

1 **Loss of SMAD3 promotes vascular remodeling in pulmonary arterial hypertension via**  
2 **MRTF disinhibition**

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31 At a Glance Commentary:

32 Scientific Knowledge on the Subject: A considerable body of clinical and experimental data  
33 point towards a critical role for TGF- $\beta$  signaling in the pathogenesis of pulmonary arterial  
34 hypertension; however, cellular mechanisms by which TGF- $\beta$  promotes lung vascular  
35 remodeling remain unknown.

36 What This Study Adds to the Field: Loss of SMAD3 presents a novel pathomechanism in  
37 pulmonary arterial hypertension that promotes both proliferation and - via disinhibition of  
38 myocardin-related transcription factor - hypertrophy of pulmonary artery smooth muscle  
39 cells, thereby reconciling the parallel induction of a synthetic and contractile smooth muscle  
40 cell phenotype that promotes lung vascular remodeling in pulmonary hypertension.

41

42 "This article has an online data supplement, which is accessible from this issue's table of  
43 content online at [www.atsjournals.org](http://www.atsjournals.org)"

44 **Abstract**

45 **Introduction:** Vascular remodeling in pulmonary arterial hypertension (PAH) results from  
46 smooth muscle cell hypertrophy and proliferation of vascular cells. Loss of bone  
47 morphogenetic protein receptor 2 (BMPR-II) signaling and increased signaling via  
48 transforming growth factor  $\beta$  (TGF- $\beta$ ) and its downstream mediators SMAD2/3 has been  
49 proposed to drive lung vascular remodeling; yet, proteomic analyses indicate a loss of  
50 SMAD3 in PAH.

51 **Objective:** We proposed that SMAD3 may be dysregulated in PAH, and that loss of SMAD3  
52 may present a pathophysiological master switch by disinhibiting its interaction partner  
53 myocardin-related transcription factor (MRTF) which drives muscle protein expression.

54 **Methods and Results:** SMAD3 was downregulated in lungs of PAH patients, and in  
55 pulmonary arteries of three independent PAH animal models. TGF- $\beta$  treatment replicated the  
56 loss of SMAD3 in human pulmonary artery smooth muscle (huPASMCs) and endothelial  
57 (huPAECs) cells. *SMAD3* silencing increased proliferation and migration in huPASMCs and  
58 huPAECs. Co-immunoprecipitation revealed reduced interaction of MRTF with SMAD3 in  
59 TGF- $\beta$  treated huPASMCs and pulmonary arteries of PAH animal models. In huPASMC,  
60 loss of SMAD3 or BMPR-II increased smooth muscle actin expression, which was attenuated  
61 by MRTF inhibition. Conversely, SMAD3 overexpression prevented TGF- $\beta$  induced  
62 activation of a MRTF reporter and reduced actin stress fibers in *BMPR2* silenced huPASMC.  
63 MRTF inhibition attenuated PAH and lung vascular remodeling in sugen/hypoxia rats.

64 **Conclusion:** Loss of SMAD3 presents a novel pathomechanism in PAH that promotes  
65 vascular cell proliferation and - via MRTF disinhibition - hypertrophy of huPASMC, thereby  
66 reconciling the parallel induction of a synthetic *and* contractile huPASMC phenotype.

67

## 68 ***Introduction***

69 Pulmonary arterial hypertension (PAH) is a rare but fatal disease, characterized by extensive  
70 remodeling of pulmonary arteries and muscularization of precapillary arterioles<sup>1,2</sup>. Clinical  
71 and experimental data point towards a central role for TGF- $\beta$  signaling in PAH, in that a  
72 series of mutations in this pathway have been associated with PAH<sup>3-10</sup>, and in that TGF- $\beta$   
73 expression is increased in pulmonary arteries and lungs of PAH patients and respective  
74 animal models<sup>11,12</sup>.

75 By signaling through its canonical pathway, TGF- $\beta$ , activates receptor-induced SMADs (R-  
76 SMADs, namely SMAD2 and SMAD3), which in turn promote gene expression involved in  
77 hypertrophy and fibrosis<sup>13</sup>. On a simplistic level, the documented elevated plasma levels for  
78 TGF- $\beta$  in PAH<sup>14,15</sup> would therefore lead one to expect that SMADs downstream of TGF- $\beta$   
79 may play a crucial role in the pathogenesis of experimental or clinical PAH.  
80 Counterintuitively, however, a significant decrease in pulmonary SMAD3 expression was  
81 detected in PAH animal models as well as IPAH patients<sup>11,12</sup> in line with the fact that  
82 prolonged exposure to TGF- $\beta$  can result in loss of SMAD3<sup>16</sup>. Hence, PAH may actually be  
83 associated with a decrease in the activity of the canonical TGF- $\beta$  pathway.

84 While systemic cardiovascular diseases are commonly associated with a switch from a  
85 differentiated vascular smooth muscle cell phenotype with high expression of contractile  
86 proteins and low migration/proliferation abilities to a proliferative dedifferentiated phenotype  
87 with weak contractile protein expression and increased migration, proliferation and resistance  
88 to apoptosis<sup>17-20</sup>, vascular remodeling in PAH is characterized by *both* dedifferentiation and  
89 increased smooth muscle cell (SMC) proliferation (hyperplasia), *as well as* an overall  
90 increase in smooth muscle protein (e.g.  $\alpha$ -smooth muscle actin, SMA) expression  
91 characteristic of SMC hypertrophy<sup>21</sup>. As contractile and proliferative SMC phenotypes are

92 commonly considered mutually exclusive, the seeming contradiction of the parallel induction  
93 of both huPASMC hyperplasia *and* hypertrophy in PAH disease has so far remained  
94 enigmatic. Myocardin related transcription factor (MRTF) is a Rho GTPase responsive, actin-  
95 regulated transcriptional co-activator of serum response factor (SRF), a master regulator of  
96 muscle protein expression, including SMA that plays a crucial role in both maintenance of  
97 normal SMC homeostasis<sup>22,23</sup> and pathological vascular remodeling<sup>24</sup>. While the central role  
98 of MRTF in SMC hypertrophy renders it an attractive candidate contributor to vascular  
99 remodeling, MRTF dysregulation in PAH and its underlying mechanism has not yet been  
100 addressed.

101 MRTF dysregulation may be intrinsically linked to altered TGF- $\beta$  signalling as suggested  
102 from our previous studies in kidney epithelial cells where we found SMAD3 capable of  
103 binding MRTF, thereby inhibiting MRTF-driven activation of the SMA promoter<sup>25-28</sup>. Since  
104 SMAD3 may concomitantly exert antiproliferative effects<sup>29,30</sup>, a general loss of SMAD3 in  
105 PAH may drive both SMA expression via disinhibition of MRTF and huPASMC  
106 proliferation, and thus might explain the parallel existence of a hypertrophic *and* proliferative  
107 huPASMC phenotype in PAH<sup>31-33</sup>. To test this notion, we analysed SMAD3 expression  
108 levels in clinical samples and pre-clinical models of PAH, explored the protein-protein  
109 interaction between SMAD3 and MRTF, and their functional roles in huPASMC and  
110 pulmonary artery endothelial cells (huPAEC) relevant to the development and progression of  
111 PAH both *in vitro* and *in vivo*.

112

113 **Methods**

114 For full experimental details, including information on proliferation and migration assays,  
115 protein isolation and western blotting, RNA isolation and real-time PCR, cell transfection,  
116 co-immunoprecipitation, animal models of pulmonary hypertension and assessment of their  
117 endpoints please see the online supplement.

118 *Human tissue samples.* Human lung tissues were obtained from 7 controls who died  
119 unexpectedly from non-pulmonary causes and 7 PAH patients for which clinical  
120 characteristics are given in Supp. Table 1. Samples were collected under the ethic protocol  
121 #20773 at Laval University and used following current recommendations<sup>34</sup>.

122 *Cells.* HuPASMC and huPAEC were purchased from Lonza, Promocell or isolated from  
123 downsized non-tumorous non-transplanted donor lungs (EK 976/2010, Medical University of  
124 Vienna) and cultured in Smooth Muscle or Endothelial Cell Growth Medium 2, respectively  
125 (Promocell). Murine PASMC were obtained from *BMPR2*<sup>+/*R899X*</sup> mice which carry a mutated  
126 allele of the *BMPR2* gene, and corresponding wild type mice as described<sup>35</sup>.

127 *Transfection.* Pre-designed, commercially available siRNA sequences directed against human  
128 *SMAD3* and *BMPR2* were purchased (Dharmacon Thermo Scientific). For non-specific gene  
129 inhibition of the siRNAs used in this study, a universal negative-control siRNA sequence was  
130 used (Dharmacon Thermo Scientific). For *SMAD3* overexpression a GFP-tagged Smad3 from  
131 Clontech or a Myc-tagged Smad3 expression construct (in pCMV5B) was used. For MRTF  
132 overexpression a FLAG-tagged MRTF construct was used. Cells were transfected with  
133 100nMol/L siRNA or 0.5-2µg plasmid for 8h.

134 *Pulmonary hypertension models.* The study was approved by the animal care and use  
135 committee of St. Michael's, and experiments were performed in accordance with the "Guide  
136 for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, 7th

137 edition 1996). For PAH induction in male Sprague-Dawley rats, both the sugen/hypoxia and  
138 the monocrotaline model were applied. Hemodynamic characterization was performed via  
139 right ventricular systolic pressure (RVSP) measurements and echocardiographic imaging of  
140 right ventricular function, and vascular remodelling and Fulton index were assessed *post*  
141 *mortem*.

142 *Statistical analysis.* All data are given as means±SEM. Student's t test (two-tailed) was used  
143 to compare two groups. Where appropriate, a two-way Analysis of Variance (ANOVA) was  
144 applied with Tukey's post-hoc test. P-values < 0.05 were considered statistically significant.

145

146 **Results**147 **Smad3 is downregulated in clinical and preclinical PAH samples**

148 Total SMAD3 protein levels were significantly decreased in lung lysates from 7 PAH  
149 patients as compared to healthy controls, while SMA expression was concomitantly elevated  
150 (Fig.1A-C). Reduced SMAD3 staining in the intima and media of pulmonary arteries was  
151 also evident by immunohistochemistry in IPAH patients as compared to donor lungs (Supp.  
152 Fig.1). Similar downregulation of SMAD3 was detected in pulmonary arteries of two animal  
153 models of PAH, the monocrotaline (MCT; Fig.1D,E) and the sugen/hypoxia rat model  
154 (Fig.1I,J), whereas TGF- $\beta$  and smooth muscle actin expression were significantly upregulated  
155 in either model (Fig.1D,F,G,I,K,L). RVSP data for both PAH models are given in Fig.1H,M.  
156 To test whether chronic upregulation of TGF- $\beta$  in PAH is sufficient to explain this loss in  
157 SMAD3, we probed for the effects of TGF- $\beta$  on SMAD3 expression *in vitro*. While short-  
158 term exposure to TGF- $\beta$  for 24h did not alter SMAD3 expression (data not shown), 72h  
159 treatment with 5 ng/mL TGF- $\beta$  downregulated total SMAD3 levels in huPASMCs at both the  
160 protein and mRNA level (Fig.2A-C). Increasing TGF- $\beta$  to 10ng/mL caused no further drop in  
161 SMAD3 levels, therefore all subsequent experiments were performed at 5 ng/mL. Decreased  
162 SMAD3 protein levels were similarly detectable in PAECs after 72h exposure to 5 or  
163 10ng/mL TGF- $\beta$  (Fig.2D,E). Likewise, hypoxia (1% O<sub>2</sub>) as a trigger of pulmonary  
164 hypertension caused a significant drop in SMAD3 in huPASMCs at the protein (Fig.2F,G)  
165 and mRNA level (Fig.2H) as well as in PAECs (Fig.2I,J). Treatment with interleukin-6 or  
166 platelet-derived growth factor previously implicated in PAH pathogenesis did, however, not  
167 reduce SMAD3 expression in huPASMCs or huPAECs, suggesting that loss of SMAD3  
168 expression is not a universal response to all PAH-related stimuli (Supp. Fig.2).

169



## 170 **SMAD3 downregulation increases proliferation and migration**

171 To test for functional consequences of SMAD3 loss, we silenced *SMAD3* in huPASMC by  
172 siRNA (Fig.3A,B) and assessed fetal calf serum (FCS)-induced proliferation in three  
173 independent assays. *SMAD3*-silenced huPASMCs exhibited i) higher protein levels for  
174 proliferating cell nuclear antigen (PCNA) (Fig.3A,C), ii) increased staining for nuclear Ki-67  
175 (Fig.3D,E), and iii) higher bromodeoxyuridine (BrdU) levels (Fig.3F) as compared to control  
176 siRNA. A similar increase in FCS-induced proliferation was detectable as higher PCNA  
177 levels in *SMAD3*-silenced huPAECs relative to internal control (Fig.3G). Graph showing  
178 group data normalized towards siCTL is given in Supp. Figure 6B. Downregulation of  
179 SMAD3 also stimulated huPASMC (Fig. 3H) and huPAEC migration (Fig. 3I) towards an  
180 FCS gradient. Thus, downregulation of SMAD3 is sufficient to replicate two major features  
181 of PAH, enhanced proliferation and migration of huPASMC and huPAEC.

## 182 **SMAD3 downregulation triggers smooth muscle cell hypertrophy**

183 To test whether SMAD3 loss also triggers huPASMC hypertrophy, we assessed the effect of  
184 TGF- $\beta$  treatment on the expression of SMA, a differentiation and hypertrophy marker in  
185 SMCs<sup>36,37</sup>, and on the ratio of total protein to DNA content. TGF- $\beta$  increased SMA  
186 expression and protein/DNA ratio in a time-dependent manner, which was associated with a  
187 concomitant decrease in SMAD3 levels (Fig.4A-C,E). In line with a direct link between  
188 SMAD3 loss and SMC hypertrophy, SMAD3 protein expression correlated inversely with  
189 both SMA expression (Fig.4D) and protein/DNA ratio (Fig.4F). Consolidating the notion that  
190 loss of SMAD3 directly contributes to increased SMA expression, we next showed that  
191 *SMAD3* silencing prior to TGF- $\beta$  treatment augmented SMA expression in huPASMCs 24h  
192 after TGF- $\beta$  (Fig.4G,H), whereas 72h after TGF- $\beta$  no significant difference was observed  
193 between *SMAD3* silenced and control siRNA-transfected cells (Fig.4G,I). This observation is  
194 in accord with the fact that by 72h, TGF- $\beta$  treatment *per se* caused a drop in SMAD3

195 (Fig.4A), thus minimizing the effect of additional SMAD3 silencing (Fig.4G). Taken  
196 together, these experiments substantiate that TGF- $\beta$ -induced loss of SMAD3 promotes  
197 myogenic (hypertrophic) responses in SMC.

#### 198 **SMAD3-MRTF interaction is decreased by TGF- $\beta$ treatment**

199 In kidney tubular cells, we showed that SMAD3 interacts with MRTF<sup>26</sup> thereby inhibiting its  
200 effect on the SMA promoter<sup>38</sup>. To test for a similar scenario in PAH where TGF- $\beta$ -dependent  
201 loss of SMAD3 may accordingly potentiate SMA expression via enhanced MRTF signaling,  
202 we probed for SMAD3-MRTF interaction by co-immunoprecipitation (co-IP). At baseline,  
203 MRTF pulled down substantial amounts of SMAD3, verifying the interaction of these  
204 proteins in resting huPASMCs. TGF- $\beta$  treatment for 72h reduced the level of MRTF-  
205 associated SMAD3 (Fig.5A). This effect is likely attributable to the comparable reduction in  
206 total SMAD3 following TGF- $\beta$  as evident in the co-IP input (Fig.5B) rather than a reduced  
207 affinity between the two proteins. Interaction between SMAD3 and MRTF was similarly  
208 reduced in pulmonary arteries of MCT (Fig.5C) or sugen-hypoxia (Fig.5D) treated rats, a  
209 finding that is in line with MRTF disinhibition in PAH.

