidence of cell death in both mutant
161 lines compared to WT NC-SMC, consistent with increased SMC loss in MFS
162 aortic dilatation (**Fig. 2e;** *Supplementary Fig. 5c*).

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

174 [31,32].

175

176 **TGF-β signaling and cyclic stretching in** MF^{C1242Y} **model**

177 The MFS mouse model has implicated both TGF-β canonical and non-

178 canonical (ERK-mediated) signaling pathways in contributing to the progress

179 of the Marfan phenotype [11]. We used our human *in vitro* model to dissect

180 TGF-β pathways at both early and mature stages. We observed that

181 phosphorylated levels of a TGF-β canonical signaling component, such as P-

182 SMAD2, and non-canonical components, including P-ERK1/2 and P-p38,

183 were increased in MF^{C1242Y} and MF^{G880S} NC-SMC at PTD12 stage compared

184 to WT, while no differences were detected between WT and MF fibroblast

185 lines (**Fig. 3a,b** and *Supplementary Fig. 6a*). At stage S30, the increase in

186 ERK1/2 phosphorylation levels was no longer detectable while P-p38 and P-

187 SMAD2 levels remained elevated (**Fig. 3b**). These findings are consistent with

188 recent observations of developmental stage-dependent differences in

189 signaling in the MF mouse[15].

190 When we looked at the transcriptional levels of MMPs, downstream targets of

191 TGF-β signaling, we observed that while fibroblast lines did not show

192 significant differences (*Supplementary Figure 6b*), MMPs expression levels

193 were increased in both MF NC-SMC mutants at PTD12 (*Supplementary Fig.*

194 *6c*) and at mature stages (S30; **Fig. 3c**). The tissue specific inhibitors of

195 MMPs (TIMPs), showed a biphasic expression pattern with increased

196 expression at an early stage (*Supplementary Fig. 6d*), but with lower TIMP

197 expression levels at S30 than in WT (**Fig. 3d**) suggesting increasing

198 proteolysis with prolonged culture. To confirm increased MMP activity in the

199 MF NC-SMC at S30, we also performed a FITC-gelatin degradation
200 experiment, which showed extensive proteolytic activity in MF NC-SMC
201 compared to the WT (**Fig. 3e,f,** *Supplementary Fig. 6e*).
202

203 **SMC cyclic stretch exacerbates the MF phenotype**

204 WT and MF SMC were mechanically stretched for 24h using 10% cyclic
205 stretching at 1Hz to mimic aortic hemodynamic forces. After 24h, both WT
206 and MF^{C1242Y} NC-SMC were aligned parallel to the direction of the stretch
207 (**Fig. 4a**). SM markers showed increased expression in MF^{C1242Y} NC-SMC
208 (**Fig. 4b**). Also, *MMP9* and *MMP10* were significantly increased in MF^{C1242Y}
209 NC-SMC in response to stretching (**Fig 4c**). Furthermore, we observed that
210 Collagen type I (COL-1) expression and deposition were higher in MF NC-
211 SMC compared to WT and further increased upon stretching (**Fig. 4d-e**),
212 which may lead to greater stiffness of the ECM[33]. Moreover, we observed
213 increased binding of phalloidin-FITC and immunostaining for Vinculin in
214 MF^{C1242Y} SMC in static conditions compared to WT, which were both further
215 incremented after stretching, suggesting that MF^{C1242Y} SMC have higher
216 density of stress-fibres (Phalloidin) and focal adhesions (Vinculin) than WT
217 SMC (**Fig. 4f-h**). Remarkably, we also observed higher expression of p38 in
218 MF^{C1242Y} SMC in response to cyclic stretch at both mRNA and protein levels
219 (**Fig. 4i,j**).
220

