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Contribution of TRPC channels in human and experimental pulmonary arterial hypertension

Short title: TRPC channels in PAH

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33 **ABSTRACT**

34 Pulmonary arterial hypertension (PAH) is due to progressive distal pulmonary artery (PA) obstruction
35 leading to right ventricular hypertrophy and failure. Exacerbated store-operated Ca^{2+} entry (SOCE)
36 contributes to PAH pathogenesis, mediating human PA smooth muscle cells (hPASCs) abnormalities.
37 The transient receptor potential canonical channels (TRPC family) are Ca^{2+} -permeable channels
38 contributing to SOCE in different cell types, including PASCs. However, the properties, signaling
39 pathways, and contribution to Ca^{2+} signaling of each TRPC isoform are unclear in human PAH.

40 We studied *in vitro* the impact of TRPC knockdown on control and PAH-hPASCs function. *In vivo*,
41 we analyzed the consequences of pharmacological TRPC inhibition using the experimental model of
42 pulmonary hypertension (PH) induced by monocrotaline (MCT)-exposure.

43 Compared to control-hPASCs cells, in PAH-hPASCs, we found a decreased TRPC4 expression,
44 overexpression of TRPC3 and TRPC6, and unchanged TRPC1 expression. Using the siRNA strategy,
45 we found that the knockdown of TRPC1-C3-C4-C6 reduced the SOCE and the proliferation rate of
46 PAH-hPASCs. Only TRPC1 knockdown decreased the migration capacity of PAH-hPASCs. After
47 PAH-hPASCs exposure to the apoptosis inducer staurosporine, TRPC1-C3-C4-C6 knockdown
48 increased the percentage of apoptotic cells, suggesting that these channels promote apoptosis resistance.
49 Only TRPC3 function contributed to exacerbated calcineurin activity. In the MCT-PH rats' model, only
50 TRPC3 protein expression was increased in lungs compared to control rats, and *in vivo* "curative"
51 administration of a TRPC3 inhibitor attenuated PH development in rats.

52 These results suggest that TRPC channels contribute to PAH-hPASCs dysfunctions, including SOCE,
53 proliferation, migration, apoptosis resistance, and could be considered as therapeutic targets in PAH.

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56 **Keywords: TRPC, PAH, calcineurin, Pyr3, apoptosis, proliferation, migration, contraction**

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62 **New and Noteworthy**

63 TRPC3 is increased in human and experimental pulmonary arterial hypertension (PAH).

64 In PAH pulmonary arterial smooth muscle cells, TRPC3 participates in the aberrant store-operated Ca^{2+}
65 entry contributing to their pathological cell phenotypes (exacerbated proliferation, enhanced migration,
66 apoptosis resistance, and vasoconstriction).

67 Pharmacological *in vivo* inhibition of TRPC3 reduces the development of experimental PAH.

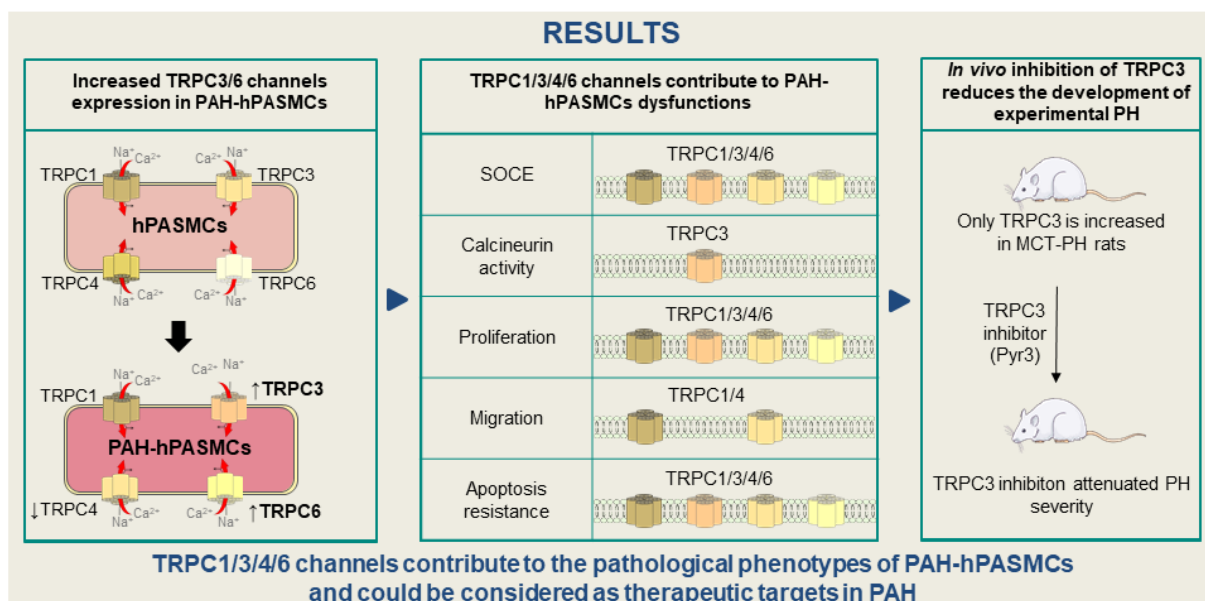
68 Even if other TRPC acts on PAH development, our results prove that TRPC3 inhibition could be
69 considered as an innovative treatment for PAH.

70

71

72 **GRAPHICAL ABSTRACT**

TRPC channels as therapeutic targets for pulmonary arterial hypertension



73

74 **INTRODUCTION**

75 Pulmonary arterial hypertension (PAH) is a rare and progressive cardiovascular disease resulting from
76 obstruction of the distal pulmonary artery (PA), leading to elevated pulmonary vascular resistance
77 (PVR), right ventricular (RV) hypertrophy, and ultimately RV failure (RVF) and death¹. According to
78 the new guidelines, PAH is defined by a mean PA pressure >20 mmHg, a PVR >2 Wood units, and a
79 PA wedge pressure ≤15 mmHg at rest². PAH is a multifactorial syndrome that can be explained by
80 dysfunction of all cell types composing the pulmonary circulation, particularly PA smooth muscle cells
81 (PASMC), including vasoconstriction, excessive migration, apoptosis resistance, and
82 hyperproliferation¹. Despite adequate therapeutics that prolong patient survival, lung transplantation is
83 the ultimate therapy for eligible PAH patients, meaning that identifying novel targets is needed to
84 improve patient management.

85 We demonstrated that exacerbated intracellular calcium concentration ($[Ca^{2+}]_i$) contributes to PASMC
86 dysfunction in PAH³. Among Ca^{2+} channel families, store-operated Ca^{2+} channels (SOC) may play a
87 critical role in regulating PASMC function^{3,4}. Three essential components constitute SOCs: the
88 endoplasmic/sarcoplasmic reticulum (ER/SR) Ca^{2+} sensor belonging to the stromal interaction molecule
89 family (STIM1-2) and two Ca^{2+} channels, namely TRPC (Transient Receptor Potential Canonical) and
90 Orai (Orai1-3)³. TRPC channels are non-selectively permeable to cations, including potassium (K^+),
91 sodium (Na^+), calcium (Ca^{2+}), and magnesium (Mg^{2+})⁵. The TRPC subfamily consists of seven
92 members, from TRPC1 to TRPC7, and TRPC2 is a pseudogene in humans⁶. TRPC contributes to the
93 proliferation and migration of systemic vascular endothelial cells and smooth muscle cells⁷. Gene
94 polymorphism in the *TRPC6* gene was identified in a few PAH patients, predisposing them to develop
95 PAH⁸. The double *trpc1/trpc6* knockout mice are protected from chronic hypoxia (CH)-induced PH⁹.
96 The triple *trpc1/trpc3/trpc6* knockout mice have decreased hypoxic pulmonary vasoconstriction without
97 any consequence on pulmonary vascular obstruction induced by CH exposure¹⁰. However, in human
98 PASMCs (hPASMCs), the role of each expressed TRPC channel in PAH pathological phenotypes
99 remains elusive.

100 In the present study, we investigated the expression and function of TRPC isoforms in human PA and
101 human PASMCs isolated from control (control-hPASMCs) and PAH patients (PAH-hPASMCs) and
102 their contribution to PAH-hPASMC phenotypes (proliferation, migration, contraction, apoptosis

103 resistance). We also evaluated *in vivo* the contribution of TRPC3 to the development of experimental
104 PH caused by monocrotaline (MCT)-exposure in rats. Overall, we show that all expressed TRPC
105 channels contribute to cellular dysfunction occurring in PAH-hPASMCs and that targeting TRPC could
106 constitute an innovative therapeutic approach in PAH.

107

108

109 **MATERIALS AND METHODS**

110 Chemicals

111 MCT, staurosporine, endothelin-1, histamine, PDTC, cytosine arabinoside, and nifedipine were
112 obtained from Sigma-Aldrich. MCV1 (Calbiochem) and U46619 were obtained from R&D Systems.
113 Fura-2-AM was obtained from Thermo Fisher Scientific. KB-R7943 and Pyr3 were obtained from
114 TOCRIS. LW6 was obtained from CliniSciences.

115

116 Human lung specimens

117 Human lung specimens were obtained from lung transplantation in 12 patients with PAH and from
118 lobectomy or pneumonectomy for localized lung cancer from 10 patients as control subjects. Female
119 PAH patients represent 70% of primary cells. PAs (diameter:3–5 mm) were isolated from non-tumoral
120 areas in the lung specimens of control subjects. Transthoracic echocardiography was performed
121 preoperatively in control subjects to rule out PH. Patients with PAH underwent genetic counseling and
122 were provided written informed consent for genetic analysis. The demographic information of PAH and
123 control patients are presented in **Supplemental Table 1**.

124 Patients were part of the French Network on Pulmonary Hypertension, a program approved by our
125 institutional ethics committee, and provided written informed consent (Protocol N8CO-08- 003, ID
126 RCB: 2008-A00485-50, approved on June 18th, 2008). Patients studied were part of a program approved
127 by our institutional Ethics Committee and had given written informed consent (ID RCB: 2018-A01252-
128 53, approved on June 18, 2006). All human tissues were obtained with written informed consent from
129 transplant recipients or families of organ donors following the Declaration of Helsinki.

130

131 Human PASMC culture from PAH and control patients

132 hPASMCs were isolated from pulmonary arteries (diameter:3–5 mm) obtained during lung
133 transplantation for PAH patients and lobectomy or pneumonectomy of localized lung cancer from
134 control subjects. Pulmonary arteries were excised at a distance from tumor areas.

135 PAs (diameter:3–5 mm) were kept in DMEM at 4°C before their intimal cell layer and residual
136 adventitial tissue were stripped of fusing forceps. The dissected media of the vessels was then cut into
137 small pieces (3–5 mm), which were transferred into 6 well plates (one piece per well). The explants
138 adhered to the support, and to allow the PASMCs to grow out, the explants were incubated in DMEM
139 supplemented with 20 % fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 U/ml penicillin
140 and 0.1 mg/ml streptomycin, 10 U/ml Epidermal Growth Factor, insulin). After 2 weeks of incubation,
141 the hPASMCs collected in the culture medium and the vessel tissues were transferred into new cell-
142 culture flasks. Cultured hPASMCs were used between passages 3 and 5^{11,12}.