210 To probe whether loss of SMAD3 may indeed prime cells for enhanced MRTF activity we  
211 treated huPASMC with sphingosine-1-phosphate (S1P), a trigger of MRTF nuclear  
212 translocation and activation<sup>39,40</sup>. In huPASMC treated with control siRNA, S1P induced SMA  
213 expression in an MRTF-dependent manner, as this effect was blocked by the MRTF inhibitor  
214 CCG1423 (Fig.6A). The latter effect was not attributable to induction of apoptosis by  
215 CCG1423, which could be effectively ruled out for both cell types (Supp.Fig.3&4). *SMAD3*  
216 silencing amplified S1P-induced SMA expression, which was again sensitive to MRTF  
217 inhibition consistent with an intensified MRTF-dependent signaling following SMAD3 loss  
218 (Fig.6A,B). Next, we expressed the MRTF/SRF-sensitive luciferase reporter 3DA in  
219 huPASMCs and showed that SMAD3 overexpression attenuated reporter activation in

220 response to either TGF- $\beta$  stimulation (Fig.6E) or MRTF overexpression (Fig.6F),  
221 respectively, thus further validating the negative regulation of MRTF-dependent  
222 transcriptional activity by SMAD3. SMAD3 knock-down also enhanced FCS-induced  
223 proliferation in huPASMCs as assessed by BrdU assay, yet, this effect was not normalized by  
224 CCG1423 (Fig.6C). Similar results were obtained in huPAEC (Fig.6D), indicating that  
225 MRTF disinhibition contributes to hypertrophic yet not hyperplastic responses to loss of  
226 SMAD3 in lung vascular cells.

### 227 **MRTF inhibition attenuates pulmonary hypertension and vascular remodelling in rats**

228 Based on our findings that i) SMAD3 levels are decreased in clinical and preclinical PAH,  
229 and ii) that loss of SMAD3 drives MRTF-dependent SMA expression, we hypothesized that  
230 pharmacological inhibition of MRTF may alleviate vascular remodeling in experimental  
231 PAH. To address this question, PAH was induced by sugen/hypoxia, and rats were treated  
232 either in a prophylactic approach from day 0 or in a therapeutic approach from day 21 after  
233 SU5416 injection with daily injections of two different doses of CCG1423 or vehicle. As  
234 compared to untreated sugen/hypoxia animals, CCG1423-treated rats showed reduced lung  
235 vascular remodeling (Fig.7A,B) and RVSP (Fig.7C). Echocardiographic parameters of right  
236 ventricular (RV) function were, however, only normalized by prophylactic treatment with  
237 high dose CCG1423, but not with low dose prophylaxis or either therapeutic treatment dose  
238 (Fig.7D,E). This deviation of treatment effects on pulmonary hemodynamics and RV  
239 function may seem surprising; it is, however, in keeping with previous studies<sup>41</sup> which  
240 identified impaired RV function as a prominent feature of the sugen/hypoxia model that is  
241 not solely attributable to increased RV afterload, but involves additional direct or indirect  
242 effects on the RV that may be less responsive to MRTF inhibition.

### 243 **Loss of functional BMPR-II causes SMAD3 loss which in turn drives PASMC** 244 **hypertrophy**

245 Loss of functional BMPR-II signaling, either due to inherited or spontaneous *BMPR2* gene  
246 mutations or to acquired loss of BMPR-II<sup>42</sup>, is a key contributing factor in the pathogenesis  
247 of PAH<sup>43,44</sup>. To test for a link between impaired BMPR-II signaling and the SMAD3/MRTF-  
248 dependent regulation of huPASMC hypertrophy, we made use of a transgenic mouse model  
249 that overexpresses a mutated form of *BMPR2* (*BMPR2*<sup>+/*R899X*</sup>) and gradually develops PAH<sup>45</sup>.  
250 In whole lung lysates of *BMPR2*<sup>+/*R899X*</sup> mice, total SMAD3 levels were markedly reduced as  
251 compared to controls (Fig.8A), indicating that loss of SMAD3 and subsequent MRTF  
252 disinhibition may contribute to lung vascular remodeling in cases of dysfunctional or  
253 deficient BMPR-II. In line with this view, PASMC isolated from *BMPR2*<sup>+/*R899X*</sup> mice had  
254 increased SMA and PCNA expression (Fig.8B,C) as well as decreased total SMAD3 and  
255 BMPR-II protein levels.

256 To assess if impaired BMPR-II signaling may *per se* account for loss of SMAD3, we silenced  
257 *BMPR2* in huPASMCs which markedly decreased SMAD3 expression (Fig.9A-C).  
258 Consistent with the concept of MRTF disinhibition, SMA expression in response to S1P was  
259 enhanced in *BMPR2* silenced cells, and this effect was prevented by CCG1423 (Fig.9A,D).  
260 Silencing of *BMPR2* also increased TGF- $\beta$  expression in huPASMC, providing for a  
261 potential mechanistic link between BMPR-II and TGF- $\beta$  (Fig.9E-G). Interestingly, MRTF  
262 inhibition also reduced the expression of both SMAD3 and BMPR-II in siCTL-treated cells, a  
263 finding that not only corroborates the tight link of SMAD3 expression to the abundance of  
264 functional BMPR-II, but also suggests that BMPR-II expression may be negatively regulated  
265 by MRTF in a homeostatic feedback loop. To link elevated SMA expression in response to  
266 S1P in *BMPR2* silenced cells directly to the loss of SMAD3, we additionally transfected  
267 huPASMC with a SMAD3 expressing plasmid to test whether reconstitution of SMAD3 may  
268 reverse the increase in SMA expression in S1P stimulated, *BMPR2* silenced cells. The latter  
269 notion was confirmed, in that *BMPR2* silencing increased the formation of actin stress fibers

270 and phalloidin staining in S1P-stimulated huPASMC, yet this effect was antagonized and  
271 baseline phalloidin staining reduced in huPASMC overexpressing SMAD3 (Fig.10).

272

## 273 ***Discussion***

274 In the present study we identified loss of SMAD3 as characteristic hallmark in different  
275 PAH-related scenarios, and as central pathophysiological mechanism in lung vascular  
276 remodeling. Loss of SMAD3 drives PASMC hypertrophy via disinhibition of MRTF, while  
277 concomitantly stimulating PASMC and PAEC proliferation in an MRTF-independent  
278 manner. Importantly, these findings not only identify a novel shared and potentially  
279 targetable signaling pathway of vascular remodeling in PAH, but also reconcile the  
280 seemingly contradictory parallel stimulation of both contractile and synthetic PASMC  
281 phenotypes in PAH. Induction of this pathway by BMPR-II dysfunction or deficiency  
282 provides for a novel explanation for the propensity of patients with spontaneous or inherited  
283 *BMPR2* mutations to develop PAH, and – as we discuss later - resolves the conceptual  
284 dilemma how functional BMPR-II deficiency may promote vascular remodeling given that  
285 BMP/BMPR-II signaling *per se* induces PASMC differentiation.

286 In line with previous reports indicating that SMAD3 may be downregulated in experimental  
287 PAH<sup>11</sup>, we demonstrate here loss of SMAD3 in i) human lung samples of PAH patients, ii)  
288 pulmonary arteries from two preclinical models of PAH, iii) cultured huPASMC stimulated  
289 by TGF- $\beta$  or hypoxia, iv) a mouse model bearing a heterozygous knock-in allele of a human  
290 *BMPR2* mutation, or v) *BMPR2*-silenced huPASMC. As such, loss of SMAD3 emerges as a  
291 common hallmark in different scale models of PAH disease. The mechanism of SMAD3  
292 downregulation in PAH was not focus of the present work, but is potentially attributable to

293 epigenetic regulation via miR-199a-5p, which is upregulated in rat models of PH<sup>46</sup> and  
294 negatively regulates SMAD3 expression in huPAEC and huPASMCM<sup>46</sup>.

295 The role of SMAD3 in cell signaling has classically been viewed primarily in terms of its  
296 function as transcriptional modulator that, upon stimulation of the TGF- $\beta$  type I receptor,  
297 becomes phosphorylated and, together with SMAD4, translocates to the nucleus where it  
298 regulates the transcription of various TGF- $\beta$  target genes by binding to the SBE (SMAD-  
299 binding element) in their promoter region<sup>47</sup>. Here, we describe a fundamentally different role  
300 of SMAD3 in the regulation of TGF- $\beta$  induced gene expression, which is independent of its  
301 phosphorylation and nuclear translocation, but relates to its overall downregulation in  
302 response to TGF- $\beta$  or in pulmonary hypertension<sup>25,48,49</sup>. In huPASMCM and huPAEC, silencing  
303 of *SMAD3* replicated key mechanisms of lung vascular remodeling in PAH, namely cell  
304 proliferation, migration, and – in case of huPASMCM – hypertrophy. The parallel induction of  
305 both hyperplastic and hypertrophic responses by SMAD3 loss reconciles these seemingly  
306 opposing effects in a single molecule.

307 Induction of hypertrophy was attributable to a liberation of the myogenic transcription factor  
308 MRTF from a tonic SMAD3-mediated inhibition. The relevance of SMAD3-MRTF  
309 interaction in vascular homeostasis, and the role of MRTF disinhibition following SMAD3  
310 loss was documented in a series of experiments, in that SMAD3 was shown to co-  
311 immunoprecipitate with MRTF in huPASMCM at rest, yet MRTF was released from this  
312 interaction upon TGF- $\beta$  treatment due to loss of SMAD3. Notably, our co-  
313 immunoprecipitation experiments focused on MRTF-B; however, we have previously  
314 documented a similar disinhibition by SMAD3 loss for MRTF-A in kidney epithelial cells<sup>50</sup>,  
315 suggesting a potential synergistic role of both MRTFs in PAH. A similar reduction in  
316 SMAD3-MRTF interaction was detected in pulmonary arteries from sugen/hypoxia or MCT  
317 treated rats. MRTF disinhibition then promotes cell hypertrophy, as demonstrated by the

318 finding that *SMAD3* silencing amplified SMA expression in response to S1P in an MRTF-  
319 inhibitor sensitive manner. Conversely, *SMAD3* overexpression attenuated the activation of a  
320 MRTF/SRF reporter in response to TGF- $\beta$  or MRTF overexpression. The relevance of this  
321 signaling pathway in the context of PAH was confirmed in the sugen/hypoxia model, in that  
322 both prophylactic and therapeutic treatment of rats with an MRTF inhibitor attenuated lung  
323 vascular remodeling and hemodynamic changes. In contrast, MRTF disinhibition did not  
324 contribute to the proliferative effect of *SMAD3* loss, since proliferation in response to  
325 *SMAD3* silencing was not attenuated by MRTF inhibition.

326 *BMPR2* presents the single most prominent gene implicated in PAH, in that missense, frame-  
327 shift, or nonsense mutations in *BMPR2* are present in 70% of families with familial PAH, and  
328 in up to 25% of patients with sporadic idiopathic PAH<sup>14</sup>. Loss of functional BMPR-II  
329 signaling extends, however, beyond *BMPR2* mutations, as acquired loss of BMPR-II is a  
330 common trait in nongenetic forms of PAH in humans<sup>42</sup> and animal models<sup>51</sup>. The mechanism  
331 by which functionally relevant mutations or acquired loss of BMPR-II promote PAH has  
332 traditionally been attributed to a shift from a BMPR-II-dependent, SMAD1/5/8 mediated  
333 homeostatic vascular phenotype to a TGF- $\beta$ -driven, SMAD2/3-mediated vascular  
334 remodeling<sup>14</sup>. In the present study, however, we show that *SMAD3* is largely lost in PASMC  
335 carrying the *BMPR2* mutation R899X, or following *BMPR2* silencing, while loss of *BMPR2*  
336 increased TGF- $\beta$  expression. This finding is reconcilable with an initial predominance of  
337 TGF- $\beta$ /SMAD2/3 signaling in *BMPR2* mutated or silenced cells, which in turn, however,  
338 results in a loss of *SMAD3* (Fig. 11). Hence, rather than promoting a long-term increase in  
339 SMAD2/3 signaling, functional loss of BMPR-II may promote PASMC hypertrophy via  
340 disinhibition of MRTF due to loss of *SMAD3*. Two findings confirm this notion: First,  
341 *BMPR2* silencing increased S1P-induced SMA expression in huPASMC in an MRTF-  
342 dependent manner. Second, overexpression of *SMAD3* prevented the formation of actin

343 stress fibers in huPASMC in response to *BMPR2* silencing. While these findings establish  
344 loss of SMAD3 and subsequent MRTF disinhibition as important mechanisms driving  
345 PASMC hypertrophy following functional loss of BMPR-II signaling, the role of MRTF in  
346 BMPR-II signaling and lung vascular homeostasis is slightly more complex: Activation of  
347 BMPR-II by BMPs stimulates itself MRTF via the RhoA/ROCK pathway<sup>22,23</sup>, an effect that  
348 has been proposed to maintain the non-proliferative, differentiated PASMC phenotype in the  
349 healthy pulmonary vasculature<sup>22</sup>. Conversely, we demonstrate here an important role for  
350 MRTF signaling in PASMC hypertrophy and PAH development. Thus, MRTF's regulation  
351 differs under healthy (BMPR-dominated) and pathological (TGF- $\beta$  dominated) conditions. In  
352 the former case MRTF is temporarily activated by BMPR-II and contributes to the  
353 maintenance of the contractile phenotype. In the latter case, MRTF acts on the background of  
354 SMAD3 loss, i.e. in the absence of its tonic inhibitor. This results in prolonged (dysregulated)  
355 MRTF activation, provoking a hypertrophic response that coincides with hyper-proliferation  
356 as an independent consequence of SMAD3 loss. Thus, instead of the normal time-segregation  
357 between proliferative or synthetic responses, PAH is characterized by parallel existence of  
358 both.

359 The recognition of SMAD3 expression levels as important master switch in the pathogenesis  
360 of PAH not only identifies loss of SMAD3 as new cellular pathomechanism of lung vascular  
361 remodelling, but also constitutes in our view an important conceptual advance in that the  
362 concurrent stimulation of proliferative and hypertrophic signaling pathways reconciles the  
363 seemingly mutually exclusive, parallel induction of a synthetic *and* contractile huPASMC  
364 phenotype, and recognizes MRTF signaling as a double-edged sword that maintains BMP-  
365 regulated vascular homeostasis in the healthy lung, while similarly driving TGF- $\beta$  induced  
366 lung vascular remodeling in PAH.

367



368 Disclosures: Authors have nothing to disclose

369

370

For Review Only

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510

511 **Figure legends**

512 **Figure 1:** SMAD3 protein expression is decreased and SMA and TGF- $\beta$  protein expression  
513 is increased in clinical samples and preclinical models of pulmonary arterial hypertension  
514 (PAH). Representative western blots (A,D,I) and corresponding densitometric quantification  
515 (B,C,E-G, J-L) show SMAD3 and SMA expression in (A-C) total lung lysates of 7 PAH  
516 patients as compared to healthy sex- and age-matched controls (CTL; n=7), and SMAD3,  
517 SMA and TGF- $\beta$  expression in rat pulmonary arteries after (D-G) 3 weeks of monocrotaline  
518 (MCT) treatment relative to untreated controls (n=8 each), or (I-L) after Sugeng/hypoxia  
519 (Su/hyp) relative to untreated controls (n=4 each). RVSP values for MCT and Su/hyp rats  
520 used for protein expression analyses are shown (H,M). \* $P$ <0.05 vs. CTL. T-test was applied.