221 **Effect of general TGF-β inhibition on the MF phenotype**

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

224 with anti-TGF-β neutralizing antibody and Losartan, a specific AGTR1 blocker
225 (*Supplementary Fig. 7a*). In mature NC-SMC cells (S30), both treatments
226 promoted fibrillin-1 accumulation in MF^{C1242Y} NC-SMC (**Fig. 5a,b),** associated
227 with reduced canonical TGF-β activity, as measured by transfecting a 4xSmad
228 binding element (SBE4) promoter-luciferase reporter in both WT and MF^{C1242Y}
229 NC-SMC (**Fig. 5c)**. Moreover, anti-TGF-β blocking antibody and Losartan
230 treatments were both able to downregulate efficiently the expression levels of
231 MMPs and also to upregulate *TIMP3* both in mature cells (S30; **Fig. 5d**) and
232 at early stages (PTD12, *Supplementary Fig. 7b*). We therefore propose that
233 excessive TGF-β activity, through effects on MMP and TIMP expression, is
234 responsible at least in part for the reduced amount of fibrillin-1 in MFS NC-
235 SMC.
236 Notably, Losartan was more effective in reducing ECM degradation than
237 Doxycycline (DOX), a nonspecific inhibitor of MMPs, suggesting losartan may
238 be acting through additional mechanisms beyond simple MMP inhibition
239 (*Supplementary Fig. 7c,d***)**. Extracellular fibrillin-1 only partly increased
240 following DOX treatment, suggesting that the changes seen are consequence
241 of abnormalities in both degradation and deposition and that mutant fibrillin-1
242 may be more prone to proteolytic degradation than WT (*Supplementary*
243 *Fig.7e,f*). High level of TGF-β1 is likely to be caused by ECM breakdown, as it
244 decreases upon DOX treatment (*Supplementary Fig. 7g*).
245 Despite beneficial effects on fibrillin-1 deposition and matrix proteolysis, anti-
246 TGF-β blocking antibody treatment failed to rescue the impaired proliferation
247 of MF^{C1242Y} NC-SMC, while Losartan provided partial rescue (**Fig. 5e**).
248 Furthermore, the increased apoptosis seen in MF^{C1242Y} NC-SMC was

249 unaffected upon Losartan treatment as shown by AnnexinV analysis (**Fig. 5f)**.
250 Likewise, a poly-caspase assay also showed no benefit with losartan on
251 MF^{C1242Y} NC-SMC cell death (*Supplementary Fig. 8a*).
252 Taken together, these results suggest that ECM turnover in MFS is regulated
253 through pathways that are distinct from those regulating SMC proliferation and
254 death.
255

256 **Emerging roles of p38, KLF4 and β1 integrin in MFS model**

257 To better understand the contribution of different TGF-β signaling pathways in
258 the development of the MFS phenotype, NC-SMC were treated with inhibitors
259 of either the canonical pathway (SB431542), ERK1/2 phosphorylation
260 (PD98059) or p38-MAP kinase (SB203580) (**Fig. 6** and *Supplementary Fig.*
261 *9a,b*). Analysis of fibrillin-1 deposition by immunostaining showed that
262 inhibition of p38 phosphorylation was surprisingly effective in rescuing the
263 phenotype, comparable to Losartan in both MF^{C1242Y} and MF^{G880S} (**Fig. 6a,b**
264 and *Supplementary Fig. 10a,b*), while inhibition of canonical Smad signaling
265 or ERK1/2 by SB431542 or PD98059 respectively had no significant effects
266 (**Fig. 6a,b** and *Supplementary Fig. 10a,b*). Comparable results were found for
267 effects on cell proliferation with significant rescue of suppressed proliferation
268 only by the p38-MAP kinase inhibitor, SB203580 and Losartan (**Fig. 6c**).
269 To investigate further the proliferative mechanisms affected in MF NC-SMC,
270 we looked at potential regulators, including KLF4 (Krüppel-like factor 4) and
271 observed a dramatic increase in KLF4 mRNA levels in MF lines compared to
272 WT NC-SMC (**Fig. 6d** and *Supplementary Fig. 11a*). Both inhibition of p38
273 upon SB203580 treatment and efficient knock-down of KLF4 using siRNA

274 (siRKLF4) were able to increase mRNA levels of the proliferation marker,
275 *CCND1* (cyclinD1), which were significantly lower in MF^{C1242Y} NC-SMC
276 compared to WT (**Fig. 6e**). Moreover, both *TP53* (tumor suppressor protein
277 p53) and *CDKN1A* (*p21*; Cyclin-Dependent Kinase Inhibitor 1A), which
278 promote cell cycle arrest, were upregulated in MF cells and reduced in
279 response to SB203580 and SiKLF4 treatments (**Fig. 6f,g**). Therefore,
280 silencing KLF4 had also a positive effect on MF^{C1242Y} and MF^{G880S} NC-SMC
281 proliferation (*Supplementary Fig. 11b,c*). Moreover, silencing of KLF4
282 increased the deposition of fibrillin-1 in the matrix compared to the control
283 scramble siRNA (siScr; **Fig. 6h,i**).
284 Similarly to KLF4 siRNA approach, a specific inhibitor of the MEK5/ERK5
285 pathway, BIX02189, exclusively reduced KLF4 mRNA and protein levels in
286 MF^{C1242Y} NC-SMC (**Fig. 6j** and *Supplementary Fig. 11d*). Conversely,
287 SB203580 and Losartan, did not affect total KLF4 mRNA and protein levels,
288 suggesting that p38 and KLF4 contribute independently to the same signaling
289 pathway that controls SMC proliferation (**Fig. 6j** and *Supplementary Fig. 11d*).
290 Consequently, inhibiting the MEK5/ERK5 pathway by BIX02189 restored
291 normal proliferation rate and mRNA levels for TP53, *CCND1* and *CDKN1A* in
292 MF^{C1242Y} NC-SMCs comparable to WT (**Fig. 6c** and *Supplementary Fig. 11e*).
293 Notably, both SB203580 and KLF4 siRNA reduced the apoptotic activity of
294 both MF mutant lines (**Fig. 7a** and *Supplementary Fig. 10d*). This may
295 indicate that both p38 and KLF4 contribute to the same regulatory apoptotic
296 mechanism, which is exclusive of SMC, since no difference in cell death was
297 seen between WT and MF fibroblasts (*Supplementary Figure 10c*). Whereas,
298 MF^{C1242Y} NC-SMC apoptotic levels were found unchanged or increased upon