143 Characterization of cultured control and PAH-hPASMCs was assessed by positive staining with
144 antibodies against smooth muscle cell α -actin, desmin, and vinculin¹³.

145

146 Animal models

147 The facility is licensed by the French Ministry of Agriculture (agreement N° C92-019-01). This study
148 was approved by the Committee on the Ethics of Animal Experiments (CEEA26 CAP Sud). Animal
149 experiments were performed following the guidelines of Directive 2010/63/EU on September 22nd,
150 2010, of the European Parliament on the protection of animals used for scientific purposes and complied
151 with the French institution's animal care and handling guidelines.

152 *In-vivo* experiments were performed according to clinical trial standards. Rats were randomly distributed
153 between groups, and all methods and analyses were performed with researchers blinded to the
154 conditions. All rats received a number known only by the experimenter who delivered the treatment.
155 Before sacrifice (day 21), all rats underwent an evaluation by closed-chest right heart catheterization.
156 This experiment was performed by a blinded experimenter who did not know the correspondence
157 between rat number and treatment. Hemodynamic parameters were analysed in a blinded fashion.

158 PH was induced by a single MCT injection (60 mg/kg, s.c.) in male Wistar rats (4 weeks old). MCT was
159 dissolved in 1 N HCl and neutralized with 1 N NaOH. Control animals received the same volume of
160 saline solution. Pyr3 was dissolved in DMSO. MCT-exposed Wistar rats were treated with Pyr3(1

161 mg/kg/day in DMSO, daily i.p. injection) from week 2 (W2) to week 3 (W3). Control animals received
162 the same volume of DMSO.

163

164 Hemodynamic measurements and tissue collection

165 Rats were placed under general anesthesia and spontaneous breathing with an isoflurane Rodent
166 Anesthesia System (Minerve Esternay, France) (maintenance: isoflurane 2 % in room air).
167 Hemodynamics parameters, such as heart rate (HR), right ventricular (RV) systolic pressure (RVSP;
168 mmHg), and cardiac output (CO; mL/min), were blindly measured in unventilated anesthetized rats
169 using a closed-chest technique. Under profound anesthesia (5 % isoflurane), animals were euthanized.
170 After catheterization, tissues were collected, and Fulton's index was calculated as the ratio of RV weight
171 to left ventricular (LV) plus septal (S) weight ($RV \div LV + S$).

172

173 siRNA transfection

174 PASMCS were transfected in suspension by incubating 4×10^5 cells in a solution containing 500 μ L of
175 Opti-MEM (Gibco, ref 31985-062), 3 μ L of Lipofectamine RNAiMax (Invitrogen, ref 13778-150), and
176 100 nM of a specific *Silencer*TM Select siRNA against TRPC1 (siTRPC1, #s14411, Thermo Fisher
177 Scientific), or TRPC3 (siTRPC3, #s14413, Thermo Fisher Scientific), or TRPC4 (siTRPC4, #s229618,
178 Thermo Fisher Scientific), or TRPC6 (siTRPC6, #s14421, Thermo Fisher Scientific) as well as a
179 *Silencer*TM Select negative control (siControl) according to the manufacturer's protocol. All experiments
180 were performed 72 h after PAH-hPASMCS transfection.

181

182 Isometric tension measurement

183 Rat intralobar main PAs were mounted on an emkaBATH4 modular tissue bath system (EMKA
184 Technology, Paris, France) coupled with IOX software (EMKA Technologies). As determined in
185 preliminary experiments, rat PAs were set at an optimal length by equilibration against a passive load
186 of 0.3 g. After adding Krebs solution, vessels were contracted with 100 mmol/L K⁺-containing solutions
187 (K100). Once a plateau was reached, the vessels were washed with Krebs solution for 30 min. DMSO
188 or Pyr3 (1 μ mol/L) was pre-treated before the dose response to U46619 (0.1 nM to 3 μ M). The relaxation
189 responses were expressed as the percentage of the pre-contractile response obtained with U46619.

190

191 Reverse transcription-quantitative PCR (RT-qPCR)

192 Total gene expression was quantified using qPCR following the standard protocol for ready-to-use
193 qPCR SignArrays (AnyGenes) on a StepOne Plus Real-Time PCR System (Life Technologies)¹⁵.
194 Predesigned probe sets used in the experiments were Human TRPC1 NM_001251845.1; Human TRPC3
195 NM_001130698.2; Human TRPC4 NM_016179.4; Human TRPC6 NM_004621.6; Human Orai1
196 NM_001277961.3; Human STIM1; Human β -Actin NM_001101.

197

198 Pulmonary vessel remodeling analysis

199 The lungs were fixed in 4 % paraformaldehyde, embedded in paraffin, and serially sectioned (5 μ m). A
200 pulmonary pathologist performed the histopathological evaluation of the lungs, blinded to treatment
201 group assignments. The morphometric analyses were performed on sections stained with hematein-
202 eosin-Safran (HES). The lung samples from all rat groups were analyzed by conventional light
203 microscopy using quantitative semiautomated software (NIS-BR; Nikon, Champigny sur Marne,
204 France). Vascular remodeling was assessed in all the pulmonary vessels of size 50 μ m to 100 μ m
205 identified in 20 randomly selected microscopic fields per tissue section. The wall thickness was
206 calculated using the following equation: (External diameter - Internal diameter) / (External
207 diameter) \times 100.

208

209 Cardiac histological assessment

210 Hearts were fixed in 4 % paraformaldehyde, paraffin-embedded, and serially sectioned (5 μ m). To assess
211 heart fibrosis, heart sections were stained with Sirius red (0.1 %). Pictures were recorded at 10X on an
212 EVOS microscope (Invitrogen). RV fibrosis was quantified using Image J software.

213 The level of cardiomyocyte hypertrophy was assessed by analyzing RV sections stained with FITC-
214 conjugated wheat germ agglutinin (WGA) (50 μ g/ml) and then observed on an EVOS microscope
215 (Invitrogen). Pictures were recorded at 40X, and RV cardiomyocytes' diameters were determined using
216 Image J software ¹⁶.

217

218 Intracellular Ca^{2+} measurements

219 hPASMCs were plated on 18-mm glass coverslips and loaded with 2 $\mu\text{mol/L}$ Fura-2-AM dissolved in
220 DMSO plus 20% pluronic acid (Life Technologies) and then incubated at room temperature for 30 min
221 in darkness in Krebs solution (in mmol/L: 135 NaCl, 5.9 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, 10 D-
222 glucose) at pH 7.4. Loaded cells were washed twice with the physiological solution before imaging.
223 Ca²⁺ images were obtained using a microscope (Olympus IX71) equipped with a Sutter Fluo Lambda
224 421 LED system (Sutter Instrument Company, Novato, CA, USA), which rapidly changed the excitation
225 wavelengths between 340 nm and 380 nm. Emission was measured at 510 nm.
226 The Ca²⁺-free solution contained 1 mmol/L EGTA instead of 2 mmol/L CaCl₂ + nifedipine (L-type Ca²⁺
227 channel inhibitor) + KB-R7943 (Na⁺/Ca²⁺ exchanger inhibitor). Ca²⁺ imaging measurements were
228 performed at room temperature. Due to the high transfection efficiency (90% of transfected cells),
229 cytosolic Ca²⁺ measurements were performed on the whole cell population. Image acquisition in selected
230 cells and analysis was performed with MetaFluor[®] 7.8 imaging software (Molecular Devices).

231

232 Immunoblot analysis

233 Total protein from human or rat lungs or isolated PA tissue samples or cell samples from rats or humans
234 (diameter:3–5 mm) were prepared in lysis buffer containing 1% Igepal, 20mM Tris HCl, 137 mM NaCl,
235 10% Glycerol, 2 mM EDTA, 1 mM Na₃VO₄, leupeptin 10 $\mu\text{g}/\mu\text{l}$, pepstatin 10 $\mu\text{g}/\mu\text{l}$, aprotinine 10
236 $\mu\text{g}/\mu\text{l}$ and protease inhibitor cocktail (aprotinin, leupeptin, and PefaBloc [Roche, Meylan, France]).
237 Protein lysates (40 μg) were separated on SDS-PAGE and transferred to a nitrocellulose membrane¹⁷.
238 After blocking, membranes were incubated in Tris Buffered Saline + tween and 5 % nonfat milk
239 overnight at 4°C with primary antibodies at adequate concentrations (**Supplemental Table 2**).

240 Blots were then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse diluted
241 1:10000 (Cell Signaling) or with HRP-conjugated goat anti-rabbit diluted 1:5000 (Cell Signaling),
242 respectively. Antibodies were revealed using Enhanced chemiluminescence reagents (Perkin Elmer).
243 ImageJ Software was used to quantify the level of protein expression. The list of antibodies used is
244 presented in **Supplemental Table 2**. β -Actin was used as a loading control.

245

246 Immunofluorescence staining

247 Paraffin-embedded lung sections (5 μ m) were dewaxed in toluene, and antigens were retrieved in Power
248 Universal Antigen Retrieval buffer pH 9,5 (Leica ref PV6125 BR02-1000) for 20 minutes at 95°C. Slices
249 were saturated with human (10 %) and donkey (10 %) sera in phosphate-buffered saline for 1 hour at
250 room temperature.

251 Free aldehyde groups from Paraformaldehyde fixation were quenched for 10 min with 50 mM NH₄Cl
252 solution. Sections were saturated with human (10 %) and goat (10 %) sera in PBS for 1 h at room
253 temperature. Primary and secondary antibodies were used, as reported in Supplemental Table 3. Primary
254 antibodies were detected with secondary antibodies from goat anti-rabbit (1/200). Slides were
255 counterstained with 4',6'-diamidino-2-phenylindole (DAPI)¹⁸. Mouse IgG and rabbit pre-immune serum
256 (both species of primary antibodies used in this study) were applied to lung sections, followed by anti-
257 rabbit Alexa Fluor 594. Sections were viewed under an LSM 700 microscope (Carl Zeiss, Le Pecq,
258 France) equipped with 405-, 488-, 555-, and 639-nm lasers (Carl Zeiss). Images were recorded and
259 analyzed with ZEN software (Carl Zeiss).

260

261 Wound healing assay

262 We used the wound healing assay to evaluate cell migration. After 48 h of starvation (medium without
263 growth factors: FBS, EGF, and insulin), human PSMCs were plated in a culture insert (Cat. No. 90209;
264 Ibidi) at a density of 1.2×10^4 cells per well in a fresh medium with cytosine arabinoside (10 μ mol/L).
265 After allowing cells to attach for 24 h, we removed the culture insert and washed the cells with
266 phosphate-buffered saline to remove non-adherent cells. For siRNA treatment, cells were treated with
267 control siRNA or siRNA against TRPC1 or TRPC3 or TRPC4, or TRPC6 24 h before starvation (72 h
268 before removal of culture inserts). Cell migration into the wound space was quantified using image J.
269 Cell motility/invasion was assessed by the percentage of wound closure 15 h after initiation of wound
270 healing ($[(\text{area T0} - \text{area T15}) \div \text{area T15}] \times 100$).