521

522 **Figure 2:** Stimulation with transforming growth factor- $\beta$  or hypoxia decreases SMAD3  
523 expression in pulmonary vascular cells. **A-E:** Representative western blots (A,D),  
524 corresponding densitometric quantification (B,E; n=4-5 independent experiments each) and  
525 mRNA expression levels as determined by real-time PCR (C) show SMAD3 expression in  
526 human pulmonary artery smooth muscle cells (huPASMC; A-C) and human pulmonary  
527 artery endothelial cells (huPAEC; D,E) after 72h stimulation with either 5 or 10 ng/mL  
528 transforming growth factor (TGF)- $\beta$ , or vehicle control (CTL), respectively. \* $P$ <0.05 vs.  
529 CTL. **F-J:** Representative western blots (F,I), corresponding densitometric quantification  
530 (G,J; n=2-3 independent experiments each) and mRNA expression levels as determined by  
531 real-time PCR (H) show SMAD3 expression in huPASMC (F-H) and huPAEC (I,J) after 72h  
532 stimulation with either 5 ng/mL TGF- $\beta$  or vehicle control (CTL) in normoxia (21% O<sub>2</sub>) or  
533 hypoxia (1 %O<sub>2</sub>), respectively. \* $P$ <0.05 vs. CTL; Two-way ANOVA was applied with  
534 Tukey's post-hoc test.

535



536 **Figure 3:** SMAD3 downregulation increases proliferation and migration in pulmonary  
 537 vascular cells. **A-C:** Representative western blots (A) and corresponding densitometric  
 538 quantification of SMAD3 (B) and proliferating cell nuclear antigen (PCNA; C) levels (n=4  
 539 independent experiments each) show increased PCNA levels upon stimulation with fetal calf  
 540 serum (FCS; 5% for 24h) in human pulmonary artery smooth muscle cells (huPASC)MC)  
 541 treated with SMAD3-specific (siSMAD3) relative to control (siCTL) siRNA. \* $P < 0.05$  vs.  
 542 CTL; # $P < 0.05$  vs. siSMAD3+FCS. **D-F:** Representative fluorescence microscopic images of  
 543 huPASC)MC immunostained for Ki-67 (red; marked by arrows) and counterstained with 4',6-  
 544 diamidino-2-phenylindole (DAPI; blue) (D), corresponding quantification of proliferating  
 545 (Ki-67<sup>+</sup>) cells per image field (E) and quantitative measurement of bromodeoxyuridine  
 546 (BrdU) incorporation into huPASC)MC (F) show increased FCS-induced proliferation in  
 547 siSMAD3-treated relative to siCTL-treated huPASC)MC (n=3-4 independent experiments  
 548 each). \* $P < 0.05$  vs CTL; # $P < 0.05$  vs. siCTL+FCS. **G:** Densitometric quantification (n=3-4  
 549 independent experiments each) shows increased PCNA levels upon FCS stimulation in  
 550 siSMAD3-treated relative to siCTL-treated human pulmonary artery endothelial cells  
 551 (huPAEC; n=3-4 independent experiments each). \* $P < 0.05$  vs. CTL; # $P < 0.05$  vs.  
 552 siCTL+FCS. **H,I:** Bar graphs show migration of siSMAD3-treated or siCTL-treated  
 553 huPASC)MC (H) or huPAEC (I) in response to FCS stimulation (n>3 independent experiments  
 554 each). \* $P < 0.05$  vs. CTL; # $P < 0.05$  vs. siSMAD3+FCS,  $P \leq 0.05$  vs. siCTL+FCS. Two-way  
 555 ANOVA was applied with Tukey's post-hoc test.

556

557 **Figure 4:** SMAD3 downregulation triggers smooth muscle cell hypertrophy. **A-C:**  
 558 Representative western blots (A) and corresponding densitometric quantification of smooth  
 559 muscle actin (SMA; B) and SMAD3 (C) levels (n=4-5 independent experiments each) show  
 560 time-dependent increase in SMA expression and parallel loss of SMAD3 in human

561 pulmonary artery smooth muscle cells (huPASMC) treated with transforming growth factor  
562 (TGF)- $\beta$  (5 ng/mL). \* $P$ <0.05 vs. CTL. **D**: Expression levels of SMA and SMAD3 at identical  
563 time points post TGF- $\beta$  treatment follow a near-linear inverse relationship as determined by  
564 linear regression analysis (dotted line). **E**: Group data show protein/DNA ratios in  
565 huPASMC at different time points post TGF- $\beta$  treatment. **F**: Protein/DNA ratios and SMAD3  
566 protein expression levels at identical time points post TGF- $\beta$  treatment follow an inverse  
567 relationship. **G-I**: Representative western blots (**G**) and corresponding densitometric  
568 quantification (n=4-5 independent experiments each) show increased SMA expression in  
569 huPASMC treated with SMAD3-specific (siSMAD3) relative to control (siCTL) siRNA at  
570 24h of TGF- $\beta$  treatment (**H**; lanes with dotted circumference in **G**), yet not at 72h (**I**; lanes  
571 with continuous circumference in **G**). \* $P$ <0.05 vs. CTL(B, C; E) or siCTL (H);respectively,  
572 n.s., not significant. Two-way ANOVA was applied with Tukey's post-hoc test.

573  
574 **Figure 5**: SMAD3-MRTF interaction is decreased by transforming growth factor- $\beta$   
575 treatment. **A,B**: Human pulmonary artery smooth muscle cells (huPASMC) were treated for  
576 72h with transforming growth factor (TGF)- $\beta$  (5 ng/mL) or vehicle (CTL),  
577 immunoprecipitated (IP) with a myocardin-related transcription factor (MRTF) antibody, and  
578 precipitated pellets and supernatant (SUP; showing effective MRTF depletion by IP) were  
579 immunoblotted (IB) for MRTF-B and SMAD3 (A). Densitometric quantification reveals  
580 reduced co-immunoprecipitation (Co-IP) of SMAD3 with MRTF that is quantitatively  
581 comparable to the reduction in total SMAD3 by TGF- $\beta$  in the input used for the IP (B, n=4  
582 independent experiments). **C,D**: Pulmonary arteries isolated from monocrotaline- (MCT; C;  
583 full blot see Supp. Figure 6A) or Sugen-hypoxia (Su/hyp) treated rats and corresponding  
584 controls (CTL; n=4-5 independent experiments each) were immunoprecipitated with an  
585 MRTF-antibody, and immunoblotted for MRTF and SMAD3. Densitometric quantification

586 demonstrates reduced Co-IP of SMAD3 with MRTF in experimental models of pulmonary  
587 arterial hypertension. \* $P < 0.05$  vs. CTL. T-test was applied.

588

589 **Figure 6:** Loss of SMAD3 primes for enhanced MRTF activity. **A,B:** Representative western  
590 blots of smooth muscle actin (SMA) and SMAD3 (A) and corresponding densitometric  
591 quantification of SMA (B) levels (n=5 independent experiments each) show increased  
592 expression of SMA following myocardin-related transcription factor (MRTF) activation by  
593 sphingosine-1-phosphate (S1P; 1  $\mu\text{Mol/L}$ ) in human pulmonary artery smooth muscle cells  
594 (huPASMC) treated with SMAD3-specific (siSMAD3) relative to control (siCTL) siRNA  
595 that is sensitive to MRTF inhibition by CCG1423 (5 $\mu\text{Mol/L}$ ). \* $P < 0.05$  vs. siCTL+S1P;  
596 # $P < 0.05$  vs. siSMAD3+S1P. **C-D:** Quantitative measurement of bromodeoxyuridine (BrdU)  
597 incorporation into huPASMC (C) or huPAEC (D) shows increased proliferation in response  
598 to fetal calf serum (FCS; 5%) as compared to vehicle in both siCTL- and siSMAD3-treated  
599 huPASMC or huPAEC, and increased BrdU incorporation in siSMAD3 as compared to  
600 siCTL-treated, FCS-stimulated huPASMC or huPAECs that is, however, not reversed by the  
601 MRTF inhibitor CCG1423 (n>3 independent experiments each). \* $P < 0.05$  vs. siCTL;  
602 # $P < 0.05$  vs. siCTL+ CCG1423; § $P < 0.05$  vs. siSMAD3; \$ $P < 0.05$  vs. siSMAD3+CCG1423.  
603 Two-way ANOVA was applied with Tukey's post-hoc test. **E-F:** Group data show luciferase  
604 reporter activity of the MRTF/SRF sensitive promoter 3DA in TGF- $\beta$  treated huPASMCs  
605 overexpressing an empty-GFP or SMAD3-GFP plasmid (E; n=3 each) or huPASMCs co-  
606 expressing MRTF-FLAG with an empty-GFP or SMAD3-GFP overexpressing plasmid (F,  
607 n=3). \* $P < 0.05$  vs. CTL. T-test was applied.

608

609

610 **Figure 7:** MRTF inhibition attenuates pulmonary hypertension and vascular remodelling in  
611 rats. Representative hematoxylin & eosin stainings show lung vascular remodelling (A), and  
612 bar graphs show vascular wall thickness relative to vessel diameter in lung microvessels of  
613 <50  $\mu\text{m}$ , 50-100  $\mu\text{m}$ , and >100  $\mu\text{m}$  diameter (B), right ventricular systolic pressure (RVSP;  
614 C), and echocardiographic analysis of tricuspid anular plane systolic excursion (TAPSE; D)  
615 and pulmonary artery acceleration time (PAAT; E). Rats were either kept under normoxic  
616 conditions, or underwent treatment with Sugden/hypoxia (Su/hyp) for induction of pulmonary  
617 arterial hypertension, and were treated with either vehicle (DMSO), the myocardin-related  
618 transcription factor (MRTF) inhibitor CCG1423 (0.15mg/kg), or a five-fold higher dose of  
619 CCG1423 (0.75mg/kg; 5xCCG1423) from day 0 or as therapeutic approach from day 21  
620 after SU5416 injection (n=5-10 rats per group). \* $P<0.05$  vs normoxic vehicle control,  
621 # $P<0.05$  vs Su/hyp+vehicle.

622  
623 **Figure 8:** Loss of functional bone morphogenetic protein receptor 2 causes downregulation  
624 of SMAD3. A: Representative western blots show SMAD3 protein expression in total lung  
625 lysates of  $BMPR2^{+/R899X}$  and corresponding wild type (WT) mice (replicated in n=3  
626 independent experiments each). B,C: Representative western blots and corresponding  
627 densitometric quantification of bone morphogenetic protein receptor 2 (BMPR-II), smooth  
628 muscle actin (SMA), proliferating cell nuclear antigen (PCNA), and SMAD3 show loss of  
629 BMPR-II and SMAD3, and increased proliferation and SMA expression in pulmonary artery  
630 smooth muscle cells isolated from lungs of  $BMPR2^{+/R899X}$  mice as compared to those from  
631 WT mice (n=4 independent experiments each). \* $P<0.05$  vs. WT. T-test was applied.

632  
633 **Figure 9:** Loss of functional bone morphogenetic protein receptor 2 drives smooth muscle  
634 hypertrophy in a myocardin-related transcription factor and SMAD3 dependent manner. A-

635 **C:** Representative western blots (A) and corresponding densitometric quantification show (B)  
 636 successful *BMPR2* silencing, (C) loss of SMAD3 in human pulmonary artery smooth muscle  
 637 cells (huPASMC) treated with *BMPR2*-specific (si*BMPR2*) relative to control (siCTL)  
 638 siRNA, and (D) increased expression of smooth muscle actin (SMA) following myocardin-  
 639 related transcription factor (MRTF) activation by sphingosine-1-phosphate (S1P; 1  $\mu$ Mol/L)  
 640 in si*BMPR2*-treated as compared to siCTL-treated huPASMC that is sensitive to MRTF  
 641 inhibition by CCG1423 (5  $\mu$ Mol/L) (n=9 independent experiments each). \* $P$ <0.05 vs siCTL;  
 642 # $P$ <0.05 vs si*BMPR2*+S1P. T-Test was applied. **E-G:** Representative western blots (E) and  
 643 corresponding densitometric quantification show increased TGF- $\beta$  (F) expression after  
 644 *BMPR2* silencing (G).

645

646 **Figure 10:** Representative immunofluorescence images show siCTL- or si*BMPR2*-treated  
 647 huPASMC stimulated by S1P (1  $\mu$ Mol/L) or vehicle and transfected with a SMAD3-Myc  
 648 plasmid stained for F-actin by phalloidin alone (left; green) or (right) in combination with  
 649 anti-SMAD3 staining for SMAD3 (red) and diamidino-2-phenylindole (DAPI; blue).  
 650 SMAD3-overexpressing cells are outlined by dotted lines. Representative images and  
 651 quantitative analysis of mean phalloidin fluorescence intensity in SMAD3-overexpressing as  
 652 compared to adjacent non-transfected cells show reduced formation of actin stress fibers in  
 653 SMAD3-overexpressing cells at rest, after stimulation with S1P or *BMPR2* silencing, or a  
 654 combination of both (replicated in n=3 independent experiments each). \* $P$ <0.05 vs siCTL;  
 655 # $P$ <0.05 vs siCTLplasmid group; One-way ANOVA was applied with Tukey's post-hoc test.

656

657 **Figure 11:** Schematic of proposed signalling pathway.

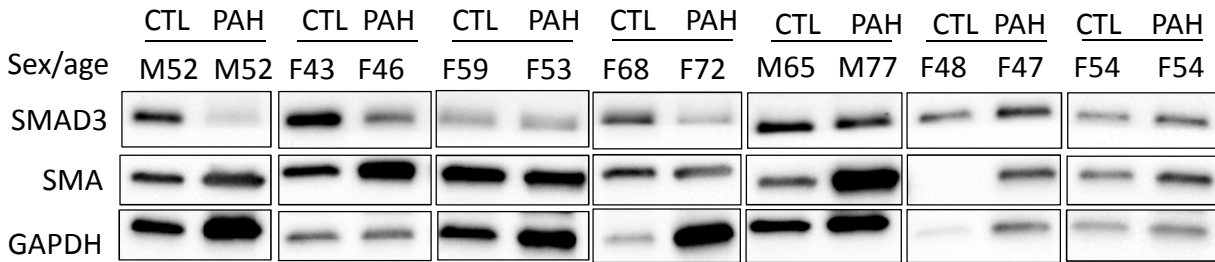
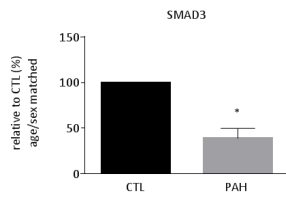
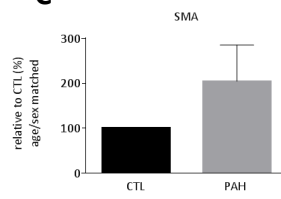
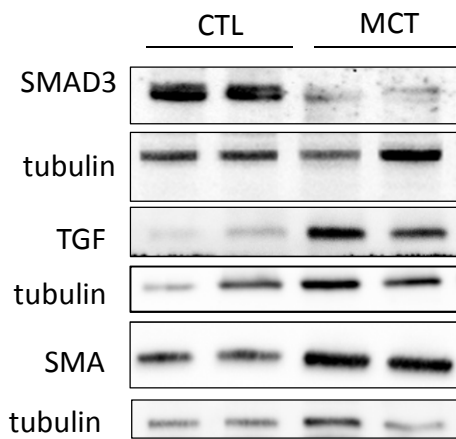
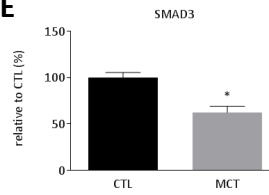
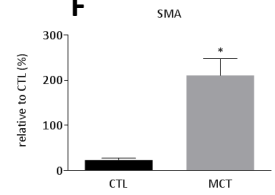
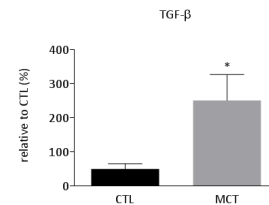
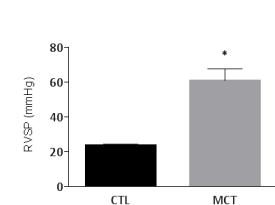
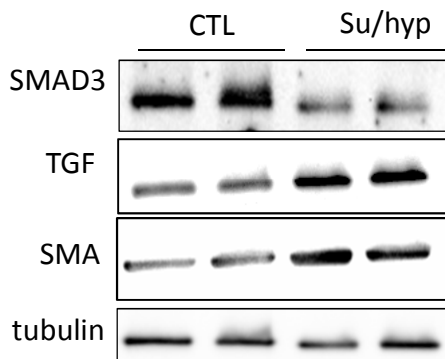
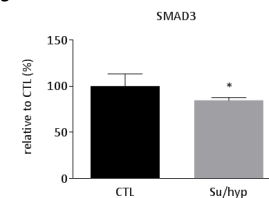
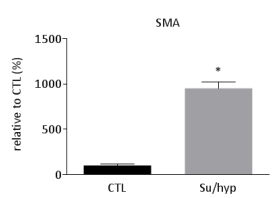
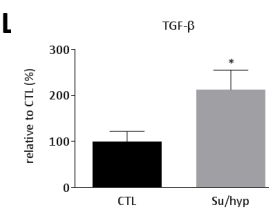
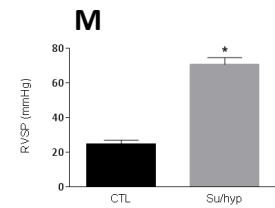
658 Short term and long term (chronic) effects of TGF- $\beta$  stimulation on pulmonary artery smooth  
 659 muscle cells. Initially, TGF- $\beta$  signaling via the classic SMAD2/3 signaling axis

660 predominates, resulting in expression of SMAD3-dependent target genes but antagonizing  
661 TGF- $\beta$  induced MRTF activation by SMAD3-MRTF interaction. Chronic TGF- $\beta$  stimulation,  
662 however, leads to downregulation of SMAD3 (red dotted line) and therefore, reduced  
663 expression of SMAD3-dependent target genes. Functional loss of BMPR-II receptor  
664 signaling due to *BMPR2* mutations or receptor downregulation, respectively, replicates this  
665 effect, presumably in part by increasing TGF- $\beta$  expression. Loss of SMAD3 then stimulates  
666 cell proliferation and migration in an MRTF-independent manner and concomitantly  
667 disinhibits MRTF (indicated by red cross) allowing MRTF to translocate into the nucleus and  
668 together with serum response factor (SRF) drive the SMA promoter, thus promoting both  
669 hypertrophy and hyperplasia in parallel.