299 Losartan and PD98059 or U0126 (selective inhibitor of MEK1/2) treatments
300 respectively, suggesting that the ERK1/2-mediated pathway has a protective
301 role for cell death in this model (**Fig. 7a** and *Supplementary Fig. 12a*).
302 Moreover, MF NC-SMC apoptosis was substantially reduced in cells with
303 plasminogen activation, implying that pericellular proteolysis is partially
304 responsible for MF SMC death[34] (*Supplemental Fig. 12b*).
305 Finally, a new insight about the MF cell death mechanisms comes from the
306 stretch studies. Upon cyclic stretching, MF^{C1242Y} NC-SMC expressed higher
307 levels of *KLF4*, *CDKN1A* and *TP53*, which may contribute to MF apoptosis
308 (**Fig. 7b**). In addition, β1 integrin, an essential adhesion molecule, increased
309 in response to cyclic stretch at both mRNA and protein levels (**Fig. 7c,d**). A
310 blocking anti-β1 integrin antibody specifically downregulated p38 phospho-
311 protein levels and consequently *TP53* expression (**Fig. 7e,f**). The hypothesis
312 that β1 integrin may play a role in promoting apoptosis, potentially mediated
313 by p38 in MF NC-SMC is consistent with the AnnexinV study that showed
314 decreased apoptotic levels in MF NC-SMC upon anti-β1 integrin blockade
315 (**Fig. 7g).** And since β1 integrin was more expressed in NC-SMC compared to
316 the other lineages, these findings may be a potential explanation of why NC-
317 SMC are more vulnerable to fibrillin-1 mutation (*Supplementary Fig. 13a,b)*.
318

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

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

324 To verify that the MF^{C1242Y} hiPSC-derived SMC phenotype is caused solely by
325 the C1242Y mutation, we generated a CRISPR/Cas9 isogenic MF hiPSC line
326 where the nucleotides in the mutant allele were replaced with WT nucleotides,
327 which we refer to as CRISPR MF corrected (*Supplementary Fig. 14a,b*). In
328 parallel, we generated as control an isogenic CRISPR/Cas9 MF hiPSC line
329 where the WT nucleotides were inserted in the WT allele, therefore retaining
330 the C1242Y mutation (CRISPR MF mutant; *Supplementary Fig. 14b-d*).
331 CRISPR MF corrected showed a WT fibrillin-1 phenotype compared to
332 MF^{C1242Y} and CRISPR MF mutant (**Fig. 8e,f**). In CRISPR MF corrected NC-
333 SMC, the phosphorylated levels of TGF-β pathway effectors were lowered to
334 levels comparable to WT (**Fig. 8g**). In addition, TGF-β levels in the
335 supernatant, the mRNA levels of MMP10, and the extent of matrix
336 degradation of CRISPR MF corrected NC-SMC were significantly reduced
337 (**Fig. 8h** and *Supplementary Fig. 15a-f*).
338 In conclusion, our hiPSC-derived SMC have proven to be a robust model for
339 MFS and a unique tool for the identification of new targets, including p38 and
340 KLF4, which may represent novel therapeutic opportunities for MFS treatment
341 (**Fig. 8i**).
342