271

272 Calcineurin activity

273 The activity of PP2A was assessed using the Ser/Thr phosphatase assay system (Cat. No. V2460,
274 Promega). The experiments were performed according to the manufacturer's recommendations. The

275 optical density values obtained with the molybdate-malachite green-phosphate complex were read at
276 600 nm with a FlexStation 3 Microplate Reader (Molecular Devices).

277

278 PASMCs induced-apoptosis measurement

279 To induce apoptosis, we treated hPASMCs with staurosporine at 1 $\mu\text{mol/L}$ for 20 min or 50 nmol/L
280 overnight. Cells were washed with cell staining buffer (Cat. No. 420201, BioLegend) and gently
281 detached with trypsin and a cell scraper. After centrifugation (400 g, 4°C, 10 min), the supernatant was
282 removed, and cells were stained with the FITC-Annexin V Apoptosis Detection Kit (Cat. No 640914,
283 BioLegend) according to the manufacturer's recommendations. Cells were acquired by flow cytometry
284 (MACSQuant, Miltenyi Biotec), with 75,000 cells counted in each sample and analyzed using Flowlogic
285 software (Inivai Technologies).

286

287 PASMCs proliferation measurement

288 To evaluate cell proliferation, we quantified BrdU incorporation in cells undergoing DNA replication
289 using the DELFIA cell proliferation kit (AD0200, PerkinElmer), according to the manufacturer's
290 recommendations. Different treatments were used, including control siRNA, siRNA against TRPC1 or
291 TRPC3 or TRPC4, or TRPC6. 48h after cell synchronization (0 % SVF) to stimulate cell proliferation,
292 the medium contained 10 % of serum in the presence of 1 $\mu\text{mol/L}$ BrdU for 24 h¹⁴. The fluorescence
293 signal in the 96-well plate was read with a FlexStation 3 Microplate Reader (Molecular Devices).

294

295 Statistical analyses

296 Analyses were performed using GraphPad Prism software (GraphPad, version 9.0 for Windows) and R
297 statistical computing software. After checking the distribution of our samples with the Shapiro–Wilk
298 Test, differences between the two or more groups were assessed using an unpaired t-test or one-way
299 ANOVA, followed by Tukey's post hoc analysis for multiple comparisons. When conditions of
300 parametric tests were not met, we used either a Mann-Whitney test or Kruskal-Wallis test, followed by
301 Dunn's post hoc analysis for multiple comparisons. All values are reported as mean \pm standard error of
302 the mean. Representative images/figures were chosen to represent the mean of each quantification. For
303 all experiments, a *P*-value of <0.05 was considered statistically significant.

304

305 **RESULTS**

306 *Increased TRPC1 and TRPC3 protein expression in pulmonary arteries isolated from PAH patients*

307 We performed immunofluorescent staining in lung sections from control and PAH patients to visualize
308 the localization and expression of TRPC1, TRPC3, TRPC4, and TRPC6 channels. **Figure 1A** shows
309 that TRPC1, TRPC3, TRPC4, and TRPC6 are expressed in PASMCs and pulmonary artery endothelial
310 cells (PAECs). As highlighted by the increased staining intensity, TRPC1, TRPC3, and TRPC6 appear
311 overexpressed in PASMCs from remodeled PA in PAH patients. In contrast, TRPC4 staining intensity
312 suggested increased TRPC4 expression in PAECs from PAH patients and not in PASMCs. As
313 immunostaining is not quantitative, we performed immunoblot analyses on isolated human PA from
314 control and PAH patients. Immunoblot showed a higher expression of TRPC1 and TRPC3 in isolated
315 PAH-PA compared to the control condition, while TRPC4 and TRPC6 protein expressions were
316 unchanged (**Figure 1B**). Since TRPC5 and TRPC7 mRNA were not detected in human pulmonary
317 arteries³, we did not investigate their role in PAH pathogenesis.

318

319 *TRPC1, TRPC3, TRPC4 and TRPC6 contribute to exacerbated SOCE in PAH-hPASMCs*

320 In PAH-hPASMCs, we found an increase in TRPC3 and TRPC6 protein expression and decreased
321 expression of TRPC4. TRPC1 protein expression was unchanged in PAH-hPASMCs (**Figure 2A**).
322 Seventy-two hours after cells transfection with selective siRNA against each TRPC isoform (siTRPC1,
323 siTRPC3, siTRPC4, or siTRPC6), each siRNAs used reduced the counterpart mRNA (**Figure 2B**) and
324 protein (**Figure 2C**) without effect on other TRPC or Orai1 or STIM1 expression (**Figure S1**).
325 Then, we elicited the contribution of each TRPC on SOCE by depleting Ca²⁺ from the sarcoplasmic
326 reticulum by applying 1 μmol/L thapsigargin (SERCA pump inhibitor) for 10 min in Ca²⁺ free medium
327 and then by adding 2 mmol/L extracellular Ca²⁺ reached medium. We confirmed the 2-fold SOCE
328 increase in PAH-hPASMCs compared to control-hPASMCs (**Figure 2D-E**), as recently described⁴.
329 In control-PASMCs, only siTRPC1 decreased the SOCE (by 40 %) (**Figure 2D**). However, in PAH-
330 hPASMCs, the knockdown of each TRPC isoform, TRPC1, TRPC3, TRPC4, or TRPC6, reduced SOCE
331 amplitude by 50%, 50%, 60%, and 30%, respectively (**Figure 2E**).

332 TRPC channels are known to be involved in receptor-operated Ca^{2+} entry. We performed experiments
333 to measure the contribution of each TRPC isoform on Ca^{2+} signaling mediated by GPCR activation
334 induced by endothelin 1 (50 nmol/L) or histamine (10 $\mu\text{mol/L}$) stimulations (**Figure S2**). Ca^{2+} signaling
335 induced by both stimulations was unchanged in control and PAH-hPASCs (Figure S2A-C). Only the
336 knockdown of TRPC1 reduced endothelin 1-mediated Ca^{2+} signaling (Figure S2B-D), while none of
337 siTRPC affected histamine-induced Ca^{2+} signaling in PAH-PASCs.

338 SOCE plays a crucial role in regulating calcineurin/NFAT activity, and we and others demonstrated
339 exacerbated calcineurin activity in PAH-PASCs⁴. We, therefore, measured the consequence of TRPC
340 knockdown on calcineurin activity. We found that only TRPC3 knockdown reduced calcineurin activity
341 in PAH-hPASCs, indicating that increased TRPC3 expression/function contributes to calcineurin
342 over-activity in PAH-PASCs (**Figure 2F**).

343 To understand the increase of TRPC3 and TRPC6 in PAH-PASCs, we evaluated the contribution of
344 two major transcription factors involved in PAH pathogenesis, NFAT, and NF- κB , using selective
345 pharmacological inhibitors. TRPC3 protein expression was unchanged in PAH-PASCs treated with
346 NFAT inhibitor (MCV1), while the treatment with NF- κB inhibitor (PDTC) reduced TRPC3 expression
347 in PAH-hPASCs (**Figure 2G**). The opposite effect was observed for TRPC6. Indeed, TRPC6
348 expression was decreased with MCV1 treatment but unchanged with PDTC treatment (**Figure 2H**).
349 NFAT or NF- κB inhibition did not affect TRPC1 protein expression, while NF- κB inhibition reduced
350 TRPC4 protein expression (**Figure S3A-B**). The selective inhibition of HIF1- α (LW6) did not change
351 TRPC3 and TRPC6 expression in PAH-hPASCs (**Figure S3C**).

352

353 TRPC channels contribute to the exacerbated proliferative phenotype of PAH-hPASCs

354 We studied the contribution of each TRPC in control and PAH-hPASCs proliferation, migratory
355 capacity, and apoptosis resistance. In control-hPASCs, only TRPC1 knockdown decreased the
356 proliferation (**Figure 3A**). Interestingly and in association with the contribution of all TRPC to SOCE
357 of PAH-hPASCs, the proliferative capacity of these cells was decreased by about 40% in siTRPC1 or
358 siTRPC3 or siTRPC4, or siTRPC6 treated PAH-hPASCs (**Figure 3B**). In correlation with the impact
359 of each TRPC isoform in the regulation of PAH-hPASCs proliferation, we found that siTRPC6
360 reduced the activation of P42/44 assessed by the level of P42/44 phosphorylation (**Figure 3C**). These

361 results could partly explain the overactivation in P42/44 already described in PAH-PASMCs¹¹. No
362 changes in the expression and activation of P42/44 were found in PAH-hPASMCs treated with siTRPC1
363 or siTRPC3, or siTRPC4 (**Figure S4A**). We also found increased cell cycle inhibitor P21^{CIP1/Waf1} (P21)
364 protein expression in PAH-hPASMCs treated with siTRPC3 or siTRPC4 (**Figure 3E**). This cell cycle
365 inhibitor is repressed in PAH-PASMCs¹¹. These results suggest that TRPC3 and TRPC4 contribute to
366 the PAH-hPASMCs excessive proliferative phenotype by regulating P21. In addition, in siTRPC1 or
367 siTRPC6 treated PAH-hPASMCs, we found an increase in the Cas9/proCaspase9 ratio, without any
368 significant changes in siTRPC3 or siTRPC4 treated cells (**Figure 3E, Figure S4B**), suggesting that
369 TRPC1 and TRPC6 contribute partly to the apoptosis resistance phenotype of PAH-hPASMCs.

370

371 TRPC1 and TRPC4 channels contribute to the aberrant migratory capacity of PAH-hPASMCs

372 As increased PASMC migration is a feature of PAH pathogenesis⁴, we analysed the contribution of
373 TRPC isoform to the migratory capacity of control-PASMCs and PAH-PASMCs. In control-PASMCs,
374 only the knockdown of TRPC1 caused a decrease in cell migration. In contrast, the knockdown of
375 TRPC1 or TRPC4 decreased the migratory capacity of PAH-PASMCs (**Figure 4A-B**), indicating that
376 both channels are involved in enhanced PAH-hPASMCs migration.

377

378 Reduction of TRPC expression worsens staurosporine-induced apoptosis in PAH-hPASMCs

379 To evaluate the contribution of TRPC channels in the resistance to apoptosis of PAH-PASMCs, we
380 exposed cells to 50 nmol/L staurosporine (an apoptosis inducer) overnight to perform Annexin V-
381 Propidium iodide staining and detect cell apoptosis. As illustrated in **Figure 5**, in PAH-hPASMCs, the
382 knockdown of TRPC1 or TRPC3 or TRPC4, or TRPC6 increased the number of Annexin V-positive
383 cells and simultaneously decreased the number of live cells (**Figure 5A-C**). Moreover, in staurosporine-
384 exposed PAH-hPASMCs transfected with siTRPC1 or siTRPC3, or siTRPC4 or siTRPC6, the
385 Caspase9/proCaspase9 ratio was increased, indicating that their knockdown increases apoptosis via
386 Caspase9 activation (**Figure 5D**).