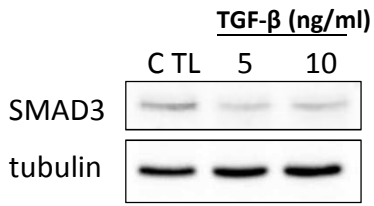
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671

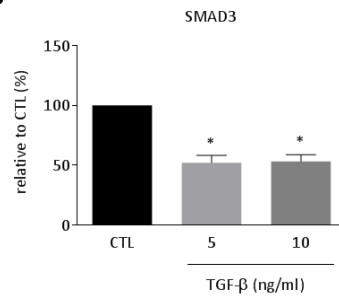
672

**A PAH (human)****B****C****D MCT****E****F****G****H****I Sugden/hypoxia****J****K****L****M**

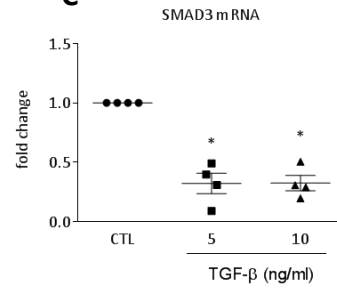
**A huPASC**



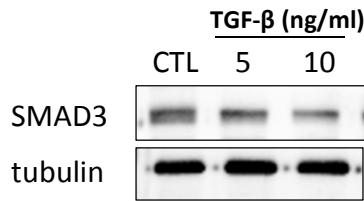
**B**



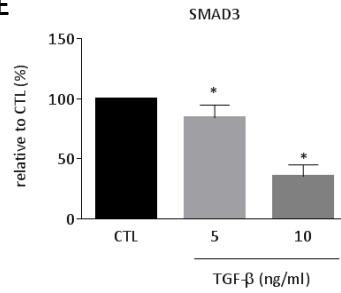
**C**



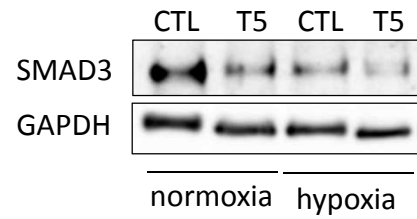
**D huPAEC**



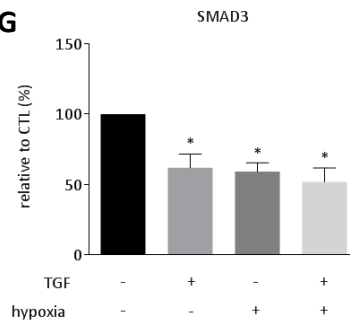
**E**



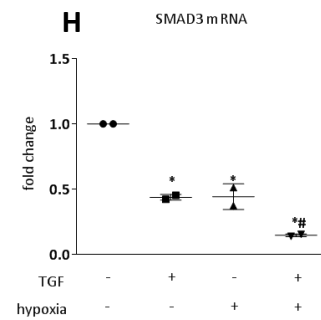
**F huPASC**



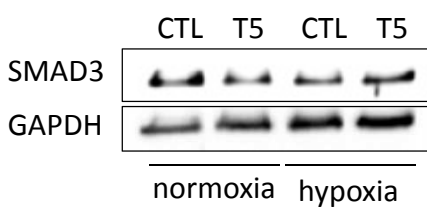
**G**



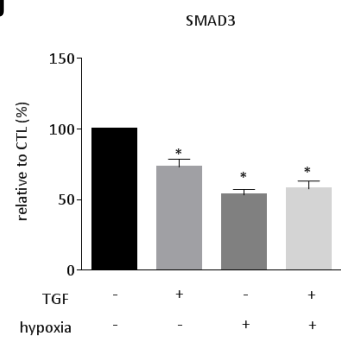
**H**



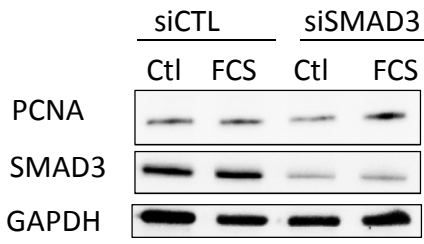
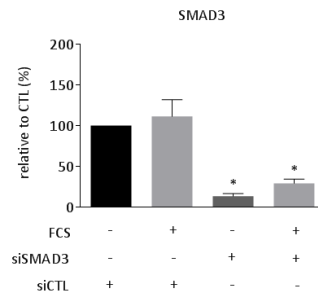
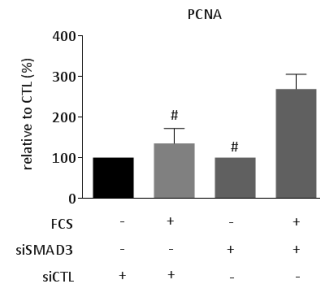
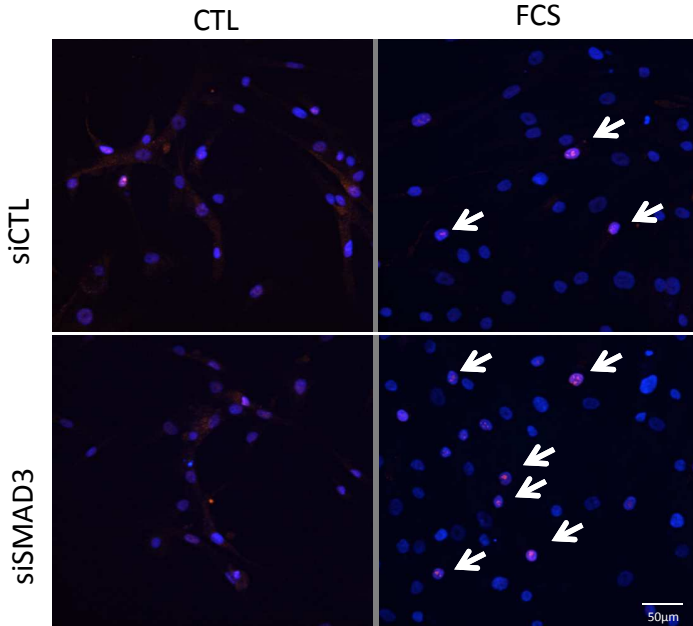
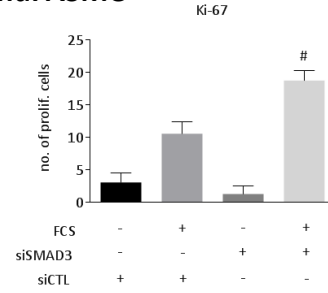
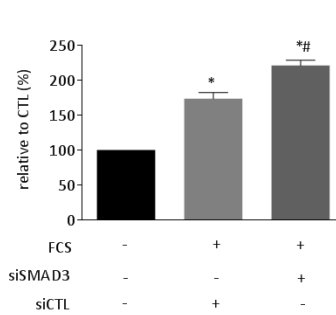
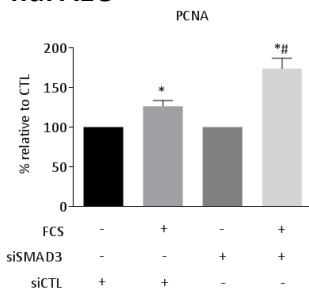
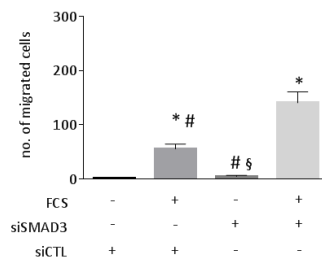
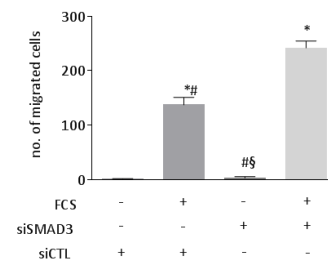
**I huPAEC**



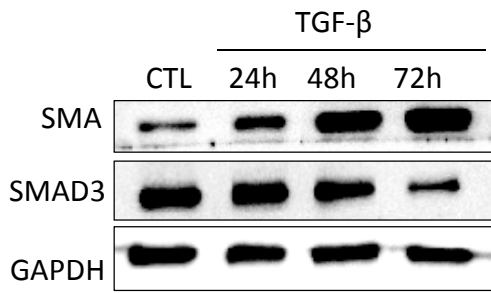
**J**



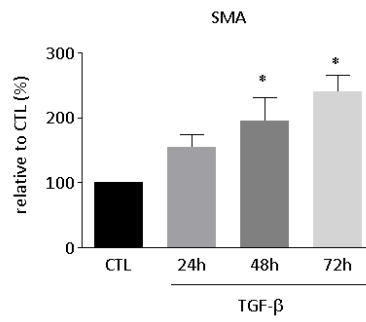


**A huPASC****B****C****D huPASC****E huPASC****F huPASC****G huPAEC****H huPASC****I huPAEC**

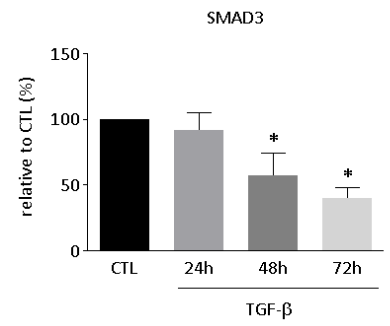
**A huPASC**



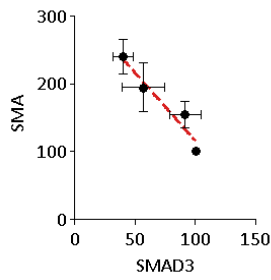
**B**



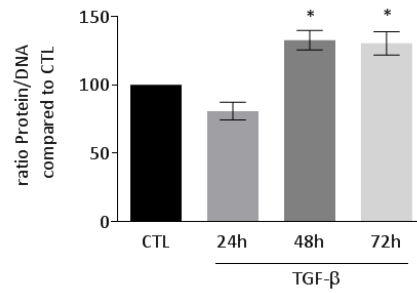
**C**



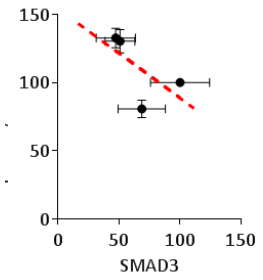
**D**



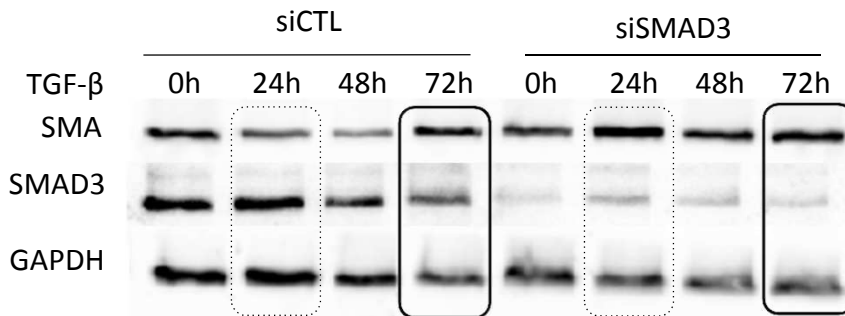
**E**



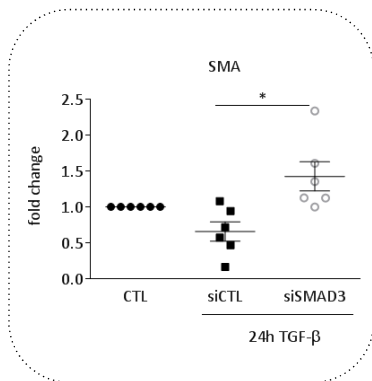
**F**



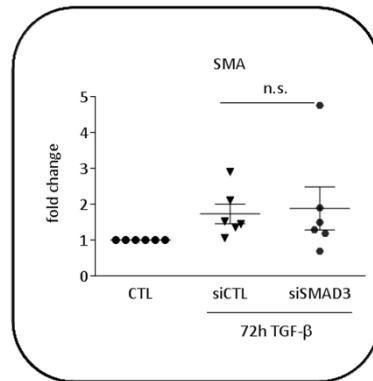
**G huPASC**

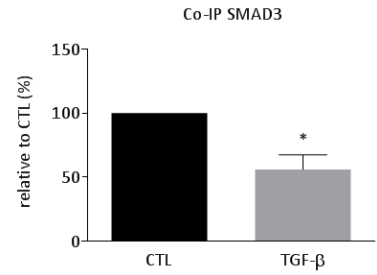
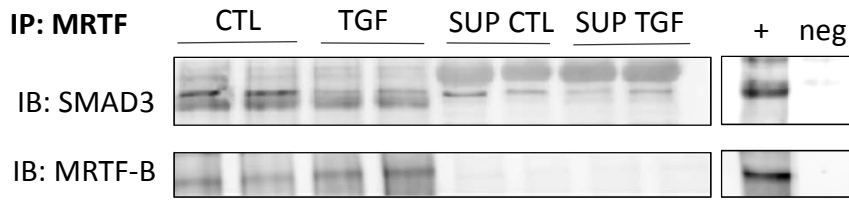
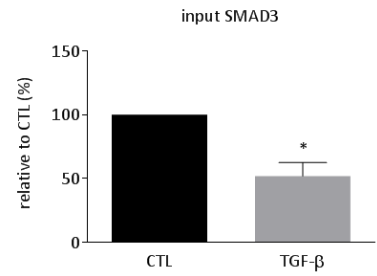
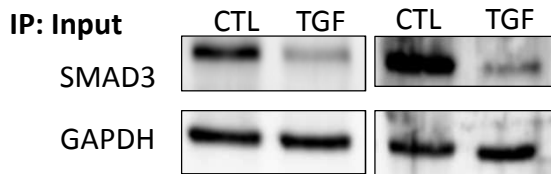
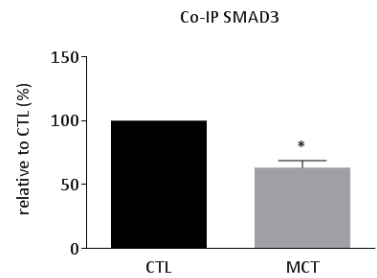
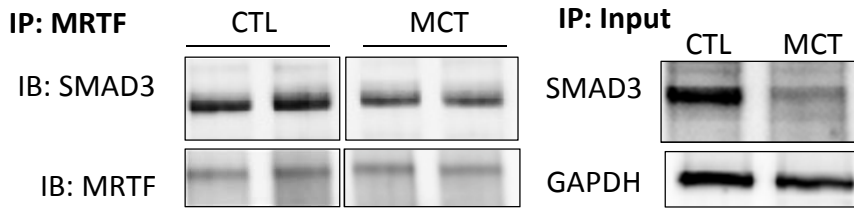
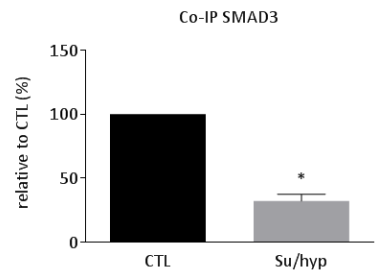
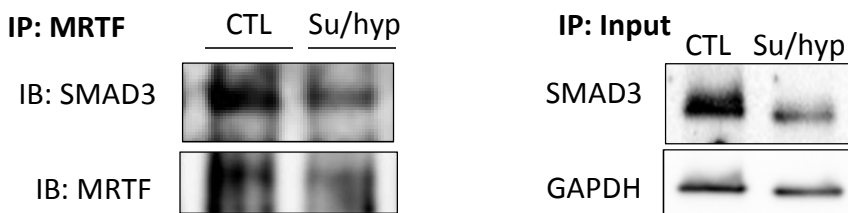