343 **Discussion**

344 This study describes the first Marfan Syndrome (MFS) patient-derived hiPSC
345 model that faithfully mimics the human vascular phenotype, which is the main
346 cause of premature death in MFS patients[7]. Recently, Quarto and colleagues
347 described the MFS skeletal phenotype using a human iPSC model[35].
348 However, our model recapitulates the key aspects of MFS vascular pathology
349 and highlights the complexity of the downstream signaling pathways
350 identifying a key role for p38 and KLF4 in disease development.
351 A crucial step in these studies was to generate vascular SMC of specific
352 embryonic origins to model different aortic regions as aneurysms in MFS are
353 found principally in the aortic root, ascending aorta and arch, regions invested
354 by NC-SMC[36,37]. Interestingly, our model indicated that SMC of neural crest
355 origin, which deposit higher levels of extracellular fibrillin-1 (Fig. 2a,b) and
356 appear to be more proliferative when compared to the other two lineages, are
357 most severely affected by the disease. Thus, NC-SMC appear to possess
358 intrinsic properties, for instance differential expression of β1 integrin, that
359 make them more susceptible to *FBN1* mutations, reinforcing the importance of
360 using lineage-specific systems for vascular disease modeling.
361 Abnormal TGF-β activation and signaling have been documented in MFS
362 mouse models[12,19,38] and has been proposed as a common factor in MFS-
363 related disorders, such as Loeys-Dietz syndrome[39–41]. As TGF-β
364 bioavailability seems to be affected by inhibition of MMP activity via
365 Doxycycline, we suggest that increased proteolysis is responsible for high
366 TGF-β levels detected in our MF model[42]. Importantly, blocking TGF-β,
367 whether by using TGF-β neutralizing antibody or the AGTR1 blocker,

368 Losartan, attenuated or prevented aortic root dilatation in mice[43]. However,
369 recent randomized clinical trials in patients with MFS have been disappointing
370 with losartan showing no benefit over either placebo or beta-blockade[16,44,45].
371 The rationale for using Losartan as a TGF-β blocker is that Angiotensin II can
372 regulate TGF-β1 mRNA and protein expression and potentially TGF-β
373 activation[46,47] and cross-talk with TGF-β signaling by promoting the
374 phosphorylation of MAP kinases[48]
375 Interestingly, in C1039G MFS mice, TGF-β-mediated ERK1/2 activation was
376 driving aneurysm formation[19]. However, other studies have shown that TGF-β
377 neutralization either exacerbated or mitigated TAA progression depending on
378 whether the treatment was initiated before or after aneurysm formation[15,49].
379 Indeed, dual treatment with losartan and late TGF-β blockade entirely blocked
380 aortic aneurysm development in the mgR mouse accompanied by a dramatic
381 increase in phosphorylated ERK1/2[15]. These findings suggest that the link
382 between ERK1/2 phosphorylation and disease development is not simple.
383 Interestingly, p38 activation has been documented in *Fbn1*-null mice (mgN)
384 with a more severe aortic phenotype[50] and systemic p38 blockade has been
385 found to normalize P-smad2 levels in the mgN mouse.
386 Correspondingly, we demonstrated that increased TGF-β activity in MFS SMC
387 was associated with increased phosphorylation of endogenous SMAD2,
388 ERK1/2 and p38 at an early stage as reported in MFS mouse models[19,50],
389 while at a more mature stage, ERK1/2 phosphorylation reduced to WT levels,
390 which may indicate the need for distinct therapies at different disease time-
391 points, as also suggested by Ramirez and colleagues using the mgR mouse
392 model[15].

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 pathogenesis[15].
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 patients[33].
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 β1 integrin

as a potential mediator of this process.

Our findings suggest that β1 integrin, which is known to interact with fibrillin-1[57], appears to have an important role in regulation of MF SMC death, potentially through p38 as shown previously[58] 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.

Acknowledgements

The authors thank Ludovic Vallier, the hiPSC core facility at the Anne McLaren Laboratory and especially Imbisaat Geti for help in generating the MF hiPSC lines. We thank Nicola Figg for the sectioning and staining of the teratomas, Jane Sterling for help with skin biopsies and Jeremy Skepper at the Cambridge Advance Imaging Centre at the University of Cambridge for the TEM images. We also thank Ziad Mallat for useful comments on the manuscript. This work was supported by Evelyn Trust, the NIHR Cambridge

Biomedical Research Centre and the British Heart Foundation
(FS/13/29/30024, RM/l3/3/30159, FS/11/77/29327).