387

388 Increased TRPC3 expression/function in PH- induced by MCT exposure in rats

389 We investigated the expression of each isoform in the lung from MCT-PH rats (3 weeks after PH
390 induction). Contrary to humans, immunoblot analyses from lungs MCT-PH rats revealed unchanged
391 TRPC1 protein expression and reduced TRPC4, TRPC5, and TRPC6 compared to control animals
392 (**Figure S5**). In contrast, TRPC3 expression was increased in MCT-PH rats' lungs (**Figure 6A**).
393 Similarly, to human lung tissues, immunofluorescent staining showed that TRPC3 was expressed in
394 PASMCs from control and MCT-PH rats with a higher fluorescence intensity in pulmonary arteries from
395 MCT-PH rats (**Figure 6B**).

396 Then, using Pyr3, a selective inhibitor of TRPC3¹⁹ at 1 $\mu\text{mol/L}$, we evaluated the involvement of TRPC3
397 in SOCE of PASMCs isolated from MCT-PH rats. As shown in **Figure 6C-D**, TRPC3 pharmacological
398 inhibition reduced SOCE in MCT-PASMCs and PAH-hPASMCs.

399 As PA vasoconstriction is a hallmark of human and experimental PAH, we evaluated the role of TRPC3
400 in regulating pulmonary arterial constriction. TRPC3 inhibition by Pyr3 (1 $\mu\text{mol/L}$) induced a shift to
401 the right of the dose-response curve for thromboxane A2 receptor agonist (U46619 at 0.1 to 3 $\mu\text{mol/L}$),
402 meaning that TRPC3 contributes to PA constriction induced by thromboxane A2 mimetics (**Figure 6E-**
403 **F**).

404

405 *In vivo, TRPC3 pharmacological inhibition reduces the development of MCT-induced PH in rats.*

406 Since we found that TRPC3 over-expression/function contributes to aberrant PAH-hPASMCs
407 phenotypes and that only TRPC3 is overexpressed in the lung of MCT-PH rats, we next measured the
408 consequence of *in vivo* TRPC3 pharmacological inhibition on the severity of PH induced by MCT
409 exposure (60 mg/kg). We administered Pyr3 or vehicle (DMSO) daily intraperitoneal injection at 1
410 mg/kg during the last week of PH induction (**Figure 7A**). In comparison with MCT+DMSO animals, in
411 the MCT + Pyr3 group, we found an improvement of the RV hypertrophy evaluated by measuring the
412 Fulton index (**Figure 7B**), a reduction of the RV systolic pressure (**Figure 7C**), an increase of the cardiac
413 output (**Figure 7D**) as well as an improvement of the total pulmonary resistance (**Figure 7E**). Moreover,
414 *in vivo*, TRPC3 inhibition reduced MCT-exposure-induced pulmonary vessel obstruction (**Figure 7G**).
415 At the cardiac level, RV fibrosis (**Figure 7F**) and RV cardiomyocyte cross-section area (**Figure 7G**)
416 were improved in MCT + Pyr3 group compared with MCT + DMSO group. All these results

417 demonstrate the benefit of pharmacological inhibition of TRPC3 in the PH experimental model induced
418 by MCT exposure.

419 **DISCUSSION**

420 In the present study, we provide evidence of the contribution of TRPC channel isoforms to the
421 development of PAH-hPASMCs pathological phenotype. First, we found that PAH-hPASMCs
422 overexpressed TRPC3 and TRPC6. Second, the use of specific siRNA demonstrated that TRPC1,
423 TRPC3, TRPC4, or TRPC6 contribute to aberrant SOCE, exacerbated proliferation, and apoptosis
424 resistance of PAH-hPASMCs. In contrast, TRPC1 and TRPC4 increased the migratory capacity of PAH-
425 hPASMCs. Third, we found that only TRPC3 contributes to calcineurin/NFAT activity exacerbation in
426 PAH-hPASMCs. Fourth, we showed that TRPC3 protein/function is pathologically increased in lungs
427 from MCT-induced PH rats. Finally, *in vivo* “curative” administration of the TRPC3 inhibitor attenuated
428 PH development in the MCT-PH model.

429 We confirmed that hPASMCs expressed TRPC1, TRPC3, TRPC4, and TRPC6 isoforms, as previously
430 described³, while TRPC1, TRPC3, TRPC4, TRPC5, and TRPC6 were expressed in rodent PASMCs^{3,20}.
431 TRPC1 was overexpressed in control-hPASMCs undergoing proliferation in association with increased
432 SOCE, meaning that TRPC1 contributes to SOCE in control-hPASMCs³. In rat or human control-
433 PASMCs, hypoxia exposure increased the expression of TRPC1 or TRPC6 in association with increased
434 SOCE^{21,22}. In rats exposed to chronic intermittent hypoxia, lung TRPC1, TRPC4, and TRPC6 protein
435 expression was increased compared to rats under normoxia^{23,24}. In CH-induced PH rats, the expression
436 of TRPC1 and TRPC6 were increased in isolated PA, associated with increased SOCE²⁵. Altogether
437 these data suggest that hypoxia-inducible factor 1 alpha (HIF-1 α) could regulate TRPC expression.
438 However, we found that HIF-1 α pharmacological inhibition had no impact on the expression of TRPC3
439 and TRPC6 in PAH-hPASMCs, indicating that increased HIF-1 α activity in PAH²⁶ is not responsible
440 for TRPC channel remodeling in PAH-hPASMCs. In iPAH-hPASMCs, the knockdown of TRPC6
441 reduced the hyperproliferative phenotype²⁷. Here, we found in PAH-hPASMCs an increased expression
442 of TRPC1, TRPC3, and TRPC6, contributing equally to increased SOCE.

443 In PAH-hPASMCs, we found that NF-kB and NFAT signaling partly control the increased TRPC3 and
444 TRPC6 protein expression, respectively. In contrast to neonatal rat ventricular myocytes, where it has
445 been demonstrated that TRPC3 expression is regulated through a calcineurin/NFAT mechanism (and
446 not by NF-kB) and that TRPC3 function contributes to calcineurin activity²⁸, we found in PAH-
447 hPASMCs that calcineurin/NFAT did not control TRPC3 expression. However, the TRPC3 function

448 partly controlled calcineurin overactivity in PAH-hPASMCs. Unlike TRPC3, we highlighted that
449 TRPC6 overexpression in PAH-hPASMCs is regulated by calcineurin/NFAT and not by NF- κ B, as
450 previously described in rodent cardiomyocytes²⁹. However, in PAH-hPASMCs, the TRPC6 function did
451 not influence the calcineurin function. In cardiomyocytes, we found that aldosterone exposure mediates
452 TRPC upregulation^{30,31}. As aldosterone contributes to PAH pathogenesis in humans³² and experimental
453 PH³³, we propose that aldosterone overproduction could enhance TRPC channels expression in PAH.
454 An additional mechanism can explain the overexpression/overactivity of TRPC6 in PAH. We recently
455 observed a reduced expression of the chloride and bicarbonate transporter CFTR (cystic fibrosis
456 transmembrane regulator) in PAH-hPASMCs¹⁸, which is known to be functionally and physically
457 coupled to TRPC6^{34,35}. In human airway epithelial cells, the loss of CFTR enhances TRPC6 expression
458 and function³⁴, observed in mice pulmonary vasculature contributing to hypoxic vasoconstriction³⁵,
459 suggesting overexpression/overactivity of TRPC6 in PAH could be related to CFTR loss in PAH-
460 hPASMCs.

461 To be fully functional, TRPC channels can form homo-tetrameric channels or hetero-tetrameric channels
462 like TRPC1/3, TRPC1/4, TRPC1/5, TRPC3/4, TRPC4/5, and TRPC1/4/5³. The inhibition of TRPC
463 channels by pharmacological or knockdown approaches indicates that TRPC1, TRPC4, and TRPC5
464 participate in SOCE in several cell types³⁶. However, TRPC channels activation mainly occurs following
465 the activation of Gq-coupled receptor and tyrosine kinases receptor, coupled to phospholipase C (PLC)
466 *via* inositol trisphosphate IP₃-dependent Ca²⁺ release from the endoplasmic reticulum or through DAG
467 (diacylglycerol) synthesis. Ca²⁺ could also indirectly regulate TRPC channel function by activating
468 calmodulin and other Ca²⁺-binding proteins or phosphatidylinositol-4,5-bisphosphate (PIP₂). Protein
469 kinase C (PKC) can activate TRPC3 in human endothelial cells during ER Ca²⁺ depletion induced by
470 thapsigargin stimulation³⁷. STIM1 interacts (directly or indirectly) with TRPC channels (except
471 TRPC7), conferring to TRPC a SOCE function^{38,39}. It has also been described that TRPC1 function
472 could be dependent on the Orai1-mediated Ca²⁺ entry, permitting the recruitment of TRPC1 into the
473 plasma membrane⁴⁰. We found that STIM1 and Orai1 expressions were unaffected by the knockdown
474 of TRPC. However, we do not entirely exclude that reduced TRPC expression alters STIM1/Orai1
475 clustering.

476 In the context of PAH, where several growth factors (Transforming growth factor beta, Platelet-derived
477 growth factor, Epidermal Growth Factor) or proangiogenic molecules (Endothelin1, Thromboxane A2)
478 are known to be overexpressed, we could hypothesize that all cited molecules could activate TRPC
479 channels by inducing endoplasmic reticulum SR Ca²⁺ depletion, DAG production, PKC activation,
480 generating intracellular Ca²⁺ elevation. Here we found that all expressed TRPC in hPASMCs are
481 involved in SOCE of PAH-hPASMCs. In contrast, only TRPC1 and TRPC4 are involved in SOCE of
482 control-hPASMCs suggesting dysregulation of a protein complex or activation mechanisms, which need
483 to be elucidated in further study.

484 In rat models, TRPC1 overexpression has been demonstrated to enhance PA vasoconstriction⁴¹,
485 suggesting that TRPC1-mediated SOCE regulates PASMC contraction. In rat aortic smooth muscle
486 cells, TRPC1 is complexed to the KCa channel (Ca²⁺-sensitive K⁺ channels), promoting TRPC1 Ca²⁺
487 entry and then membrane hyperpolarisation. Plasma membrane hyperpolarisation mediated by the
488 TRPC1-KCa complex could limit smooth muscle cell contraction by reducing membrane
489 depolarization⁴². *Trpc6* deficiency in mice impaired the contractile capacity of mesangial cells⁴³. TRPC3
490 inhibitor application mediates mesenteric artery vasodilatation, suggesting that TRPC3 function
491 contributes to mesenteric constriction regulation⁴⁴. Here, we found that selective TRPC3
492 pharmacological inhibition reduced the contractile capacity mediated by thromboxane A2, suggesting
493 that TRPC3 exacerbated function in PAH-PASMCs could contribute to PA vasoconstriction.