**H**

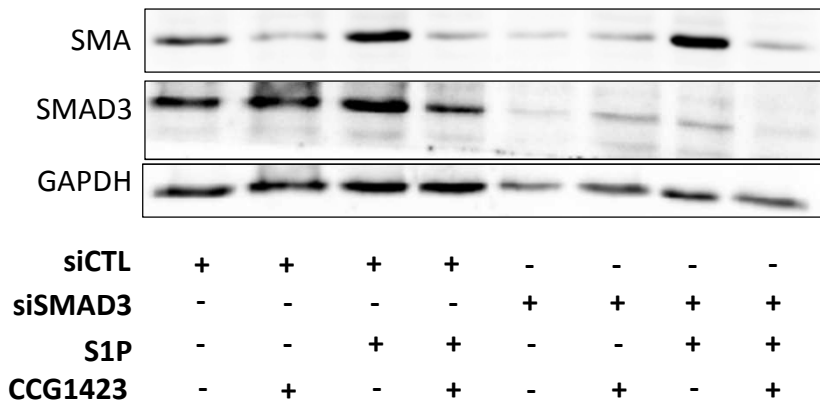


**I**

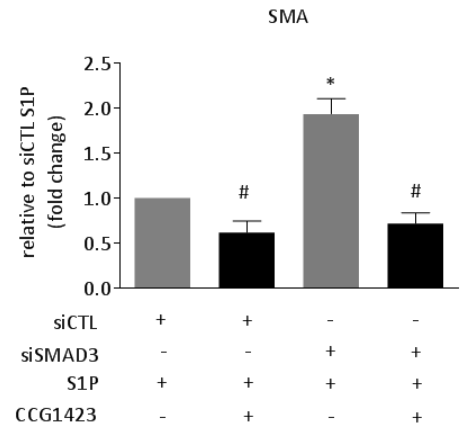


**A huPASC****B huPASC****C MCT****D Sugen/hypoxia**

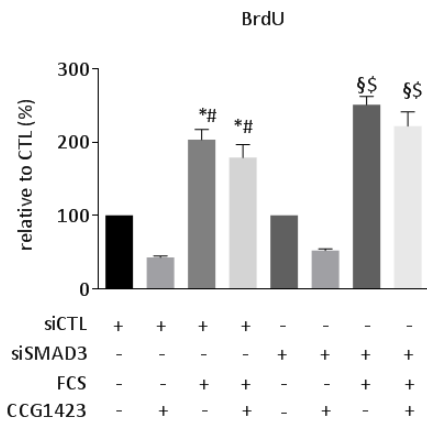
**A huPASC**



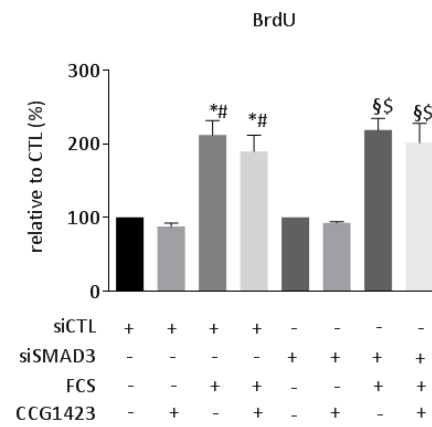
**B**



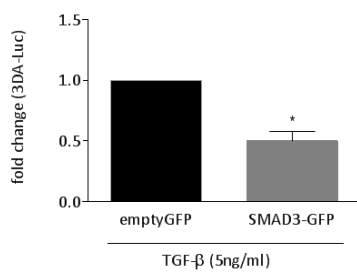
**C huPASC**



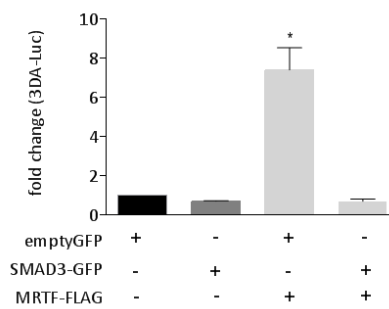
**D huPAEC**

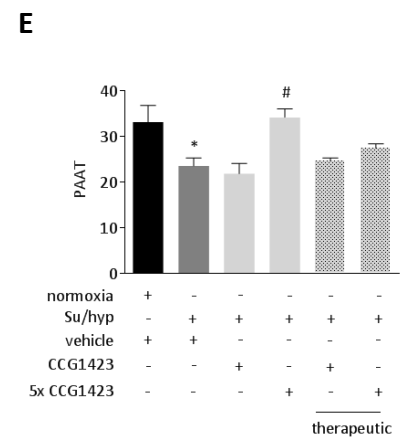
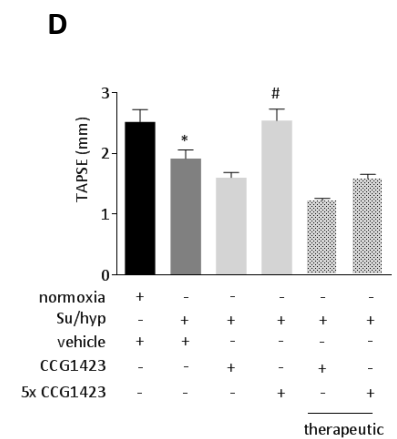
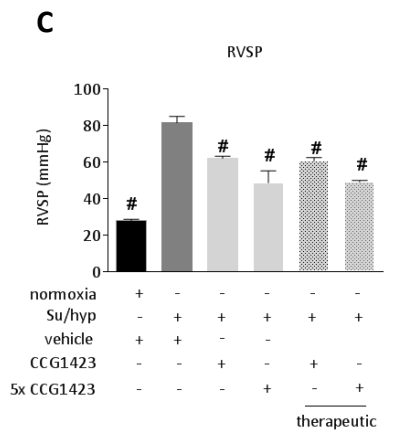
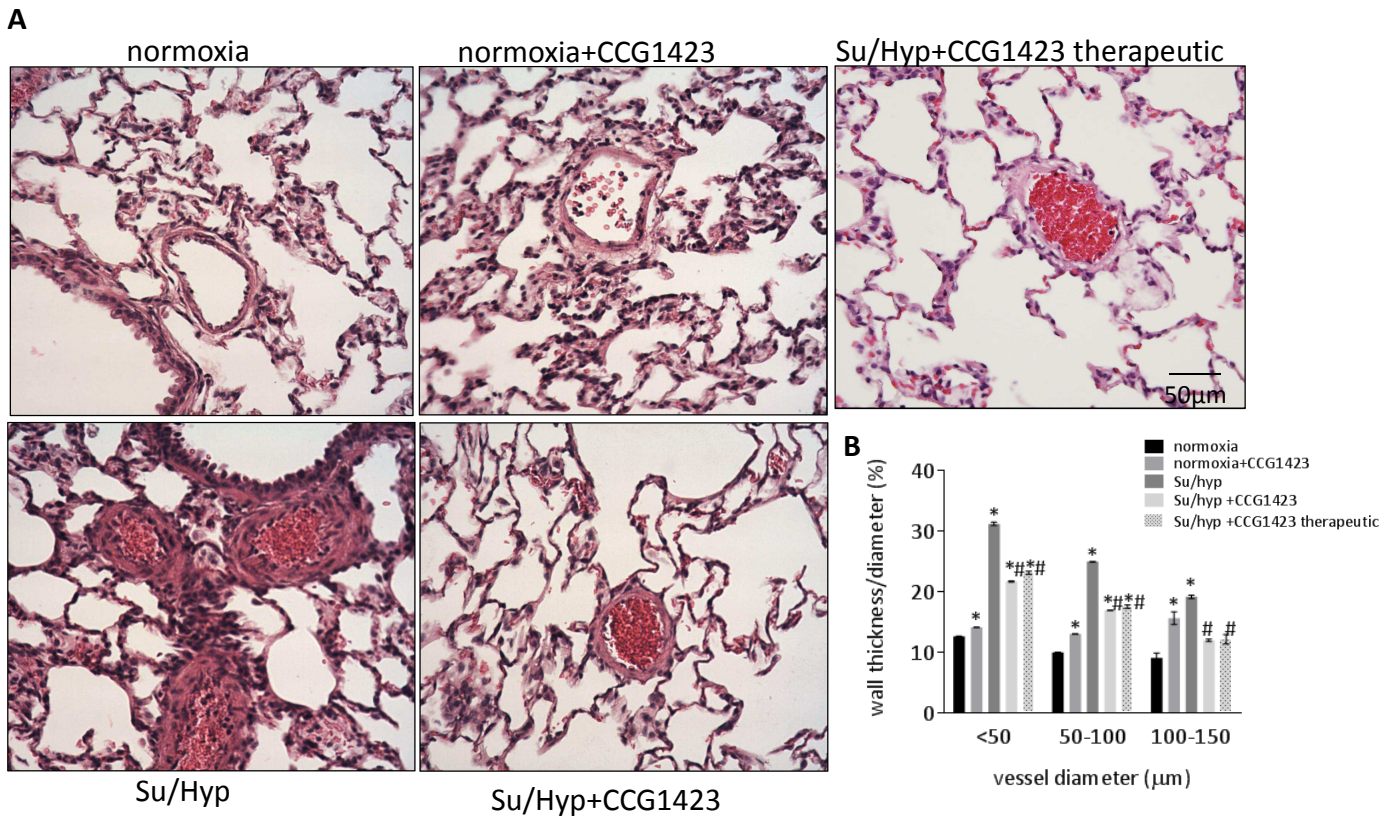


**E huPASC**

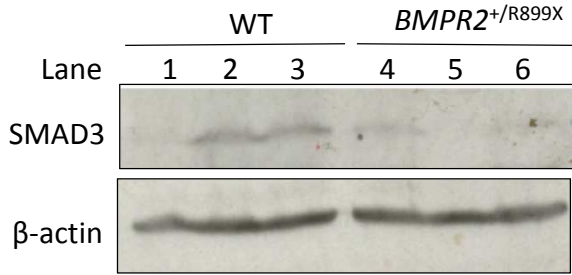


**F huPASC**

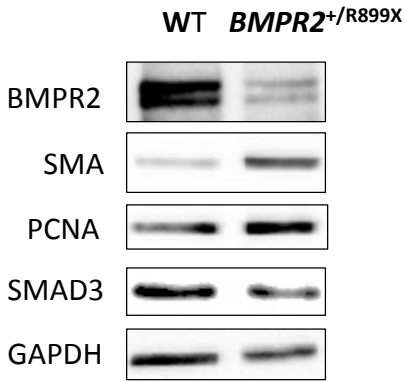




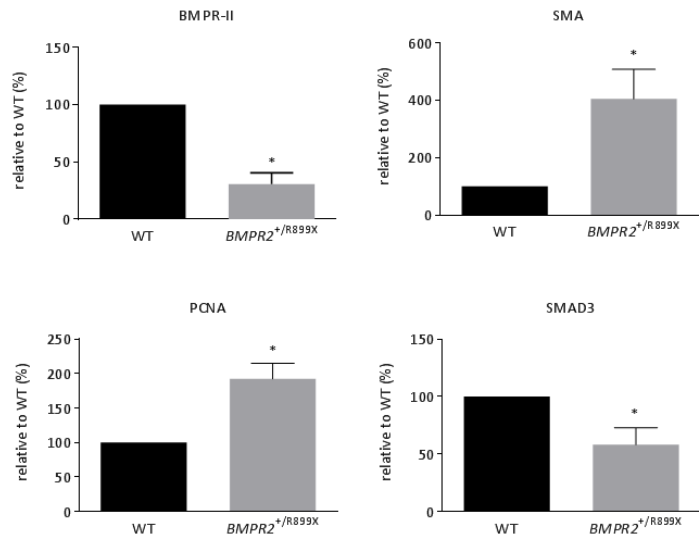
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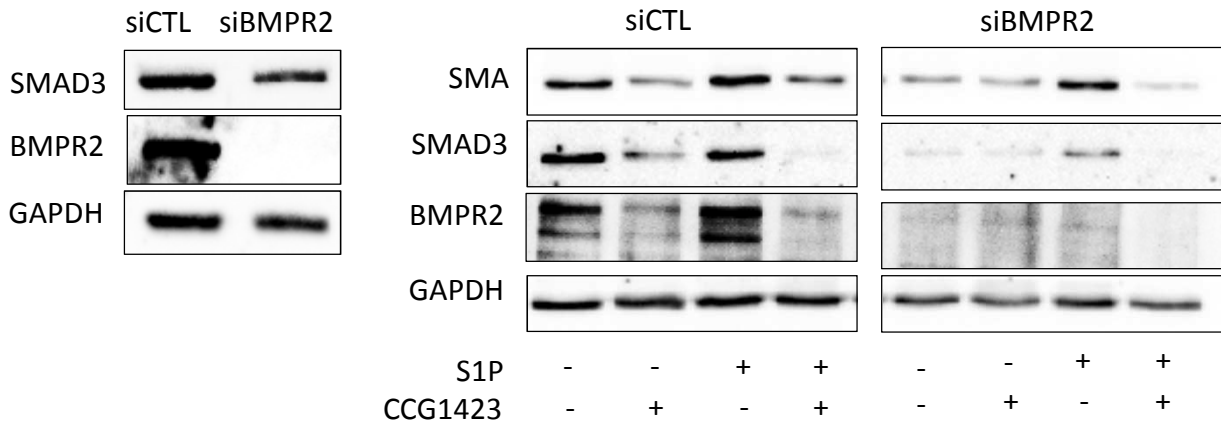
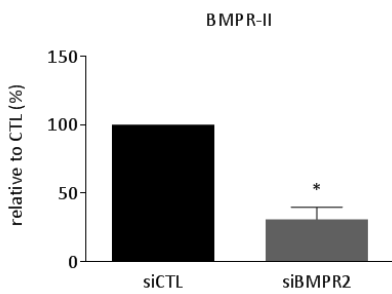
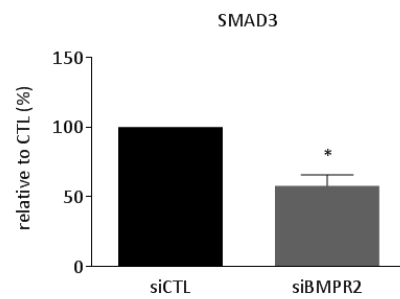
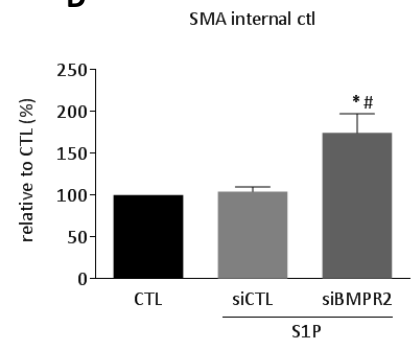
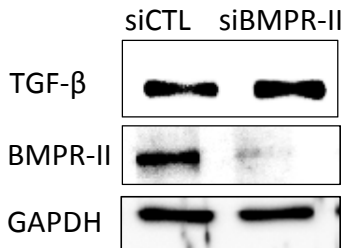
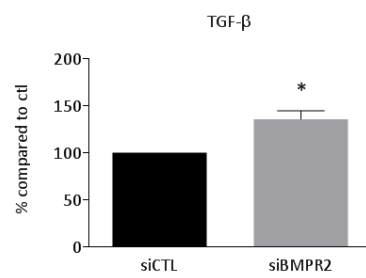
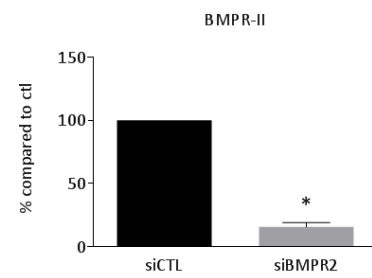


