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In vitro differentiation of porcine Aortic Vascular Precursor Cells to Endothelial and Vascular Smooth Muscle cells.

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(Article begins on next page)

1 ***In vitro* differentiation of porcine Aortic Vascular Precursor Cells to Endothelial and**  
2 **Vascular Smooth Muscle cells**

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23

24 **Running Head:** Vascular Stem-like Cells from porcine aorta

25

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31 **Keywords:** Vascular Stem Cells; Mesenchymal Stromal Cells; Endothelial Differentiation; Smooth  
32 Muscle Differentiation, Pig Animal Model

33

34 **Abstract**

35 Recent findings suggested that progenitor cells and multipotent mesenchymal stromal cells (MSCs)  
36 are anatomically and functionally associated to vascular niches and proposed that cells isolated  
37 from a vessel that display mesenchymal proprieties and that are able to differentiate to the whole  
38 component of a functional blood vessel (including endothelial and smooth muscle cells), can be  
39 defined Vascular Stem Cells (VSCs).

40 We recently isolated and characterized a population of MSC-like cells from porcine aorta (porcine  
41 Aortic Vascular Precursor Cells pAVPCs) with MSCs and pericyte-like proprieties *in vitro*. The  
42 main aim of the present work was to investigate whether pAVPCs possess VSC-like properties  
43 assessing their differentiation potential toward the endothelial and the smooth muscle lineages.  
44 pAVPCs have been isolated as previously described by us and cultured in in a medium specified for  
45 pericyte to maintain stemness and multipotency. In order to assess if pAVPCs differentiate to  
46 smooth muscle cells, pAVPCs were grown for 21 days in high glucose – DMEM + 10% Foetal  
47 Bovine Serum (DMEM + 10% FBS) (Long Term Medium – LTM), whilst in order to assess if cells  
48 are able to differentiate toward the endothelial phenotype cells were seeded in a 24 well plate (5000  
49 cells/cm<sup>2</sup>) and cultured for 21 days in Endothelial Differentiation Medium (EDM) (human  
50 Endothelial Serum Free Medium supplemented with 5% FBS and 50 ng/mL of human recombinant  
51 Vascular Endothelial Growth Factor [hVEGF]).

52 After 21 days of culture in LTM, pAVPCs showed an elongated fibroblast-like shape and seems to  
53 organize in cord-like structures. Quantification of smooth muscle cells markers showed a  
54 significant increment of the three transcripts ( $\alpha$ SMA, CNN1, SMM-hc) and immunofluorescence  
55 confirmed the presence of  $\alpha$ SMA and SMM proteins. pAVPCs cultured in EDM displayed an  
56 endothelial cell-like morphology and revealed the over-expression of the typical endothelial  
57 markers: CD31, VE-Cadherin, vWF and eNOS. Immunofluorescence of CD31 confirmed the  
58 presence and the typical distribution of this protein. In conclusion we can be defined pAVPCs as a

59 population of VSC-like cells considering that they express markers of MSCs, display the classical  
60 MSC trilineage differentiation and differentiate, *in vitro*, toward smooth muscle and endothelial  
61 cells phenotypes.

62

## 63 **Introduction**

64 Recent findings and theories suggested that tissue-specific progenitor cells and multipotent  
65 mesenchymal stromal cells (MSCs) are anatomically and functionally associated to  
66 perivascular/vascular niches (11, 38, 40-43, 52, 53). According to these theories, blood vessels  
67 distributed in the whole body, could be considered a systemic reservoir of multipotent  
68 stem/progenitor cells (5, 44). In a recent review, Lin and Lue (29) proposed that cells isolated from  
69 a vessel that display MSC proprieties and that are able to differentiate to the whole component of a  
70 functional blood vessel (including endothelial and smooth muscle cells), can be defined vascular  
71 stem cells (VSCs) (29).

72 The presence of VSCs has been demonstrated in different type of vessels including embryonic, fetal  
73 and adult aorta. Undifferentiated mesenchymal cells from human fetal aorta has been isolated by  
74 Invernici and colleagues (22, 23). The expression of endothelial and myogenic markers has been  
75 demonstrated for these cells which, if opportunely stimulated with VEGF or PDGFB, were able to  
76 give rise, respectively, to endothelial and mural cells. Other populations of progenitor cells have  
77 been isolated from human fetal aorta; in particular, Fang and colleagues (14) described a population  
78 of CD105, VEGF Receptor 2 (VEGFR2, also known as Flk1) positive and CD34 negative vascular  
79 cells which were able to differentiate to endothelial and vascular smooth muscle cells, if cultured,  
80 respectively, with VEGF or PDGFB, and to the osteogenic and the adipogenic lineages. Both cells  
81 isolated by Invernici and colleagues and by Fang and colleagues displayed high angiogenic  
82 potential *in vivo*, too (13, 22, 23).