494 Even though PAH patients died due to RV failure, RV failure is understudied compared to left
495 ventricular failure (LVF). Nevertheless, many studies demonstrated that TRPC channels are crucial for
496 Ca²⁺ mishandling in LVF⁴⁵. Indeed, a considerable amount of knowledge obtained in experimental
497 models demonstrated the crucial role of each TRPC isoform in the development of LV hypertrophy
498 (LVH) or LVF⁴⁵. In humans, failing hearts increase TRPC1, TRPC5, and TRPC6 expression⁴⁵,
499 suggesting that TRPC channels are also involved in human heart failure. In most cases, TRPC
500 upregulation leads to increasing SOCE that activates the calcineurin/NFAT pathway and/or Calmodulin
501 Kinase II pathway or the NF-κB pathway influencing ventricular remodeling⁴⁶. Selective
502 pharmacological inhibition of TRPC3 and TRPC6 has been proposed to attenuate LVH and improve LV
503 function induced by pressure overload in mice^{19,47}. In the RV, only a few studies investigated the
504 implication of SOCE in RV remodeling. We found a significant reduction of CO in MCT-DMSO rats,

505 indicating that these animals developed heart failure. We showed an improvement of CO after TRPC3
506 selective inhibition, explained by the reduction of pulmonary vascular narrowing and direct action on
507 the TRPC3 channels expressed in RV cardiomyocytes⁴⁸. In RVF induced by MCT in rats, the mRNA
508 level of *Trpc1* decreased, while the mRNA level of *Trpc6* increased⁴⁹. At the protein level, we found an
509 upregulation of TRPC1/TRPC4 expression and of the long STIM1 in RVF induced by MCT exposure
510 in rats correlated with an increased SOC current, contributing to the RV remodeling during hypertrophic
511 stress⁴⁸. In this previous study, TRPC3 protein expression was unchanged in RV from MCT-PH rats⁴⁸.
512 In the present study, we found that *in vivo* inhibition of TRPC3 improved RV hypertrophy and systolic
513 pressure in MCT-PH rat models, explained by a reduction in pulmonary vascular remodeling and, to
514 some extent, by the direct action of TRPC3 inhibition on RV myocyte function.

515 By preventing pulmonary vessel remodeling induced by CH exposure, double *trpc1/trpc6* knockout
516 mice are protected against PH⁵⁰, indicating that TRPC1 and TRPC6 are essential for CH-induced PH in
517 mice. Moreover, *in vivo*, administration of siRNA against TRPC1 in CH-exposed mice reduces
518 pulmonary arterial remodeling and RV systolic pressure⁵¹. Furthermore, *trpc1*^{-/-} mice developed partial
519 protection against PH in response to CH compared to wild-type mice⁵². *Trpc4*^{-/-} rats exposed to
520 Sugen/hypoxia exhibited similar hemodynamic parameters to wild-type rats, but *Trpc4*^{-/-} rats showed a
521 significant survival benefit, preserving cardiac output⁵³. *In vivo* TRPC6 inhibition with BI-749237
522 compound reduced PH phenotype in mice exposed to chronic hypoxia⁵⁴, suggesting that TRPC6 could
523 also be a potential target in PAH. Using the siRNA strategy, we found that TRPC3 inhibition reduced
524 several pathological phenotypes of PAH-hPASMCs *in vitro*, including aberrant proliferation,
525 exacerbated migration, and apoptosis resistance. Finally, our present results revealed that *in vivo*
526 “curative” inhibition of TRPC3 reduced the development of PH in rats MCT-PH model. These data
527 highlight that targeting the TRPC3 function could be a promising strategy for treating PAH.

528

529 Indeed, this study has some limitations. First, Pyr3 has been described to selectively and directly inhibit
530 TRPC3 channels. Since the IC₅₀ of Pyr3 for TRPC3 is 700 nmol/L, we used in the present study PYR3
531 at 1 μmol/L for our *in vivo* experiments to avoid potential side effects. Indeed, it was documented that
532 at higher concentration (3 μmol/L), Pyr3 can also inhibit TRPC6¹⁹. At 3 μmol/L, Pyr3 was also
533 suggested to reduce Orai1 Ca²⁺ channel function⁵⁵. Since Orai1 or TRPC6 inhibition reduces

534 experimental PH^{4,54}, we cannot discriminate whether Pyr3 *in vivo* exposure protects rats from PH
535 through the Orai1 and/or TRPC6 function. However, we found that Pyr3 at 1 μmol/L has similar effects
536 on SOCE in PAH-hPASMCs compared with siRNA directed against TRPC3 in transfected PAH-
537 hPASMCs. Moreover, despite significant evolution in the selectivity of pharmacological molecules
538 targeting TRPC, we were limited in using a selective inhibitor of each TRPC isoform since we found in
539 the present study that all TRPC isoforms could be an interesting target to reduce PAH-hPASMCs
540 dysfunctions.

541

542 In conclusion, our findings prove that all TRPC isoforms contribute to PAH pathogenesis by regulating
543 the PAH-PASMC phenotypes, such as hyper-proliferation, apoptosis resistance, excessive migration,
544 and contraction. Our data reveal that pharmacological inhibition of TRPC3 may be a relevant approach
545 to decrease pulmonary vascular remodeling in PAH.

546

547

548 **DATA AVAILABILITY**

549 The authors declare that all supporting data are available within the article and its Online Data
550 Supplement.

551

552 **SUPPLEMENTAL DATA**

553 **All Supplemental Material is available [10.6084/m9.figshare.23145740](https://doi.org/10.6084/m9.figshare.23145740).**

554 Supplemental Table 1

555 Supplemental Table 2

556 Supplemental Table 3

557 Supplemental Fig. S1

558 Supplemental Fig. S2

559 Supplemental Fig. S3

560 Supplemental Fig. S4

561 Supplemental Fig. S5

562

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572

573 **DISCLOSURES**

574 MH and DM have relationships with drug companies, including Actelion, Bayer, GSK, Novartis, and
575 Pfizer. In addition to being investigators in trials involving these companies, other relationships include
576 consultancy services and memberships to scientific advisory boards. The other authors have no conflicts
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578

579 **AUTHOR CONTRIBUTIONS**

580 B.M, A.S-M-W, V.C, and F.A. participated in the research design. B.M, A.S-M-W, M.D, L.P, C. E-J,
581 V.C, and F.A. conducted the experiments and analysed the data. All authors drafted the manuscript for
582 important intellectual content the manuscript. All authors reviewed and revised the final version and
583 approved the manuscript submission.

584

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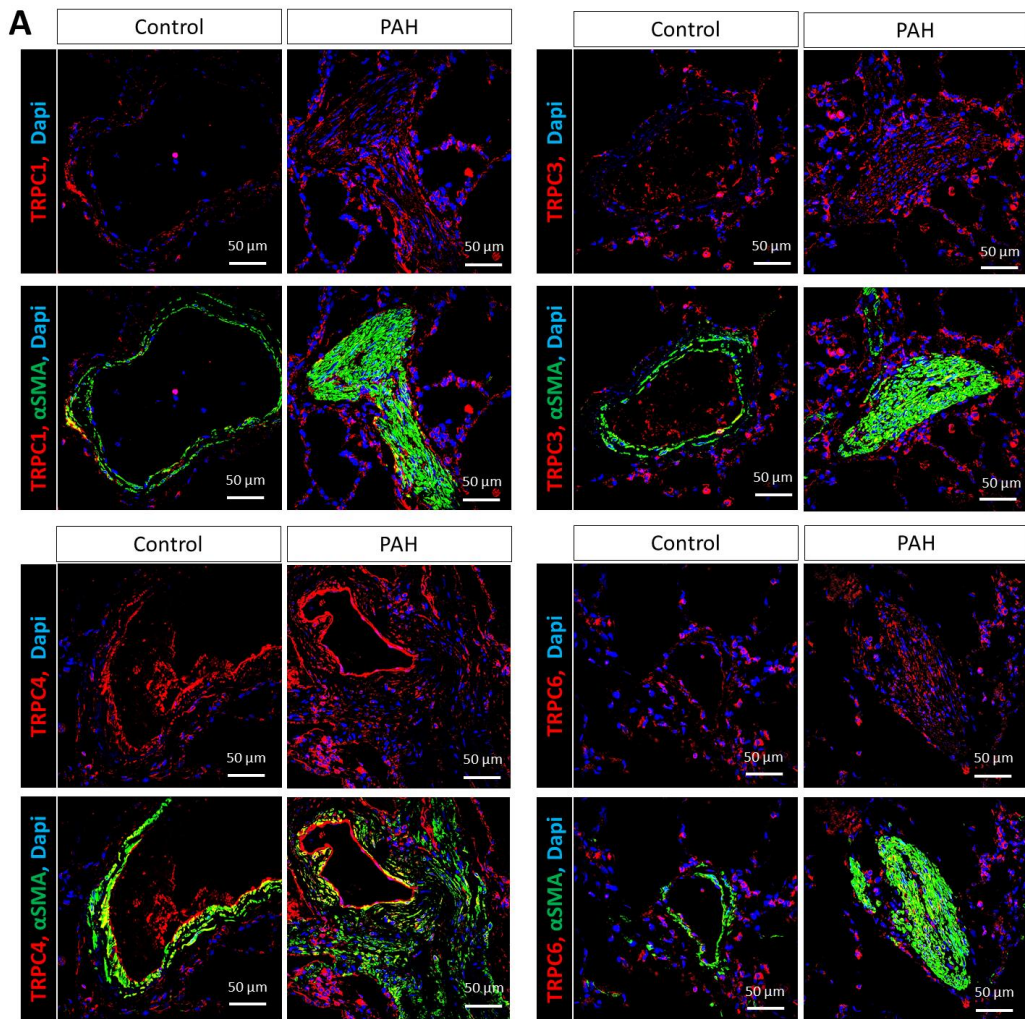
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B *Human pulmonary arteries*

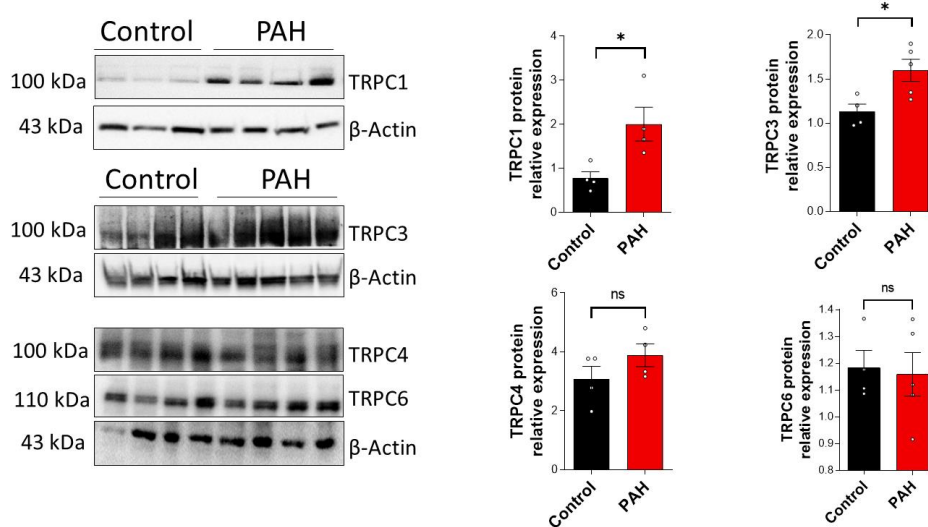


Figure 1

772 **Figure 1: Increased expression of TRPC1 and TRPC3 in pulmonary arteries (PA) isolated from**
773 **patients with pulmonary arterial hypertension (PAH).** (A) Expression and localization of TRPC1,
774 TRPC3, TRPC4, and TRPC6 by immunofluorescence staining on paraffin-embedded lung sections from
775 control and PAH patients (Dapi staining (blue), TRPC staining (red) and α SMA staining (green)). Bar
776 graph = 50 μ m. (B) Immunoblot and quantification of TRPC1 (n=4 patients), TRPC3 (n=5 patients),
777 TRPC4 (n=4 patients), or TRPC6 (n=5 patients) in pulmonary arteries from control and PAH patients.
778 Experiments presented in panel B were analysed using the Mann-Whitney test. ns = non-significant;
779 * P < 0.05.