B



C

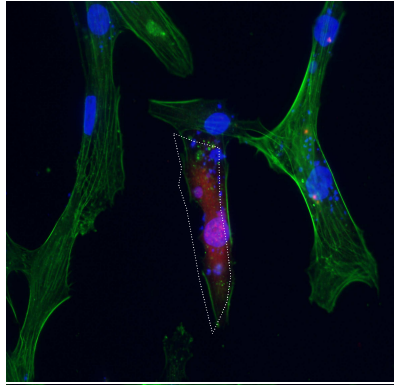
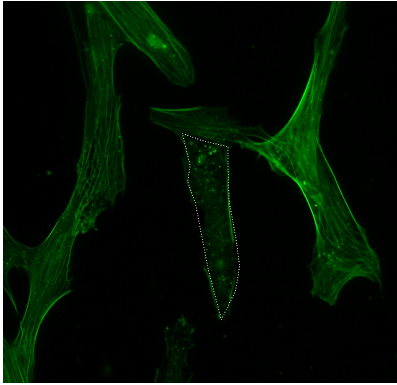


**A****B****C****D****E****F****G**

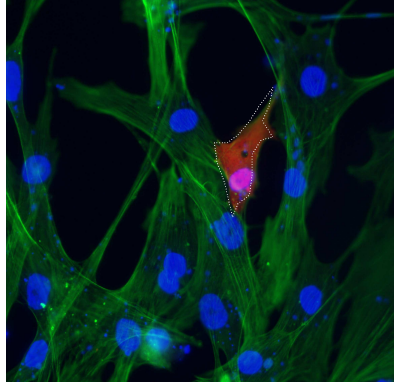
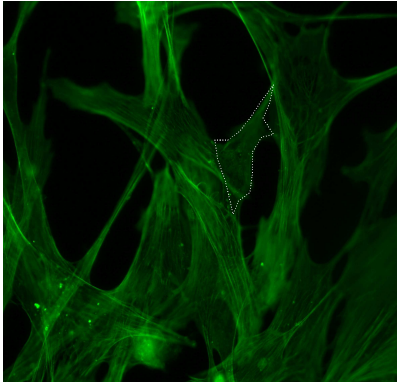
Phalloidin staining

Overlay

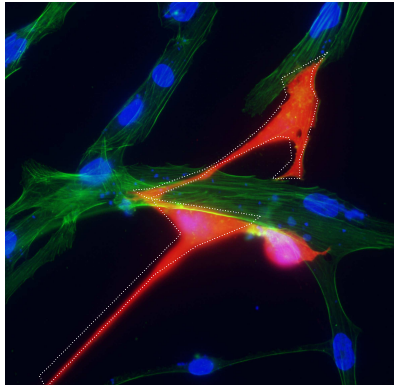
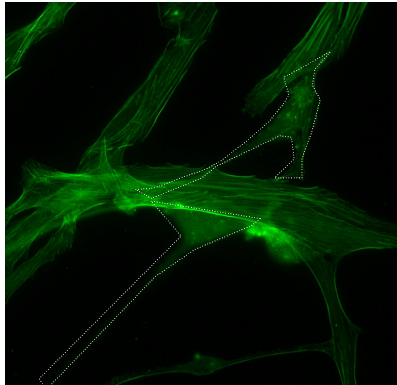
siCTL+ CTL



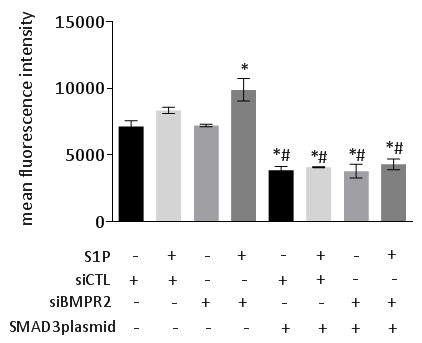
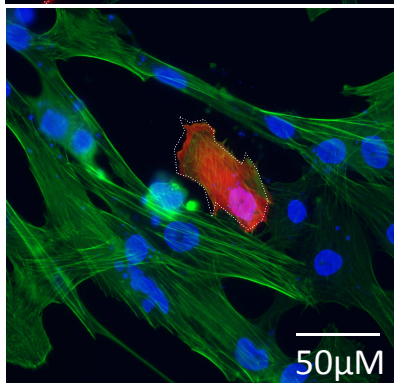
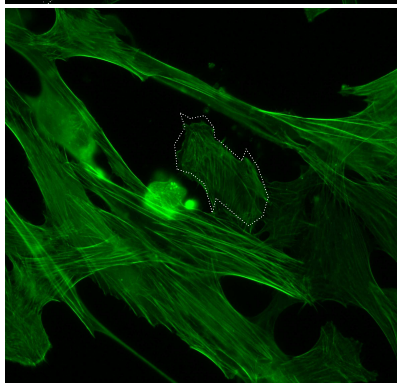
siCTL+S1P



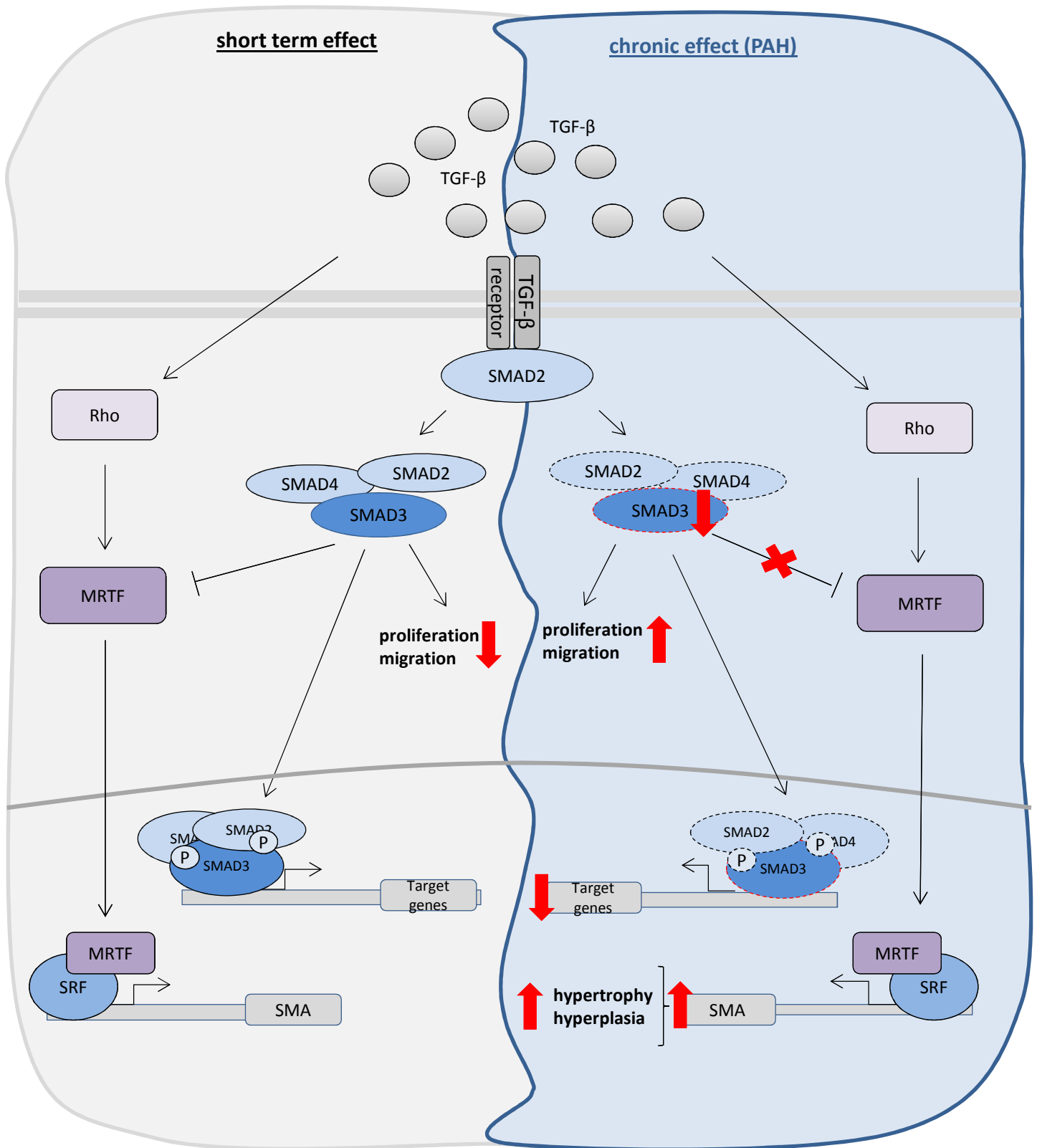
siBMPR2+CTL

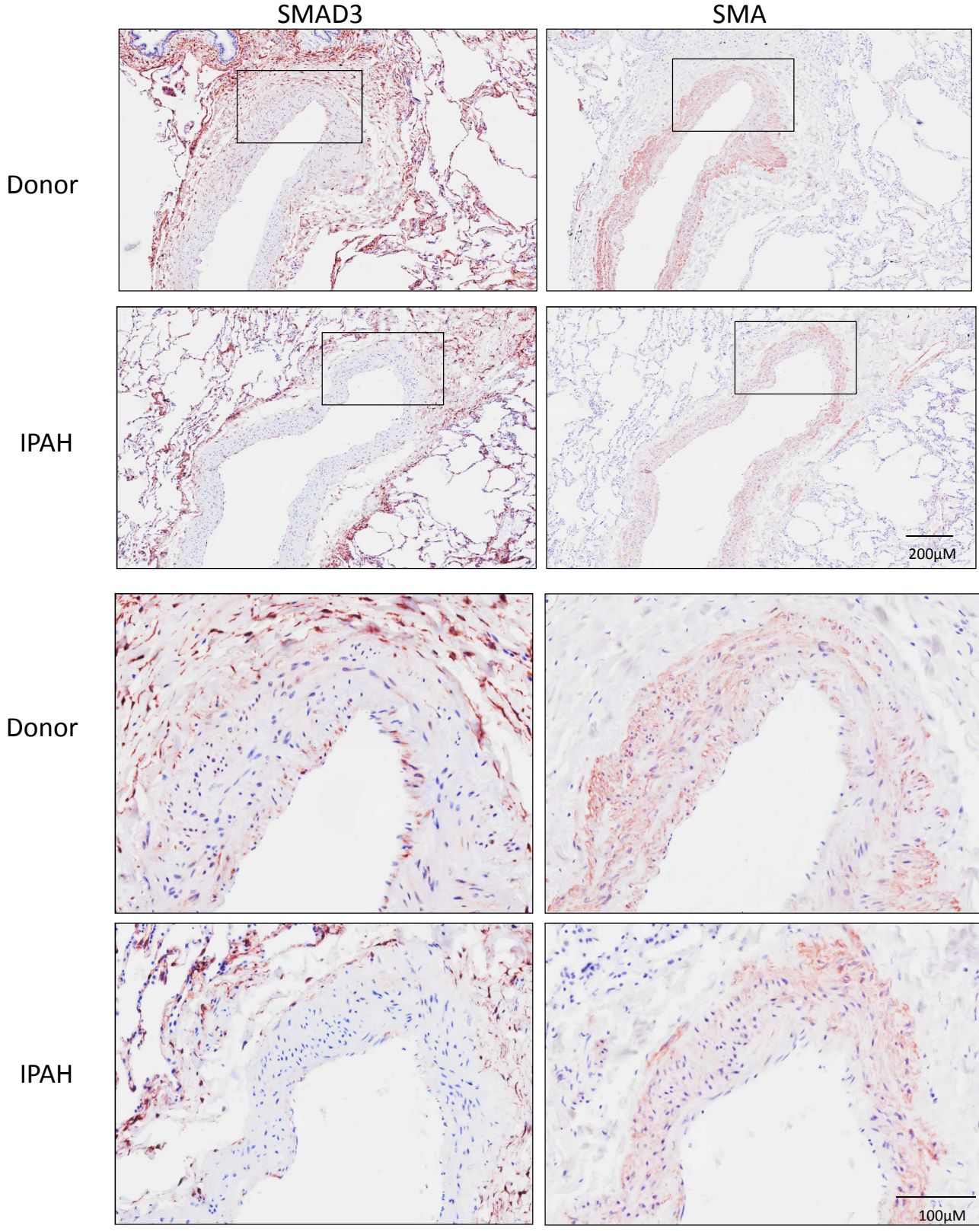


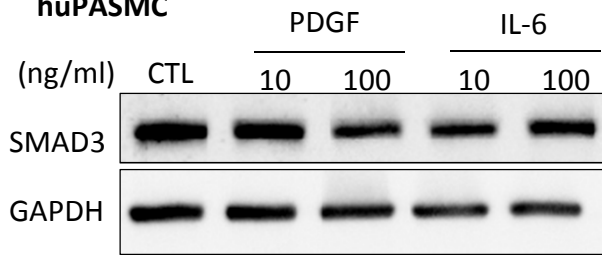
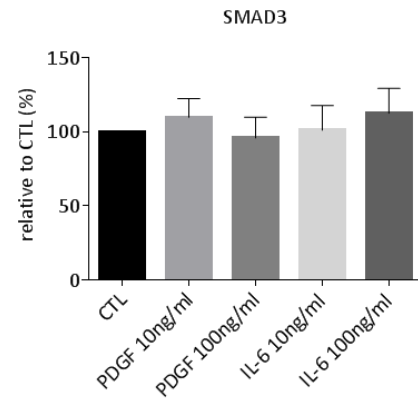
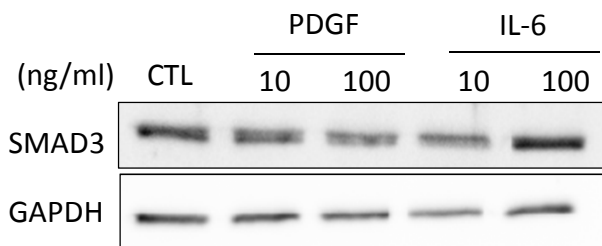
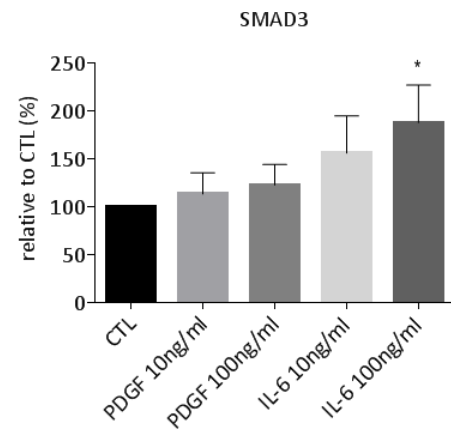
siBMPR2+S1P