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

623 **Figure 1: Generation of hiPSC and lineage-specific SMC from Marfan**
624 **MF^{C1242Y} fibroblasts show greatest perturbation of fibrillin-1 deposition,**
625 **TGF-β levels and *MMP* expression in MF^{C1242Y} NC-SMC**
626 **a)** DNA sequencing analysis of MF hiPSC showing 3725G>A heterozygous
627 mutation in *FBN1* in exon 30. **b)** Immunofluorescence staining of MF^{C1242Y}
628 hiPSC colonies for the pluripotency markers, OCT3/4, SOX2, SSEA4 and
629 TRA-1-60. Scale bar = 100 μm. **c)** Schematic representation of hiPSC
630 differentiation to three different smooth muscle cell (SMC) origins: lateral
631 mesoderm (LM), paraxial mesoderm (PM) and neuroectoderm (NE)/neural
632 crest (NC). **d)** Immunostaining analyses of extracellular fibrillin-1 in MF^{C1242Y}
633 and WT SMC of LM, PM and NC embryonic origins after 30 days of culturing
634 in serum-containing medium (S30) (scale bar=100μm). **e)** Transmission
635 electron microscopy (TEM) of fibrillin-rich microfibrils (arrows) of WT and
636 MF^{C1242Y} NC-SMC (scale bar = 500nm). **f)** Quantification of fibrillin-1 staining
637 relative to cell number in MF^{C1242Y} and WT SMC of all three embryonic
638 lineages. **g)** Levels of TGF-β1 measured by ELISA in the supernatant of
639 MF^{C1242Y} SMC and WT SMC. **h)** Cropped blot of total TGF-β1 in WT, MF^{C1242Y}
640 and MF^{G880S} SMC lysates. **I)** RT-qPCR of plasminogen activator inhibitor-1
641 (PAI-1) expression in WT and MF^{C1242Y} LM-, PM- and NC-SMCs. **j)** *MMP1*
642 and *MMP9* mRNA levels detected in WT and MF^{C1242Y}SMC lineages. The
643 relative mRNA level was normalized to its *GAPDH*/*PBGD* content. The results
644 are presented as means ± SD of three independent experiments. The
645 asterisks indicate statistically significant differences; * $p < 0.05$; ** $p < 0.01$. ns
646 = non significant.
647 **Figure 2: MF^{C1242Y} hiPSC-derived SMC exhibit functional abnormalities**

648 **consistent with the human disease phenotype.**

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

667 basal level and total Fluo-4AM loading.

668 The results are representative of three independent experiments (means ±

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

670

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

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

673 of total and phosphorylated levels of TGF-β canonical (SMAD2) and non-
674 canonical (ERK1/2 and p38) pathways in WT and MF^{C1242Y} NC-SMC at
675 PTD12 and S30. The result is representative of three independent
676 experiments. **b)** Quantification of the phosphorylated levels of SMAD2,
677 ERK1/2 and p38 relative to the total protein levels and to β-actin. **c,d)** RT-
678 qPCR expression profile of a selection of MMPs and TIMPs in MF^{C1242Y} and
679 WT NC-SMCs at S30 stage. The results for each sample were normalized to
680 its *GAPDH* and *PBGD* content. **e)** FITC-gelatin degradation assay performed
681 with WT and MF^{C1242Y} S30 NC-SMC over 48h. Cells were then fixed and
682 stained for the SMC marker, CNN1 (red). **f)** Quantification of the FITC-
683 degraded areas for S30 MF^{C1242Y} NC-SMCs compared with WT. The results
684 are presented as means ± SD of three independent experiments. The
685 asterisks indicate statistically significant differences; * $p< 0.05$; ** $p< 0.01$; ***p
686 < 0.001. ns = non significant.
687
688 **Figure 4: Effect of mechanical cyclic stretching on MF^{C1242Y} SMC**
689 **phenotype. a)** Morphology of NC-SMC before and after cyclic stretching
690 (Flexcell FX-5000, 10% elongation); double arrows indicate stretch
691 directionality. RT-qPCR mRNA profile of SM markers (*CNN1*, *ACTA2* and
692 *TAGLN*, **b**), *MMP9* and *MMP10* (**c**) and Collagen type I (*COL-1*; **d**) in WT and
693 MF^{C1242Y} NC-SMC before and after 24h stretching. Immunofluorescence
694 analysis of WT and MF^{C1242Y} SMC before and after stretching for Collagen I
695 (**e**), Phalloidin-FITC (**f**) and Vinculin (**g**). **h)** Quantification of Collagen type I,
696 Phalloidin and Vinculin staining intensity relative to cell numbers in WT and
697 MF^{C1242Y} SMC in un-stretched and stretched conditions**. i)** mRNA expression

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

703

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

723 non significant.