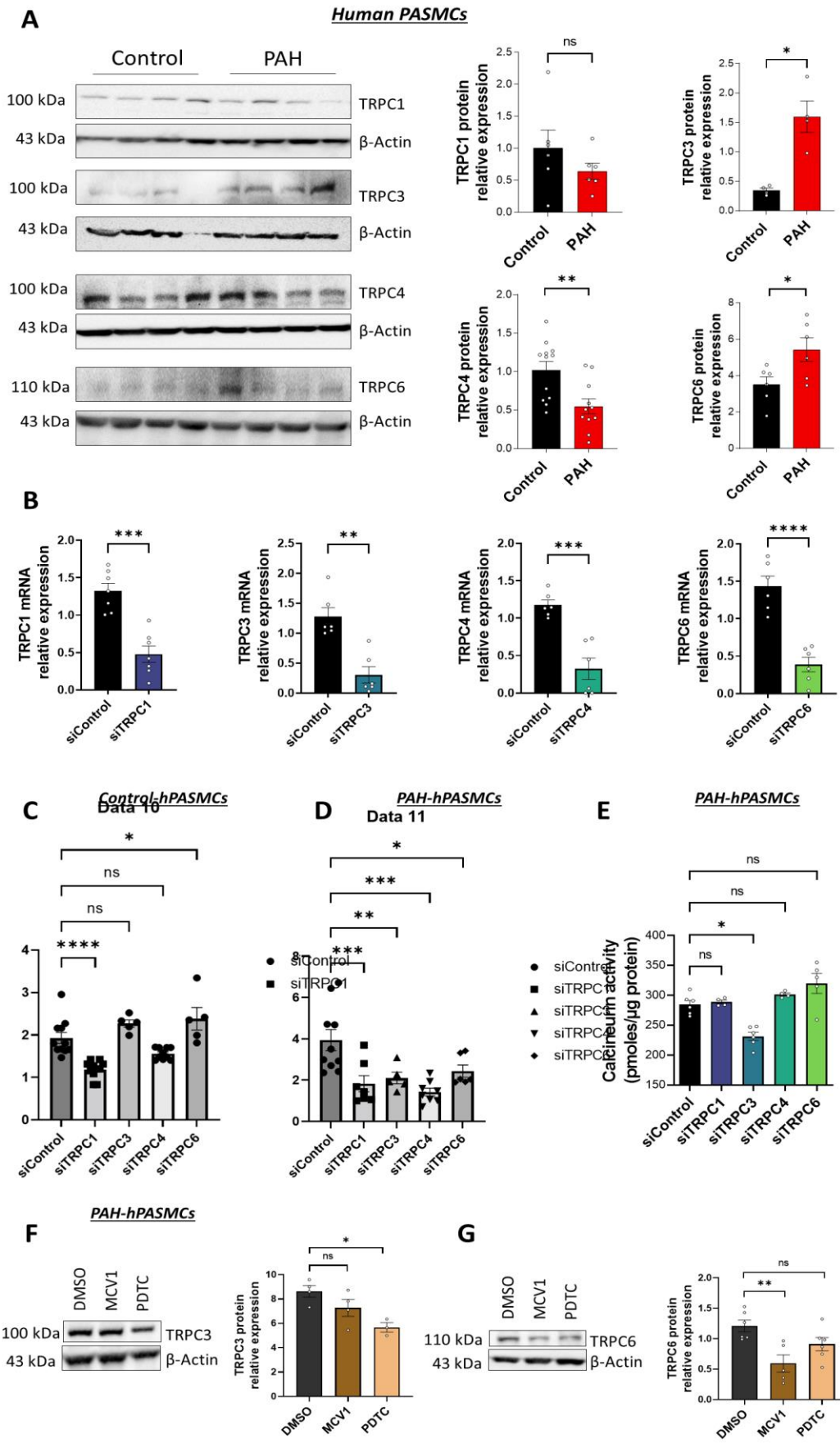


Figure 2

781 **Figure 2: TRPC channels are associated with exacerbated store-operated Ca²⁺ entry (SOCE) in**
782 **pulmonary arterial hypertension (PAH)-human pulmonary arterial smooth muscle cells**
783 **(hPASCs).** (A) Immunoblot and quantification of TRPC1 (n=6 patients), TRPC3 (n=4 patients),
784 TRPC4 (n=11 patients), or TRPC6 (n=6 patients) in PAH-hPASCs. (B) PAH-hPASCs were
785 transfected with siRNA against TRPC1 or TRPC3 or TRPC4, or TRPC6, and knockdown efficiency
786 was assessed by RT-qPCR (n = 6 for each condition). (C) Consequence of TRPC1 or TRPC3 or TRPC4,
787 or TRPC6 knockdown on their counterpart protein expression (n = 4-6 for each condition). (D)
788 Consequence of TRPC1, TRPC3, TRPC4, or TRPC6 knockdown on SOCE amplitude in control-
789 hPASCs (n=9 patients with 173 cells for siControl, n=9 patients with 134 cells for siTRPC1, n=4
790 patients with 85 cells for siTRPC3, n=6 patients with 137 cells for siTRPC4, n=4 patients with 69 cells
791 for siTRPC6). (E) Consequence of TRPC1, TRPC3, TRPC4 or TRPC6 knockdown on SOCE amplitude
792 in PAH-hPASCs. (n=8 patients with 196 cells for siControl, n=4 patients with 72 cells for siTRPC1,
793 n=4 patients with 79 cells for siTRPC3, n=6 patients with 96 cells for siTRPC4, n=8 patients with 153
794 cells for siTRPC6). (F) Consequence of TRPC1, TRPC3, TRPC4 or TRPC6 knockdown on calcineurin
795 activity in PAH-hPASCs (n=6 patients for siControl, n=4 for siTRPC1 and TRPC4, n=5 for siTRPC3
796 and TRPC6). (G) Immunoblot and quantification of TRPC3 in PAH-PASCs treated 48h with NFAT
797 inhibitor (MCV1, 1 µmol/L) and NFκB inhibitor (PDTC, 1 µmol/L) (n=4 for DMSO and MCV1 and
798 n=3 for PDTC). (H) Immunoblot and quantification of TRPC6 in PAH-PASCs treated 48 h with
799 NFAT inhibitor (MCV1 1 µmol/L) and NFκB inhibitor (PDTC, 1 µmol/L) (n=6 for DMSO and PDTC
800 and n=5 for MCV1). Experiments presented in panels A-C were analysed using an unpaired t-test or
801 Mann-Whitney test, and experiments presented in panels D-H- were analysed using the Kurskal-Wallis
802 test. ns = non-significant; **P*< 0.05, ***P*< 0.01, ****P*<0.001, *****P*<0.0001.

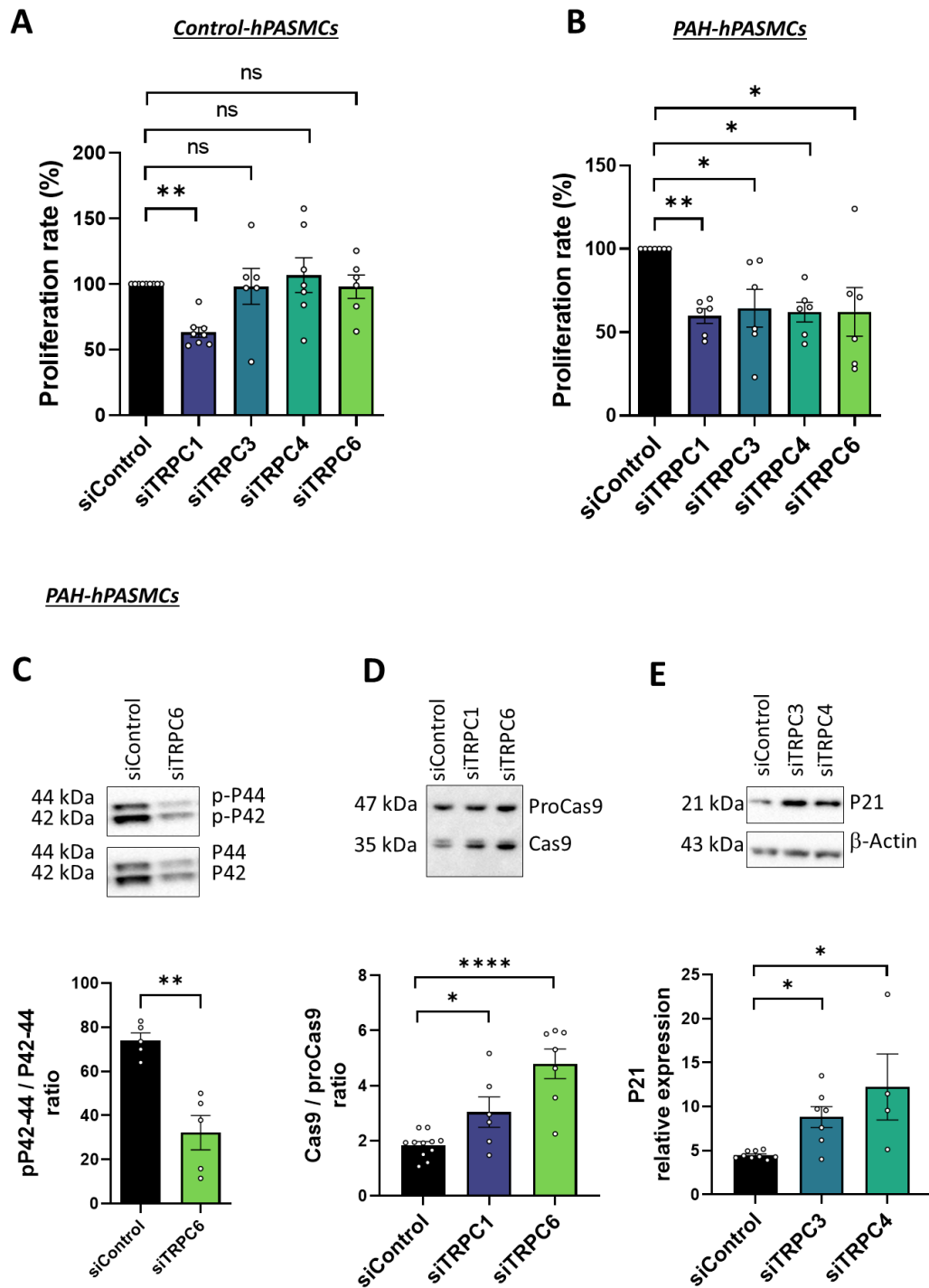


Figure 3

803

804 **Figure 3: TRPC channels are associated with increased proliferation of PAH-hPASMCs.** (A)

805 Consequence of TRPC1, TRPC3, TRPC4, or TRPC6 knockdown on the proliferation rate (BrdU assay)

806 of control-hPASMCs (n=9 patient for siControl, n=8 for siTRPC1, n=6 for siTRPC3 and siTRPC6 and

807 n=7 for siTRPC4). (B) Consequence of TRPC1, TRPC3, TRPC4, or TRPC6 knockdown on the

808 proliferation rate (BrdU assay) of PAH-hPASMCs (n=7 patient for siControl, n=6 for siTRPC1,

809 siTRPC3, siTRPC4, and siTRPC6). (C) Immunoblot and quantification of the consequence of TRPC6
 810 knockdown on the pP42-44 / P42-44 ratio (n=5 patients). (D) Immunoblot and quantification of the
 811 consequence of TRPC1 or TRPC6 knockdown on the Caspase9 / proCaspase9 ratio (n=11 patients for
 812 siControl, n=6 for siTRPC1, and n=7 for siTRPC6). (E) Immunoblot and quantification of the
 813 consequence of TRPC3 or TRPC4 knockdown on P21 expression in PAH-PASMCs (n=9 patients for
 814 siControl, n=7 for siTRPC3, and n=4 for siTRPC4). Experiments presented in panels A-B, D-E were
 815 analysed with Kruskal–Wallis test, and panel C were analysed using the Mann-Whitney test. ns = non-
 816 significant; * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.