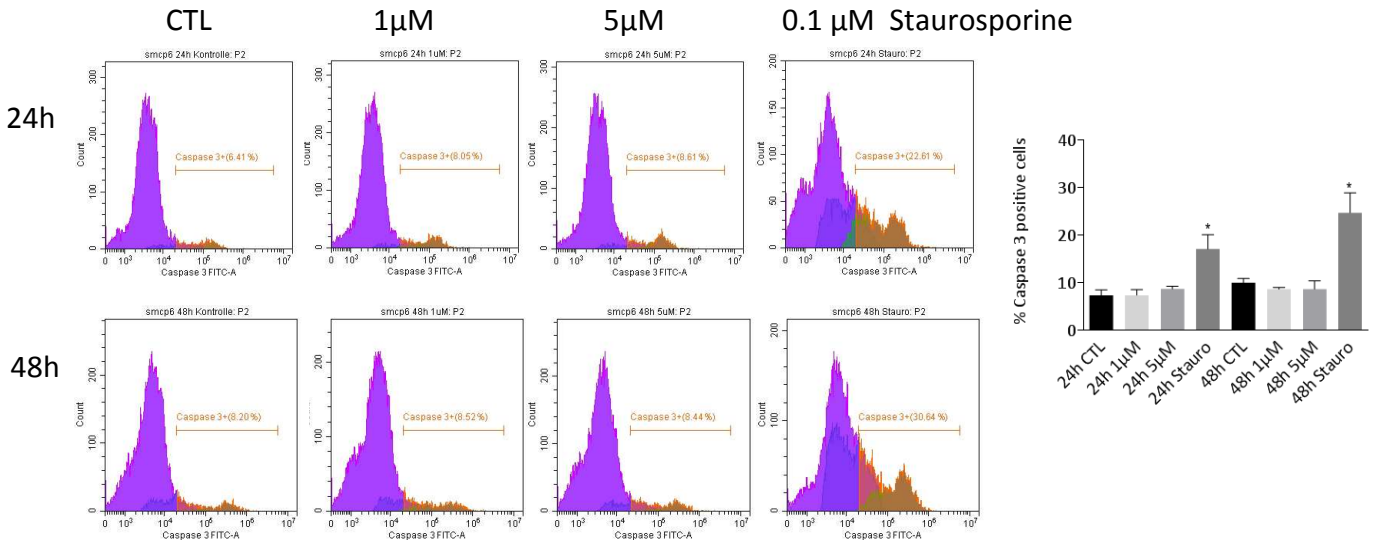




**A huPASC****B****C huPAEC****D**

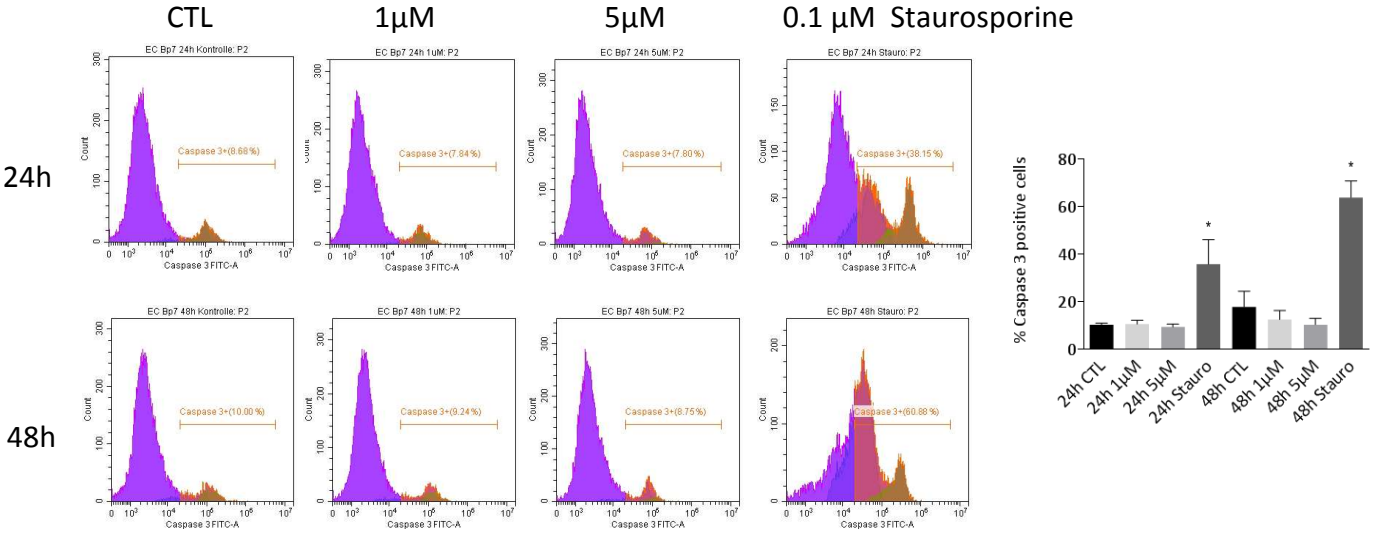
### A huPASC activated Caspase 3

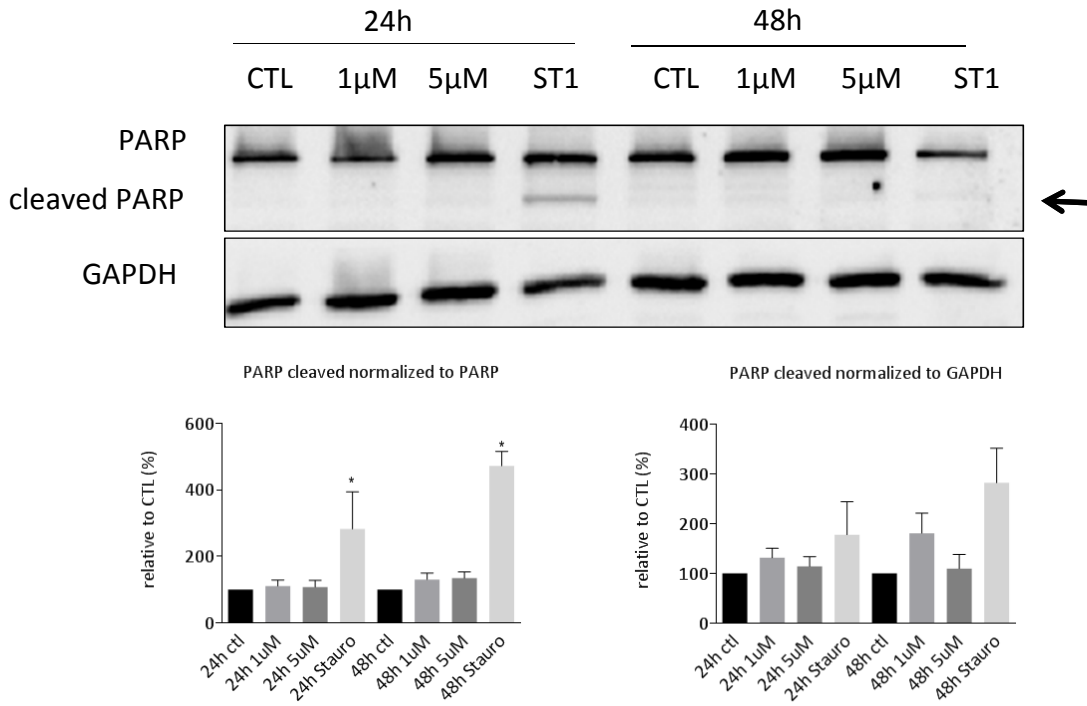
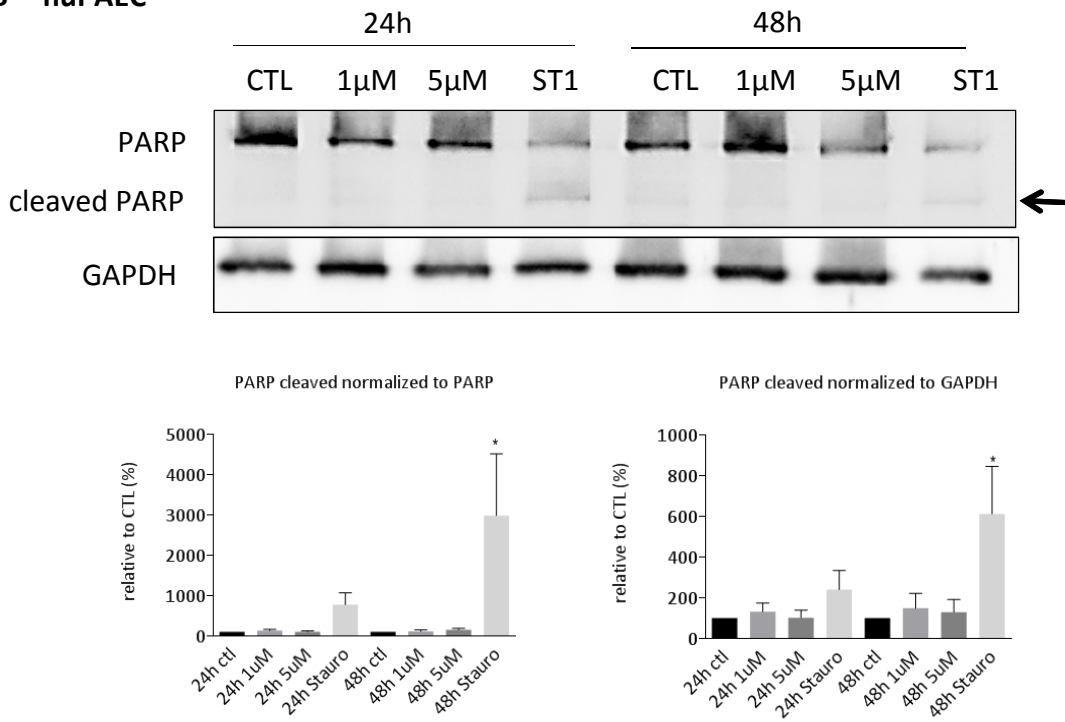
CCG1423



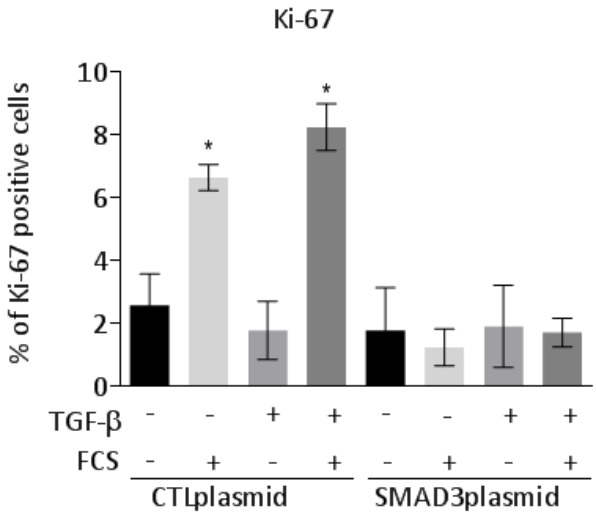
### B huPAEC activated Caspase 3

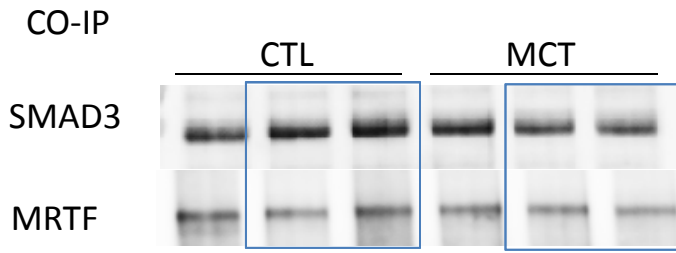
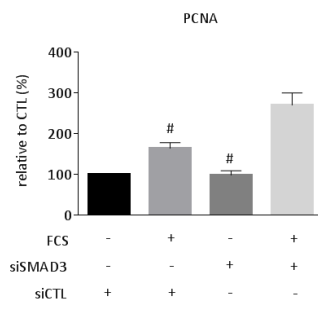
CCG1423



**A huPASC****B huPAEC**

huPASMC



**A****B**

## *Online Supplement*

### **Methods**

**Cells.** Human pulmonary arterial smooth muscle cells (huPASMC) and human pulmonary arterial endothelial cells (huPAEC) purchased from Promocell (C-12521, Heidelberg, Germany) and Lonza (CC-2530, Basel, Switzerland), respectively, were cultured either in Smooth Muscle (C-22062) or Endothelial Cell Growth Medium 2 (C-22211) both purchased from Promocel and used from passage 4-8. Murine PASMC were obtained from *BMPR2*<sup>+R899X</sup> mice which carry a mutated allele of the *BMPR2* gene, and corresponding wild type mice as previously described <sup>1</sup>, and cultured in Smooth Muscle Growth Medium 2 (Promocell).

**Protein isolation and western blotting.** For protein extraction, human (donor and PAH) as well as rat lung samples (normoxic, SU5416/hypoxia as well as MCT treated rats) were ground in liquid nitrogen. HuPASMC or huPAEC were lysed in RIPA buffer with protease inhibitor complex and phosphatase inhibitors (Roche, Basel, Switzerland). After incubation on ice for 30 minutes, samples were centrifuged (12,000g, 10 min, 4°C), and the protein concentration in the supernatant was determined by spectrophotometry (BCA assay, Pierce, Rockford, IL, USA). Final concentrations of 10 µg of protein were run on a 10% sodium dodecyl sulfate (SDS) polyacrylamide gel, followed by electrotransfer to a 0.2-µm pore nitrocellulose membrane (Bio-Rad, Bio-Rad Laboratories, Mississauga, Ontario, Canada). After blocking with 5% non-fat dry milk in TBS-T buffer (Tris-buffered saline with 0.1% Tween20), the following antibodies were applied over night at 4°C: SMAD3 (1:1000; #9523S; Cell Signaling Technology, Danvers, MA, USA), MRTF-B (1:2000; sc61074; Santa Cruz Biotechnology, Dallas, Texas, USA), SMA (1:10000; A2547; Sigma Aldrich), PCNA (1:2000; #2586S; Cell Signaling Technology, Danvers, MA, USA), BMPR-II (1:1000; 612292; BD Biosciences, Mississauga, ON, Canada); TGF-β (1:2000; ab179695; Abcam;



Cambridge, United Kingdom ); PARP (1:1000; #9542; Cell Signaling Technology, Danvers, MA, USA); GAPDH (1:5000; sc25778; Santa Cruz Biotechnology, Dallas, Texas, USA), tubulin (1:10000; #2125S; Cell Signaling Technology, Danvers, MA, USA). Secondary antibodies were purchased from Jackson ImmunoResearch and applied at a 1:5000 dilution. Final detection of proteins was performed using the Amersham ECL Prime Western Blotting Detection System (GE Healthcare, Wien, Austria).

**Transfection.** Pre-designed, commercially available siRNA sequences directed against human SMAD3 and BMPR2 were purchased (Dharmacon Thermo Scientific). For non-specific gene inhibition of the siRNAs used in this study, a universal negative-control siRNA sequence was used (Dharmacon Thermo Scientific). For SMAD3 overexpression a GFP-tagged Smad3 from cClontech or a Myc-tagged Smad3 expression constructs (in pCMV5B) was used which was a kind gift from L. Attisano (University of Toronto). , for MRTF overexpression a FLAG-tagged MRTF construct kindly provided by E.N. Olsen (University of Texas, Southwestern Medical Center, Dallas, TX) . Cells were transfected with 100nMol/L siRNA or 0.5-2 $\mu$ g plasmid for 8h using Effectene, Transfection Reagent (Qiagen, Toronto, Ontario, Canada) .