724

725 **Figure 6: Inhibition of p38 phosphorylation or AGTR1 and knock-down**
726 **of KLF4 rescue fibrillin-1 deposition and SMC proliferation defects. a)**
727 Immunofluorescence shows fibrillin-1 staining in S30 NC-SMC after 7 days
728 treatment with a range of inhibitors: CTL= control; SB431542 (ALK5 inhibitor);
729 SB203580 (p38 inhibitor); PD98059 (ERK1/2 inhibitor) and Losartan (ATR1
730 inhibitor). **b)** Quantification of fibrillin-1 staining relative to cell number (nuclei)
731 using ImageJ. **c)** MMT assay to measure NC-SMC proliferation after 48h
732 treatment with SB203580, BIX02189 (MK5/ERK5 inhibitor) and Losartan**. d)**
733 *KLF4* mRNA levels in MF^{C1242Y} NC-SMCs and WT NC-SMC in response to
734 KLF4 siRNA (siRKLF4) and scrambled siRNA (siScr) transfections. **e)** *CCND1*
735 (CyclinD1) expression **f)** *TP53* (p53 protein) expression and **g)** *CDKN1A*
736 (P21) mRNA expression levels in MF^{C1242Y} NC-SMC and WT in response to
737 SB203580 and siRKLF4. **h,i)** Immunostaining analysis and quantification of
738 WT and MF^{C1242Y} NC-SMC showing fibrillin-1 deposition in response to KLF4
739 knockdown (siRKLF4) compared to scrambled siRNA (siScr). **j)** Immunoblot
740 for total KLF4 in WT and MF^{C1242Y} NC-SMC samples transfected with
741 siRKLF4 or treated with SB203580 (p38 inhibitor) or BIX02189 (MEK5/ERK5
742 inhibitor).
743 The results are presented as means ± SD of three independent experiments.
744 The asterisks indicate statistically significant differences; * $p< 0.05$; ** $p< 0.01$.

745

746 **Figure 7: MFS SMC death is regulated by p38, KLF4 and β1 integrin. a)**
747 Flow cytometric staining for AnnexinV to determine apoptosis levels of WT

748 and MF^{C1242Y} NC-SMC following treatment with SB203580, Losartan,
749 PD98059 (ERK1/2 inhibition) and by transfection with KLF4 siRNA.
750 mRNA expression levels for *KLF4*, *CDKN1A* and *TP53* **b)** and β1 integrin **(c)**
751 in un-stretched and stretched conditions in WT and MF NC-SMC. **d)** Flow
752 cytometric analysis of β1 integrin-APC levels in NC-SMC before and after
753 stretching. **e)** Cropped western blot for SMAD2, ERK1/2 and p38 phospho-
754 proteins in control (CTL, IgG), stretched conditions and after treatment with
755 anti-β1 integrin blocking antibody (anti-β1 Ab) in WT and MF^{C1242Y} NC-SMC. **f)**
756 RT-qPCR profile of *TP53* expression in control (CTL) and anti-β1 Ab treated
757 WT and MF NC-SMC. **g)** Comparison of apoptotic levels by AnnexinV flow
758 cytometry in control (CTL) and upon treatment with anti-β1 integrin antibody
759 of WT and MF^{C1242Y} NC-SMC in resting and stretched conditions.
760 The results are presented as means ± SD of three independent experiments.
761 The asterisks indicate statistically significant differences; * p< 0.05; ** p< 0.01.
762

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

773 the WT allele), CRISPR MF corrected (MF^{C1242Y} hiPSC CRISPR-edited with
774 WT sequence into the mutant allele to correct the C1242Y mutation) and WT
775 hiPSC. **f)** Quantification of fibrillin-1 staining levels in the four groups depicted
776 in **e** normalized for cell numbers. The asterisks indicate statistically significant
777 differences; * $p< 0.05$; ** $p< 0.01$. **g)** Cropped blots of phosphorylated and
778 total SMAD2, ERK1/2 and p38 with β-actin loading controls in S30 NC-SMC
779 derived from MF^{C1242Y} hiPSC, CRISPR MF mutant, CRISPR MF corrected
780 and WT hiPSC. The result is representative of two independent experiments.
781 **h)** AnnexinV assay to determine apoptotic rate in CRISPR MF corrected
782 compared to CRISPR MF mutant. The results are presented as means ± SD
783 of two independent experiments. **i)** Schematic of the proposed mechanism
784 for regulating SMC loss and matrix breakdown in MFS.
785
786