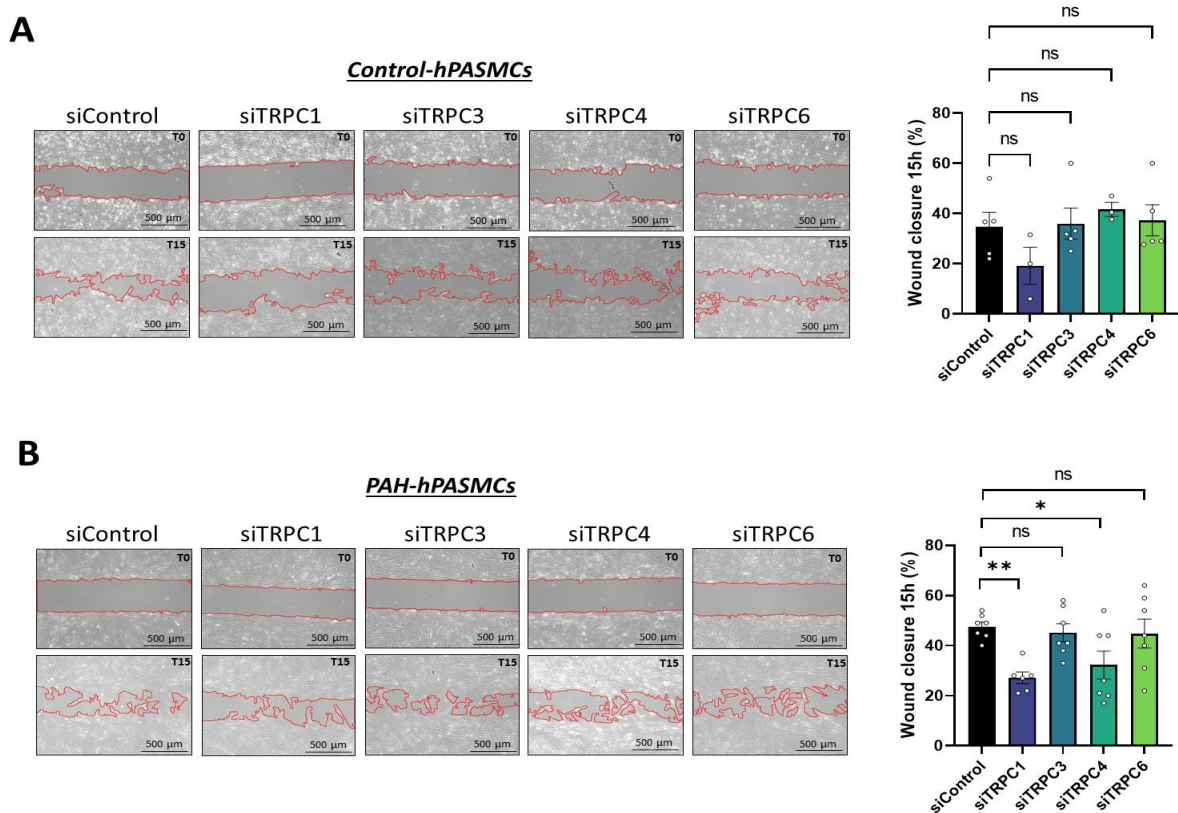


Figure 4

817
 818 **Figure 4: TRPC1 and TRPC3 channels contribute to the exacerbated migration of PAH-**
 819 **hPASMCs.** (A) Wound-closure images (15 h after initiation) and percentage wound closure of the
 820 consequence of TRPC1, TRPC3, TRPC4, or TRPC6 knockdown in control-hPASMCs (n=5 patient for
 821 siControl, siTRPC3, and siTRPC6, n=3 for siTRPC1 and siTRPC4). (B) Wound-closure images (15 h
 822 after initiation) and percentage wound closure of the consequence of TRPC1, TRPC3, TRPC4, or

823 TRPC6 knockdown in PAH-hPASCs (n=7 patient for siControl, siTRPC3, siTRPC4 and siTRPC6,
 824 n=6 for siTRPC1). Experiments presented in panel A-B with Kruskal–Wallis test. ns = non-significant;
 825 * $P < 0.05$, ** $P < 0.01$.

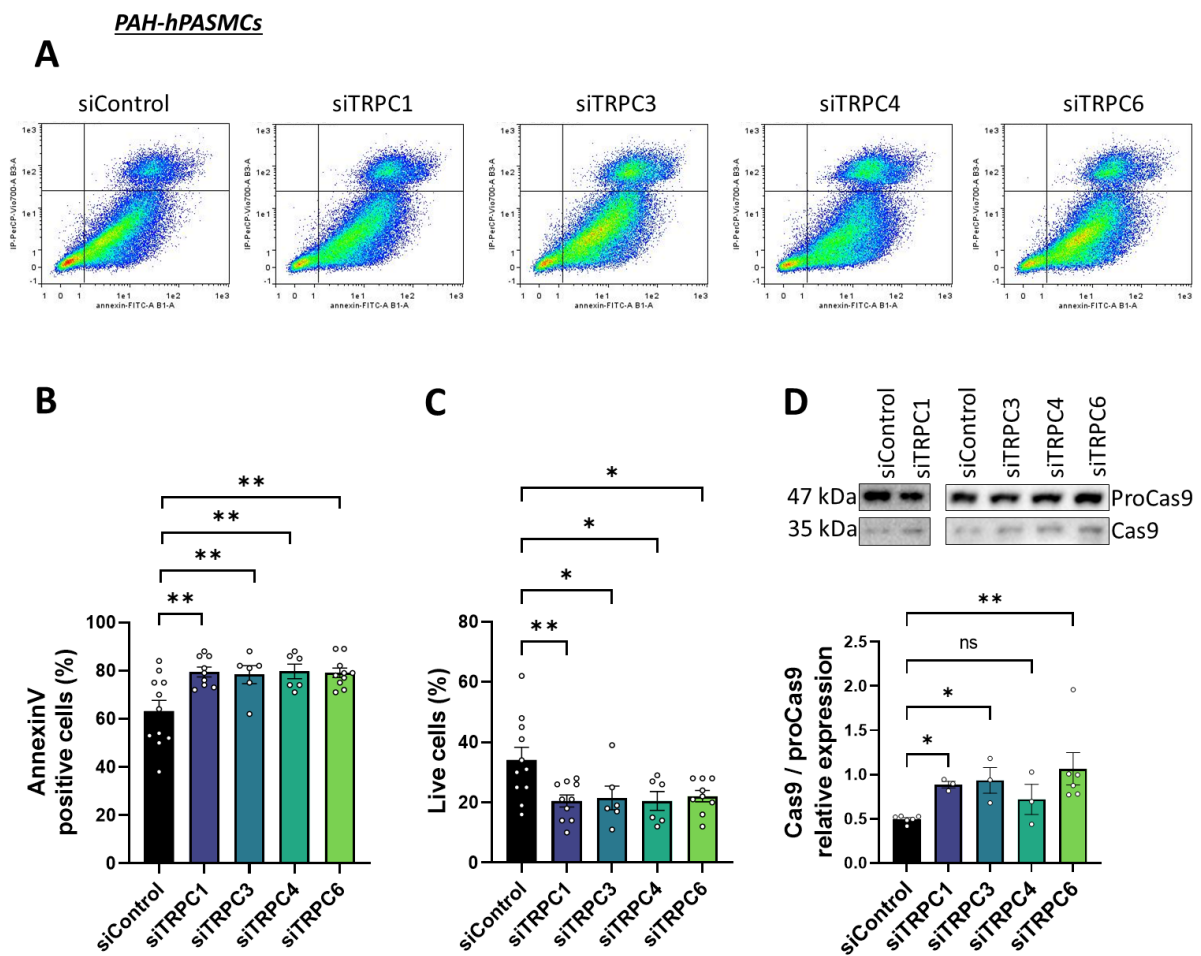


Figure 5

826

827 **Figure 5: Knockdown of TRPC channels enhances apoptosis of PAH-hPASCs exposed to**

828 **staurosporine.** (A) Dot plots of Annexin V+Propidium Iodide dual-labeling in PAH-PASCs

829 transfected with siControl, siTRPC1, TRPC3, TRPC4, or TRPC6 and exposed to 50 nmol/L

830 staurosporine overnight. (B-C) Consequence of TRPC1, TRPC3, TRPC4, or TRPC6 knockdown on the

831 percentage of Annexin V-positive and live PAH-hPASCs exposed to 50 nmol/L staurosporine

832 overnight (n=11 patient for siControl, n=9 for siTRPC1, n=6 for siTRPC3 and siTRPC4, n=10 for

833 siTRPC6). (D) Immunoblots and quantification of the consequence of TRPC1, TRPC3, TRPC4, or

834 TRPC6 knockdown on Caspase 9/proCaspase 9 ratio in PAH-PASCs exposed to 50 nmol/L

835 staurosporine overnight (n=6 patient for siControl and siTRPC6, n=3 for siTRPC1, siTRPC3, and
 836 siTRPC4). Experiments presented in panels B-D were analysed with Kruskal–Wallis test. ns = non-
 837 significant; * $P < 0.05$, ** $P < 0.01$.