**Proliferation assays.** For SMAD3 silencing, huPASCs or huPAECs were treated for 8 h with SMAD3-specific (siSMAD3) or scrambled (siCTL) small interference RNA (100nMol/L L-020067-00-0005 or D-001810-10-05, respectively; Dharmacon Thermo Scientific)). Tenthousand cells per well were seeded on 96 well plates and kept overnight in starvation medium (0% FCS). The following day cells were stimulated with basal media with or without 5% FCS and or 5  $\mu$ Mol/L CCG1423 (10010350; Cayman Chemical, Ann Arbor, Michigan; USA). Proliferation of huPASCs and huPAECs was determined by immunoblotting for proliferating cell nuclear antigen (PCNA #2586, Cell Signaling Technology, Danvers, MA, USA), by incorporation of bromodeoxyuridine (BrdU assay) according to manufacturer

instructions (#6813; Cell Signaling Technology, Danvers, MA, USA), or by immunocytochemical staining for Ki-67, respectively.

**Migration assay.** huPASCs and huPAECs were treated with siSMAD3 or siCTL as described above. The next day, 20,000 cells per 24-transwell (CA62406-198; VWR, Mississauga, Ontario, Canada) were seeded and allowed to migrate towards basal medium with or without 5% FCS for 6h. Cells were fixed with ice-cold methanol and stained with haematoxylin. Residual non-migrated cells in the compartment of the transwell were removed and migrated cells on the lower filter insert were counted manually.

**RNA isolation and real-time PCR.** Total cellular RNA was isolated from huPASCs and huPAECs by use of the RNeasy Mini Kit from Qiagen (Toronto, Ontario, Canada). A Nanodrop 2000c spectrophotometer (PeQlab) was used to quantify the concentration and the purity of the isolated total RNA. Total RNA was reverse transcribed to cDNA using an iScript™ cDNA Synthesis Kit (Bio-Rad, Mississauga, Ontario) and amplified using a Lightcycler 480 (Roche, Basel, Switzerland). The PCR reactions were performed with QuantiFast SYBR PCR kit (Qiagen, Toronto, Ontario, Canada). Gene expressions were calculated using the  $\Delta C_t$  method with GAPDH serving as control.

**Co-immunoprecipitation.** Cell lysates of TGF- $\beta$  (5 ng/mL for 72 h) or vehicle treated huPASCs, or pulmonary arteries isolated from MCT, Sugen-hypoxia, or control rats were incubated with an antibody directed against MRTF-B (1:100; Santa Cruz Biotechnology, Dallas, Texas, USA) over night at 4°C. To collect the formed complex composed of anti-MRTF-B antibody bound to MRTF-B and possible binding partners (SMAD3), protein G (or A) sepharose was added the next day for 1h at 4°C. After centrifugation (3-5 min at 2,000 rpm at 4°C) and 5x washing with cell lysis buffer, the supernatant of the sample was carefully

removed and 2x concentrated SDS-PAGE sample loading buffer with denaturing  $\beta$ -mercaptoethanol was added to the pellet. The samples were boiled at 95°C for 10 min and western blots for SMAD3 (1:1000; Cell Signaling Technology, Danvers, MA, USA) and MRTF-B (1:2000; Santa Cruz Biotechnology, Dallas, Texas, USA) protein levels were performed on the immunoprecipitated pellet, as well as the supernatant and the original input (cell lysate) as corresponding controls.

**Immunocytochemistry.** Cells were fixed with 4% methanol-free paraformaldehyde and treated for 10 min with 100 mMol/L glycerol followed by 15 min of 0.1% TritonX100 (Sigma). After 1h 5% treatment with bovine serum albumin (BSA) cells were stained with anti-SMAD3 (1:100; Cell Signaling Technology, Danvers, MA, USA) for 1h at room temperature. Alexa Fluor555 donkey-anti-rabbit (1:200; Thermo Fisher Scientific) was applied as secondary antibody. For actin staining Alexa Fluor Phalloidin488 (1:100; Thermo Fisher Scientific) was applied for 1h at room temperature. Mounting medium with DAPI was used from Vectorshield, Burlingame, Canada.. Pictures were taken with an Nikon Upright E800 microscope.

**Immunohistochemistry.** Serial cut human lung slides from donor or IPAH patients were deparaffinized and stained for SMAD3 (1:100, #9523S; Cell Signaling Technology, Danvers, MA, USA) or SMA (1:300, A2547; Sigma Aldrich) overnight in 4°C; secondary antibody staining was performed with the Vektor ImmPRESS reagent kit (Vector Laboratories Ltd, Peterborough, UK). Hematoxylin was applied for counterstaining.

**Flow cytometry:** For caspase-3 analysis, cells were treated either with 1 $\mu$ Mol/L or 5 $\mu$ Mol/L CCG1423 for 24h or 48h. Staurosporine (0.1 $\mu$ Mol/L) served as positive control. Cells and supernatant were collected, centrifuged and washed with PBS. For caspase 3 staining the

CellEvent™ Caspase-3/7 Green Flow Cytometry Assay Kit was employed according to the manufacturer's instructions (C10427; Life Technologies, Carlsbad, CA), and caspase-3 positive events were detected and analysed on a CytoFLEX using CytExpert software (Beckman Coulter Life Sciences, Indianapolis, IN). For Ki-67 analysis, cells were fixed in 1% paraformaldehyde (PFA) for 15min on ice. After 15min treatment with 0.3% saponin and subsequent PBS washing steps, cells were stained for 20min with a Ki-67 phycoerythrin-labeled antibody (Ki-67 PE set, Becton Dickinson, Vienna, Austria), and analyzed on a LSRII cytometer (Becton Dickinson, Franklin Lakes, NJ, USA, State).

**Luciferase Reporter Assays:** Assays were carried out as described previously<sup>(2)</sup>. Briefly, 70 – 80% confluent PSMCs were incubated in 0% serum for 2 hr and then co-transfected using 0.6 µL/mL of Human Artery Smooth Muscle Cell Avalanche (EZ Biosystems, LLC) according to manufacturer's instructions. Cells were co-transfected with the MRTF/SRF sensitive promoter plasmid pGL3-3DA-Luc (0.25 µg/mL) which contains the firefly luciferase gene under the control of a CArG box triplet, the control plasmid pRL-TK (0.05 µg/mL), and one of the following four constructs: empty GFP (0.5 – 2 µg, Clontech, Mountain View, CA, USA), GFP-Smad3 (0.5 – 2 µg, Clontech), pcDNA (0.1 µg, Invitrogen, Burlington, ON, Canada), FLAG-MRTFB (0.1 µg, kindly provided by E.N. Olsen, Univ. of Texas Southwestern Medical Center, Dallas, TX). Cells were treated 24h later as described for individual experiments and lysed on ice with 1x Passive Lysis Buffer (Promega, Madison, WI, USA). The luciferase assay was conducted using the Dual Luciferase Kit (Promega) as per manufacturer's instructions.

**Pulmonary hypertension models.** The study was approved by the animal care and use committee of St. Michael's (ACC #554), and all experiments were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, 7th edition 1996).

*Sugen-hypoxia model:* Male Sprague-Dawley (Charles River Laboratories, St. Constant, QC) rats with a starting body weight (bw) of 200g received a single subcutaneous injection of the VEGF receptor antagonist SU5416 (20 mg/kg bw dissolved in 0.5% carboxymethylcellulose sodium + 0.9% sodium chloride + 0.4% Tween80 + 0.9% benzyl alcohol in distilled water) and were subsequently exposed to 10% O<sub>2</sub> (hypoxia) for 3 weeks, followed by 2 weeks of normoxia (21% O<sub>2</sub>) as previously described<sup>3</sup>. In subgroups of animals, the pharmacological MRTF inhibitor CCG1423 (0.15mg/kg or 0.75mg/kg [5xCCG1423] in DMSO, Cayman Chemicals, Ann Arbor, Michigan, USA) or respective vehicle was delivered daily via intraperitoneal injections either from day 0 (prophylactic approach) or from day 21 (therapeutic approach) after SU5416 injection.

*Monocrotaline model:* Male Sprague-Dawley rats (Charles River Laboratories, St. Constant, QC) with a starting bw of 200 g were treated with either a single intraperitoneal injection of monocrotaline (MCT; 60 mg/kg, Sigma, Deishofen, Germany in distilled water, pH: 7.5) or vehicle alone. After 21 days the animals underwent hemodynamic characterization (*vide infra*) and were sacrificed for organ harvesting.

**Hemodynamic characterization.** Rats were anesthetized by isoflurane inhalation (5%). Right ventricular systolic pressure (RVSP) measurements were performed as previously described<sup>4</sup> using a closed chest technique. Briefly, a 1.4 mm pressure catheter (Millar Instruments, Houston, TX, USA) was inserted into the jugular vein and directed to the right ventricle. Pressure recordings were made over a two minute period each and analyzed with Powerlab Pro software (AD Instruments Spechbach, Germany).

Echocardiographic imaging of right ventricular function was performed in anesthetized rats using a 12-MHz probe (Hewlett Packard Sonos 5500, Philips, Ultrasound, Bothell), and tricuspid anular plane systolic excursion (TAPSE) and pulmonary artery flow acceleration time (PA time) were quantified as surrogates for pulmonary hypertension.

**Vascular remodelling.** Hematoxylin and eosin staining was performed on paraffin fixed lung tissue slides. For quantification of medial wall thickness, external and internal (lumen) area as well as vessel diameter was determined for all pulmonary arteries within the field of observation, and the difference between external and internal vessel area relative to the external area was quantified. Vessels were categorized according to external diameter in <50  $\mu\text{m}$ , 50-100  $\mu\text{m}$ , and >100  $\mu\text{m}$  pulmonary arteries.

**Fulton Index.** After exsanguination, lungs and hearts were isolated. The right ventricle (RV) was dissected from the left ventricle and septum (LV+S) and the samples were weighed separately to obtain the right-to-left ventricle plus septum weight ratio [RV/(LV+S); Fulton index] as a measure of right ventricular hypertrophy.

**Statistical analysis.** Statistical analyses were performed by use of GraphPad Prism software (GraphPad Prism 6.0; GraphPad Software Inc., La Jolla, CA). All data are presented as means  $\pm$  SEMs. Student's t-test (two-tailed) was used to compare two groups. For >2 groups, two-way Analysis of Variance (ANOVA) was applied and Turkey was used as a post-hoc test. Linear regression analysis was performed by GraphPad Prism software. *P*-values < 0.05 were considered as statistically significant.

**Supplementary Table 1:** Clinical data of patient cohort given in mean $\pm$  SEM

	<b>Control</b>	<b>PAH</b>
<b>body mass index (kg/m<sup>2</sup>)</b>	26 $\pm$ 2	22.8 $\pm$ 4.7
<b>WHO / NYHA</b>	NA	III and IV
<b>TVC (POD/RAP/PVC) (mmHg)</b>	NA	13.5 $\pm$ 5.4
<b>systolic pulmonary arterial pressure (PAP) (mmHg)</b>	NA	81 $\pm$ 14.8
<b>diastolic PAP (mmHg)</b>	NA	33 $\pm$ 4.2
<b>mean PAP (mmHg)</b>	NA	51 $\pm$ 8.8
<b>systolic blood pressure (mmHg)</b>	NA	113 $\pm$ 10.1
<b>diastolic blood pressure (mmHg)</b>	NA	69 $\pm$ 6.6
<b>mean blood pressure (mmHg)</b>	NA	88 $\pm$ 9.6
<b>cardiac output (L/min)</b>	NA	4 $\pm$ 1.1
<b>cardiac index (L/min/m<sup>2</sup>)</b>	NA	2 $\pm$ 0.5
<b>pulmonary capillary wedge pressure (mmHg)</b>	NA	10 $\pm$ 3.1
<b>SvO<sub>2</sub> mixed venous oxygen saturation (%)</b>	NA	56 $\pm$ 10.7
<b>heart rate (bpm)</b>	NA	82 $\pm$ 9.2
<b>pulmonary vascular resistance (PVR) (dyne·s·cm<sup>-5</sup>)</b>	NA	808 $\pm$ 66.3
<b>arterial oxygen saturation (SaO<sub>2</sub>) (% at rest)</b>	NA	91 $\pm$ 2.8
<b>6 min walk distance (m)</b>	NA	281 $\pm$ 101
<b>arterial oxygen saturation (SaO<sub>2</sub>) (% after 6min)</b>	NA	81 $\pm$ 7.7

**References:**

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2. Speight, P., Kofler, M., Szász, K. & Kapus, A. Context-dependent switch in chemo/mechanotransduction via multilevel crosstalk among cytoskeleton-regulated MRTF and TAZ and TGF $\beta$ -regulated Smad3. *Nat. Commun.* **7**, 11642 (2016).
3. Lang, M. *et al.* The soluble guanylate cyclase stimulator riociguat ameliorates pulmonary hypertension induced by hypoxia and SU5416 in rats. *PLoS One* **7**, (2012).
4. Tabeling, C. *et al.* CFTR and sphingolipids mediate hypoxic pulmonary vasoconstriction. *Proc. Natl. Acad. Sci. U. S. A.* **112**, E1614–23 (2015).

**Supplementary figure legends:**

**Suppl. Fig. 1:** Serial sections of human lungs from healthy donors or IPAH patients were immunohistochemically stained (in red) for SMAD3 (left) or smooth muscle actin (SMA; right). Nuclei were counterstained blue with Hematoxylin. Lower panels show enlarged areas as indicated in top panels.

**Suppl. Fig. 2:** HuPASMC (A, B) and huPAEC (C, D) were treated either with platelet-derived growth factor (PDGF; 10ng/mL and 100ng/mL) or interleukin-6 (IL-6; 10ng/mL or 100ng/mL) and probed for SMAD3 protein expression. Representative western blots are shown in A,C and quantitative data in B,D (n=7-8 each; \* $P$ <0.05 vs. control, CTL).

**Suppl. Fig. 3:** Effect of 24h and 48h treatment with either 1 $\mu$ Mol/L or 5 $\mu$ Mol/L CCG1423 on activated caspase 3 expression as measured by flow cytometry in either huPASMC (A) or huPAEC (B). Staurosporine (0.1  $\mu$ Mol/L) served as positive control. Representative



histograms and quantitative data for the percentage of activated caspase 3 positive cells are shown (n=3 each; \* $P < 0.05$  vs. corresponding time control, CTL).

**Suppl. Fig. 4:** Effect of 24h and 48h treatment with either 1 $\mu$ Mol/L or 5 $\mu$ Mol/L CCG1423 on Poly (ADP-ribose) polymerase (PARP) cleavage in either huPASC (A) or huPAEC (B). Staurosporine (0.1  $\mu$ Mol/L) served as positive control. Representative western blots for total PARP, cleaved PARP and GAPDH as loading control are shown as well as quantitative data for cleaved PARP normalized either to total PARP or GAPDH, respectively (n=4 each; \* $P < 0.05$  vs. corresponding time control, CTL).

**Suppl. Fig. 5:** Ki-67 expression is decreased in SMAD3 overexpressing huPASCs following 72h stimulation with 5ng/ml TGF- $\beta$  and 24h with 5% FCS. Ki-67 positivity was determined by flow cytometry in GFP positive cells overexpressing either GFP-linked control (CTL) plasmid or GFP-linked SMAD3 plasmid, treated with or without TGF- $\beta$  and/or FCS (n=3 each; \* $P < 0.05$  vs CTL-CTLplasmid).

**Suppl. Fig. 6:** (A) Uncropped western blot shown in Figure 5C. (B) Alternative to Figure 3C PCNA western blot quantification summary. Data was normalized to siCTL