787 ***Supplementary Material and Methods***

788 *hiPSC Derivation and Culture Conditions*

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

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

791 The generation of patient-derived hiPSC was approved by NRES Committee,
792 Cambridgeshire (ethic code; 11/EE/0053) and informed consent was obtained
793 from all the patients. Fibroblasts were cultured in DMEM (Sigma) with 10%
794 FBS (Sigma), 50 U ml^{-1} penicillin (P) and 50 μg ml^{-1} streptomycin (S). To
795 generate hiPSC, MF fibroblasts harboring *FBN1* mutations were transfected
796 using commercially available monocistronic iPSC reprogramming kit from
797 Vectalys, consisting of four vectors encoding: *OCT4, SOX2, KLF4, v-MYC*;
798 retroviral transduction was performed on 100 000 cells per one well of 6-well
799 plate in mitotically inactive mouse embryonic fibroblasts (MEF) media without
800 P/S per patient line. Five days post-transduction, the cells were resuspended
801 with trypsin, and 1×10^5 cells were and seeded onto 10-cm dishes pre-plated
802 with irradiated MEF feeders (CF-1 MEF IRR). Colonies appeared between
803 day 12 and 32 after transfection. Colonies with hESC-like morphology were
804 manually picked and transferred to 12-well plates pre-plated with inactivated
805 MEF feeders containing 10μM p160ROCK (Rho-Associated coiled-coil
806 containing Protein Kinase 1) inhibitor (Y-27632; Tocris). Two independent
807 colonies were picked and expanded from each of the MF mutant lines. All
808 hiPSC were maintained on irradiated mouse feeders in typical DMEM/F12
809 medium, supplemented with 20% Knockout Serum Replacement (Gibco), 2
810 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM β-
811 mercaptoethanol, and 4 ng/ml FGF-2 (R&D System). Medium was changed

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

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

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

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

822 *CRISPR-mediated FBN1 gene editing*

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

824 The Cas9 and single guide RNA (sgRNA) plasmid pSpCas9(BB)-2A-Puro
825 (PX459) was obtained from Addgene (plasmid 48141). The sgRNAs targeting
826 *FBN1* gene were designed according to the rule of 5'-GN_{20}NGG-3'
827 (*Supplemental Table 2*). sgRNAs were synthesized and ligated to the PX459
828 plasmid that was digested with BbsI (New England Biolabs) to obtain an
829 expression vector of Cas9 and FBN1 sgRNA. CRISPR2 guide RNA
830 (*Supplementary Table 2*) was selected for targeting using Transgenomic™
831 Surveyor™ Mutation Detection Kit (Thermo Fisher).

832 A fragment of genomic DNA from FBN1 containing 1063 BP upstream and
833 970 BP downstream flanking Exon 30 was amplified by PCR with specific
834 primers (*Supplemental Table 2*). This fragment was cloned into a
835 pUC18plasmid using PstI and SacI restriction sites. To facilitate the

836 screening, a silent mutation, which removes the putative ClaI restriction site in
837 Exon 30, was created by directed mutagenesis (QuikChange II XL Site-
838 Directed Mutagenesis Kit, Qiagen). The piggyBac PGK-PuroR-pA cassette (a
839 kind gift from Dr Pentao Liu) was inserted into the donor plasmid at 40bp into
840 the intron upstream of Exon 30. This intronic location was checked with
841 Human Splicing Finder software to avoid exon skipping[59]. KpnI and BclI
842 restriction sites were then created by direct mutagenesis (QuikChange II XL
843 Site-Directed Mutagenesis Kit, Quiagen) to generate the final donor plasmid
844 with a selectable marker in the 5' intronic region, the wildtype exon 30 and two
845 intronic homology arms for HDR (*Supplemental Figure 10A*).

846 *ii. Gene targeting in Marfans hiPSC*

847 For gene targeting, $2.5X10^6$ MF hiPSCs were electroporated with 1 ug each of
848 the donor plasmid and Cas9 sgRNA plasmid (Addgene) in 82ul Lonza Stem
849 Cell Solution + 18 μL *suplement 1* (Lonza) using CA137 program of 4D-
850 nucleofector system (Amaxa). Transfected cells were plated onto DR4 strain
851 feeders (Jackson Laboratory) and cultured in CDM BSA+10% KSR
852 supplemented with 4 ng/ml FGF-2 and 10uM of Y-27632. 36 hours after
853 transfection, puromycin selection (1μg/mL) was applied and the surviving
854 colonies were picked and expanded for PCR screening verification. Primers
855 used are listed in *Supplementary Table 2.*

856 *hiPSC differentiation protocols*

857 *i. 3 germ layers differentiation*

858 Differentiation assays were performed as previously described[25]. For
859 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 previously [60]. 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 CHIR99021 [61]. 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 described [21]. The neural crest
882 population was derived using a modified version of the previously described
883 protocol to generate neuroectoderm [25]. For mesoderm subtype differentiation,
884 we differentiated the hiPSC to 36-h early mesoderm (FlyB) with a combination