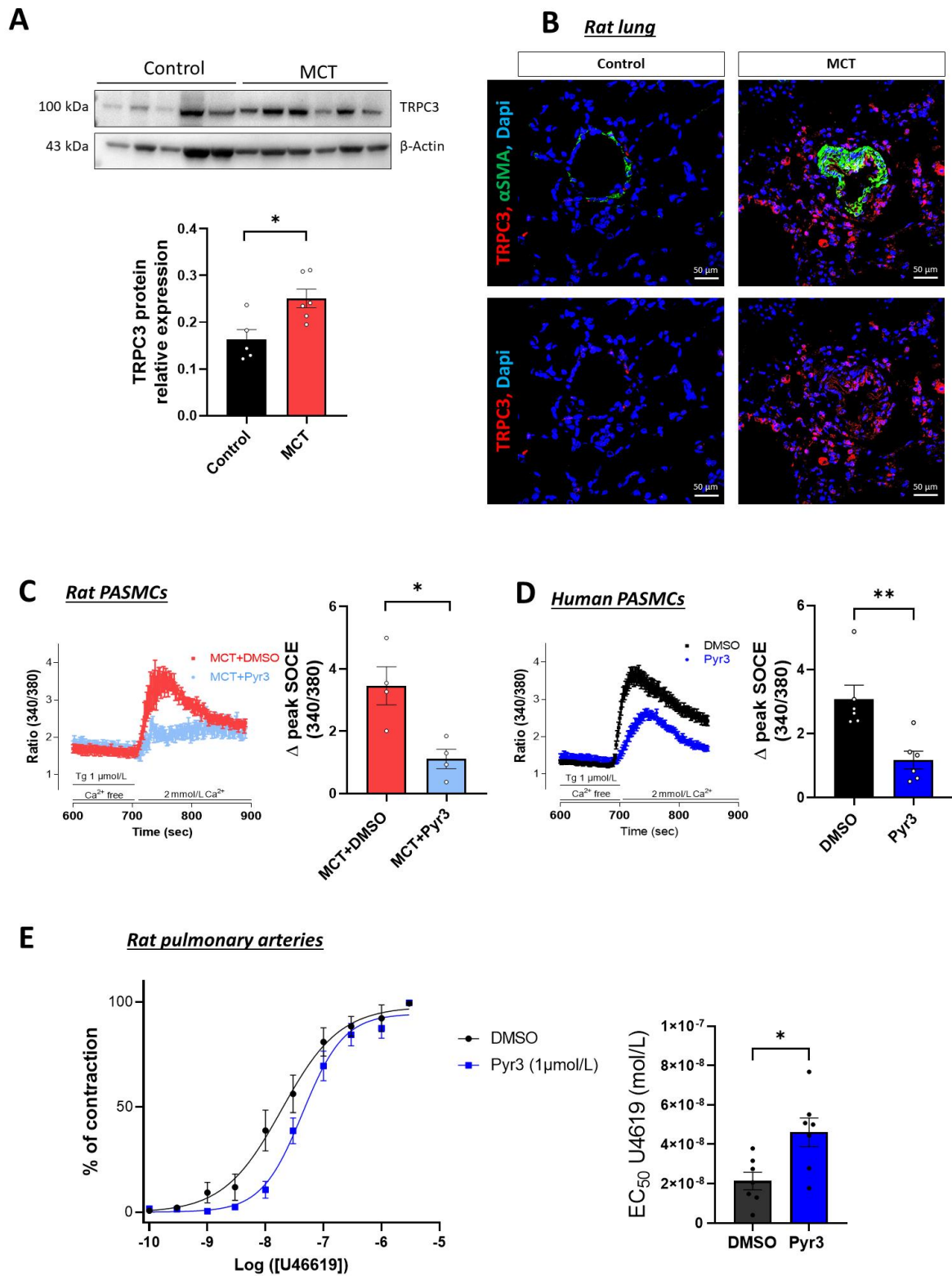


Figure 6

839 **Figure 6: TRPC3 expression is increased in experimental pulmonary hypertension (PH)-induced**
840 **by monocrotaline (MCT)-exposure in rats, and TRPC3 pharmacological blockade reduced SOCE**
841 **in MCT-PASMCs.** (A) Immunoblot and quantification of TRPC3 expression in lung tissue from control
842 and MCT rats (n=5 for control rats and n=6 for MCT rats). (B) Localization and expression of TRPC3
843 immunofluorescence staining of paraffin-embedded lung sections from control- and MCT-rats at three
844 weeks (Dapi staining (blue), TRPC3 staining (red) and α SMA staining (green)). Bar graph=50 μ m. (C)
845 SOCE and SOCE amplitude in MCT-rat PASMCs from rats treated with DMSO or Pyr3 (1 μ mol/L,
846 TRPC3 inhibitor) (n=4 rats with 45 cells for DMSO and n=4 with 46 cells for Pyr3). (D) SOCE and
847 SOCE amplitude in PAH-hPASMCs treated with DMSO or Pyr3 (1 μ mol/L) (n=6 rats with 67 cells for
848 DMSO and Pyr3). (E, left panel) Dose-response curve (normalized to K100) was established by applying
849 increasing concentrations of U46619 (a thromboxane A2 mimetic) on isolated rat pulmonary arteries in
850 the presence of DMSO or Pyr3 (1 μ mol/L). (E, right panel) Corresponding quantification of EC₅₀ values
851 (n=4 rats). Experiments presented in panels A-E were analysed using the Mann-Whitney test. ns= non-
852 significant, * P < 0.05, ** P <0.01.

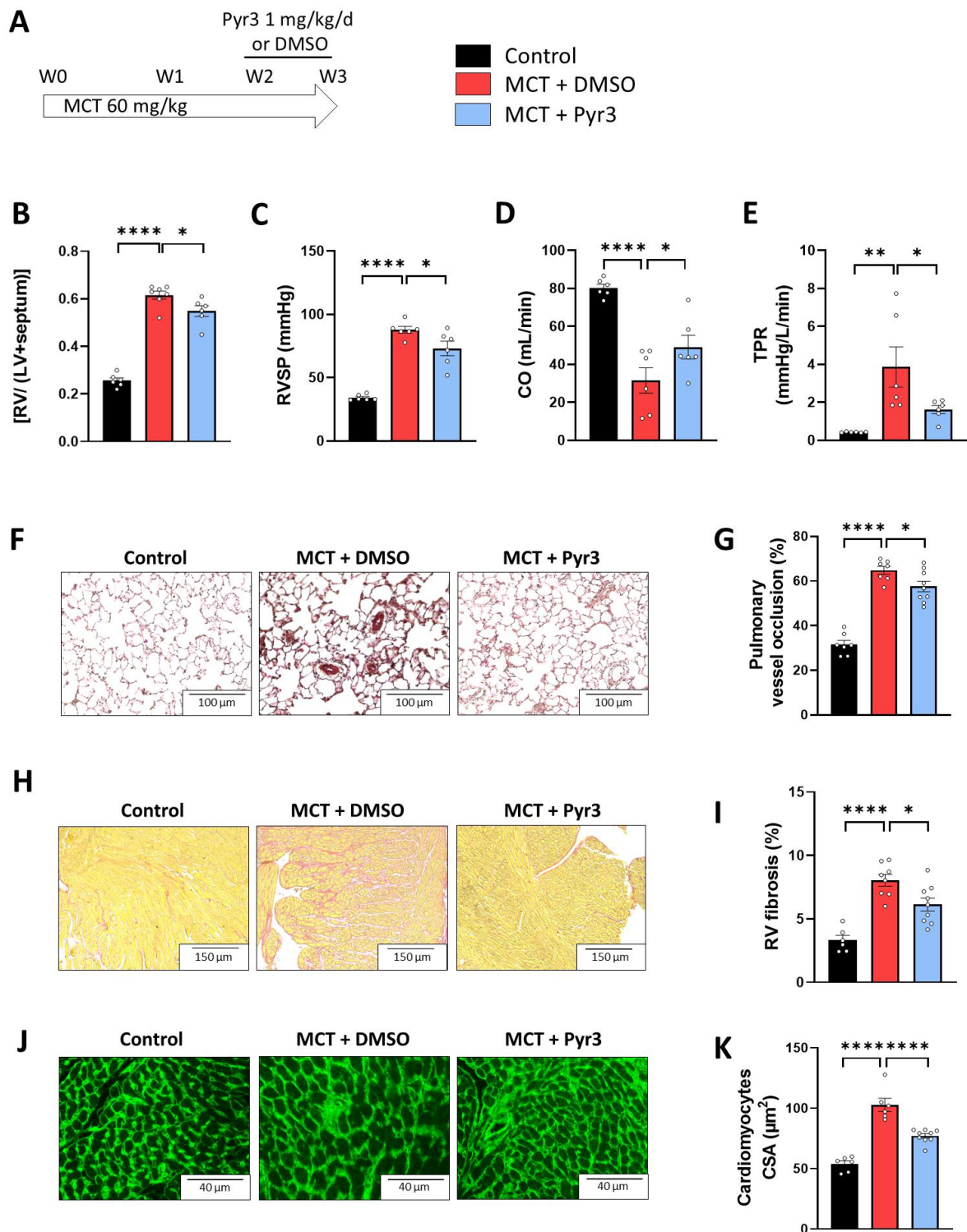


Figure 7

853

854 **Figure 7: *In vivo* pharmacological inhibition of TRPC3 reduces the development of MCT-induced**

855 **PH at pulmonary and right ventricular (RV) levels. (A) *In vivo* experimental design. Rats were**

856 **injected subcutaneously with MCT at week 0 (W0) (60 mg/kg). DMSO or Pyr3 (1 mg/kg/day) was**

857 **administered 7 days from week 2 (W2) to week 3 (W3) by intraperitoneal injection. (B) Fulton's index**

858 (RV ÷ LV + septum) (n=6 rats for Control and MCT + Pyr3 group, n=7 rats for MCT + DMSO group).
859 (C) Right ventricular systolic pressure (RVSP) in mmHg (n=6 rats). (D) Cardiac output (CO) in mL/min
860 for Control (n=6 rats), MCT + Pyr3 (n=7 rats), and MCT + DMSO (n=7 rats) group. (E) Total pulmonary
861 resistance (TPR) in mmHg/L/min (RVSP/CO) (n=6 rats). (F) Representative hematoxylin/eosin/Safran
862 staining of paraffin-embedded lung sections from the control, MCT + DMSO, and MCT + Pyr3 groups.
863 Scale bar, 100 µm. (G) Pulmonary vessel occlusion (%) in control (n=7 rats), MCT + DMSO (n=9 rats),
864 and MCT + Pyr3 (n=9 rats) groups. (H) Representative interstitial fibrosis identified with Sirius red
865 staining in RV compartment from control, MCT + DMSO, and MCT + Pyr3 groups. Scale bar, 200 µm.
866 (I) Quantification of the percentage of fibrosis in RV tissue from control (n=6 rats), MCT + DMSO (n=8
867 rats), and MCT + Pyr3 (n=9 rats) groups. (J) Representative immunofluorescence images of RV sections
868 stained with fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin (WGA, 50 µg/mL,
869 green) from the control, MCT + DMSO, and MCT + Pyr3 groups. Scale bar, 40 µm. (K) Quantification
870 of cardiomyocytes cross-section area (CSA, n=30 cardiomyocytes measurement per rat for the control
871 (n=6 rats), MCT + DMSO (n=6 rats), and MCT + Pyr3 (n=9 rats)). Experiments presented in panels B-
872 E and G, I, and K were analysed using a one-way Anova test. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$.
873