885 of FGF2 (20 ng/ml), LY294002 (10 μM) and BMP4 (10 ng/ml). Subsequent
886 mesoderm subtype specification was further obtained with FGF (20 ng/ml)
887 and BMP4 (50 ng/ml) for lateral mesoderm (LM) and FGF (20 ng/ml) and Ly
888 (10 μM) for paraxial mesoderm (PM) for a further 4 days. To produce neural
889 crest (NC), we modified the previously described protocol for neuroectoderm
890 differentiation obtained by using fibroblast growth factor 2 (FGF2, 12 ng/ml)
891 and the activin/nodal inhibitor SB431542 (10 μM) and passaged the cells first
892 at day 4 and then repeatedly in FGF2 and SB431542 (; *Serrano F, Bernard*
893 *WG, Granata A, Iyer D, Kim M; in submission*). After obtaining the
894 intermediate populations, cells were trypsinized and cultured in SMC differen-
895 tiation medium CDM-PVA containing PDGF-BB (10 ng/ml, Peprotech) and
896 TGF-β1 (2 ng/ml, Peprotech) for at least 12 days (PT). For long-term cultures,
897 SMCs were subsequently grown in MEM medium (Sigma M5650) containing
898 10% fetal bovine serum (Sigma F7524) up to 10 passages. Each intermediate
899 was tested for the expression of specific genes (NKX2.5 for LM, TBX15 or
900 MEOX1 for PM and p75 for NC) by RT-PCR and immunostaining.

901 *TGF-β1 ELISA*

902 hiPSC-SMC were cultured in serum-free medium for 24h. Levels of TGF-β1 in
903 culture supernatant were estimated by using DuoSet Sandwich ELISA
904 according to the manufacturer's instructions (DY240-05; R&D System). The
905 samples were treated with the activation reagent (1N HCl) for 10 min at room
906 temperature followed by addition of the neutralization reagent (1.2 N
907 NaOH/0.5 M HEPES). Treated samples were transferred to ELISA plates
908 coated with the capture antibody overnight at 4°C and incubated for 2h at RT.
909 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-β1 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 (10μg/ml) to visualize death cells.

926 *ii. AnnexinV assay*

927 For AnnexinV staining, 1X10^{6} cells/ml were harvested and resuspended in 1X
928 annexin-binding buffer and incubated with 5μl 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 (10μM; 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 µg/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 µM, Molecular Probes) in normal extracellular solution (NES; 140 mM NaCl, 5

958 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM glucose and 10 mM HEPES, pH
959 7.3) for 1 h at RT. Cells were the washed for 15 min at RT. Intracellular
960 calcium flux was monitored as time series with acquisition rates of 1 frame
961 every 0.2ms over a period of 1 min using a Zeiss LSM 700 confocal
962 microscope, before and after addition of carbachol (100 µM, Sigma). The total
963 Fluo4-AM uptake was measured by treating the cells with 0.1% Triton to
964 permeabilise the cellular membranes. For both WT and MF SMCs, ten cells
965 were randomly picked from a field of view and the fluorescent trace was
966 analysed using ImageJ software.

967 *FITC-gelatin degradation assay*

968 Cells were plated into m-slide 8 glass bottom wells (Ibidi) previously coated
969 with FITC-gelatin (1mg/ml; Life Technologies G13187), air dried, rehydrated
970 with water for 15 min and fixed with glutaraldehyde for 30 min. Cells were
971 grown on FITC-gelatin-coated wells for 48h and then fixed with 4% PFA,
972 permeabilised with 0.5% Triton X-100, washed twice with PBS and blocked in
973 1% FBS blocking solution. Cells were then stained with a CNN1 specific
974 antibody (1:2000; Sigma) overnight at 4°C followed by Alexa Fluor 588-tagged
975 secondary antibody (1:500, Molecular Probes Invitrogen) and DAPI (10 µg/ml,
976 Vector Laboratories) for 1h at RT. Fluorescence intensity was analysed with
977 ImageJ software and expressed as relative to cell numbers (DAPI staining). 5
978 images were taken for each well of each of the three independent. The results
979 are presented as means ± SD of three independent experiments.

980 *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 α1 (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 µg/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 ± 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 ± SD of two independent experiments.

1020 *Luciferase assays*

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

1022 MLCEs 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 200μg/ml
1025 G418. The day after, MLCEs were treated with conditioned media from WT
1026 and MF SMC for 12h. Control cells were left untreated. After, MLCEs were
1027 lysate with 20μl of Passive Lysis Buffer 1X (Luciferase system; Promega
1028 E1501). Luciferase assay reagent (100 μl: Promega) was added to each well
1029 by injector and the relative luciferase unit (RLU) was read by 2030 Multilabel

Reader (Perkin Elmer).

ii. *SBE4 Reporter Luciferase assay*

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

Cyclic Strain

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

Static stretching

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

1054 of 2x10^5 and left to attach for 4hrs at 37ºC. 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 ± 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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