83 Adult aorta is a good source of vascular mesenchymal stromal cells, too, as reported by Pasquinelli  
84 and colleagues (35) which isolated from tunica media and adventitia, respectively, CD34 positive  
85 and c-kit positive progenitor cells. Both cell populations were positive for the expression of MSCs  
86 markers *in vitro* and, in particular, a VEGF treatment induced a phenotypical shift to the endothelial  
87 lineage with the upregulation of the expression of Flk1 and von Willebrand Factor (vWF).

88 Moreover, these cells were able to form capillary like structure in an *in vitro* angiogenesis assay  
89 (35).

90 Although the high potential of MSCs and precursor cells from vasculature in field of regenerative  
91 medicine (6, 10, 12, 24), several paper described these cells as potentially involved in pathogenesis  
92 of different diseases (20). Using a Knock Out (KO) mouse model of Apolipoprotein E (ApoE), Hu  
93 and colleagues showed that adventitial progenitor cells contributed to atherosclerotic lesion in vein  
94 grafts (21). Instead, in 2010, Juchem and colleagues described an high pro-thrombogenic potential  
95 of intimal pericytes in endothelial-denuded vascular region which support the hypothesis that these  
96 cells are involved in the pathogenesis of atherosclerosis, thrombosis and saphenous vein graft  
97 disease (26). Moreover, in 2012, in a well performed work by Tang and colleagues, it has been  
98 described a population of stem cells resident in the blood vessel wall, named multipotent vascular  
99 stem cells, that were able to differentiate to a MSC-like phenotype and subsequently to the smooth  
100 muscle one, spontaneously. In that work it has been also shown that in response of vascular injuries  
101 these cells become proliferative and differentiate into smooth muscle and chondrogenic cells  
102 contributing to vascular remodeling and neointimal hyperplasia (42).

103 Considering the potential of perivascular cells in field of regenerative medicine (10) and  
104 cardiovascular physio-pathology (20) and the usefulness of pig as animal model in these fields (15,  
105 17, 27, 39, 46, 50, 51), we recently isolated and characterized a population of MSC-like cells from  
106 porcine aorta. We named these cells porcine Aortic Vascular Precursor Cells (pAVPCs) for their  
107 differentiation potential and for their pericyte-like proprieties *in vitro* (49).

108 Despite pAVPCs has been properly characterized for their morphological, phenotypical and  
109 functional properties as pericytes-like cells (49), we observed (unpublished data) that they tend to  
110 lose their trilineage multipotency and to undergo senescence during *in vitro* culture.

111 Therefore in the present work we decided to modify the previous protocol (49) and we cultured  
112 pAVPCs in a medium specified for pericyte (Pericyte Growth Medium) and we verified in this new  
113 culture condition MSC-like and pericyte-like properties.

114 The main aim of the present work was to investigate whether pAVPCs cultured in Pericytes Growth  
115 Medium possess VSC-like properties assessing their differentiation potential toward the endothelial  
116 and the smooth muscle lineages in order to define these cells Vascular Stem Cells

117

## 118 **Material and methods**

119

### 120 *Cells isolation and culture*

121 pAVPCs have been isolated as previously described by us (49) from 3-mo-old pigs, euthanized for  
122 other experimental purposes, to generate three primary cell culture replicates. In line with the  
123 reduction rule, an animal-sharing approach was used; the aorta donor animals were the controls of  
124 an experimental trial conducted according to relevant Italian and international guidelines. All  
125 procedures on pigs were reviewed and approved in advance by the Ethics Committee of the  
126 University of Bologna (Bologna, Italy) and were then approved by the Italian Ministry of Health.

127 Briefly, the cells were isolated, from the media layer of the aortas through a collagenase IA  
128 digestion. The cells were cultured overnight (15-16 h) in high glucose (hg) DMEM (GIBCO – Life  
129 Technology Corporation, Carlsbad, CA, USA) to which 10% FBS and 10X antibiotic-antimycotic  
130 (hgDMEM-10X) were added in a 5% CO<sub>2</sub> incubator at 38.5°C. The culture medium was then  
131 replaced with hgDMEM + 10% FBS (GIBCO) + 1X antibiotic-antimycotic (GIBCO) (hgDMEM-  
132 1X).

133 After 3 days of culture with hgDMEM-1X medium, the cells were serum starved overnight (24 h)  
134 with hgDMEM + 1X antibiotic-antimycotic. After serum starvation the cells were cultured in  
135 hgDMEM:M199 (GIBCO) (1:1) to which 10% FBS and 1X antibiotic-antimycotic (DM medium)  
136 were added until 60-65% confluency was reached. The cells were trypsinized and cultured to  
137 passage (P) 6 in Pericytes Growth Medium (PGM – Promocell, Heidelberg, Germany). The cells  
138 were expanded to the further passage when a 60-65% confluency was reached. Cell doubling and  
139 doubling time between passages were calculated as previously described (49).

140

#### 141 *Transcriptional Characterization of pAVPCs cultured in PGM*

142 Cultured cells at P3 were transcriptionally analyzed through qPCR for mesenchymal stromal cells  
143 (MSCs) (CD105, CD90, CD73, CD56, CD106), pericytes (neural/glial antigen 2 [NG2], Nestin,  
144 platelet derived growth factor (PDGF) receptor  $\beta$  [PDGFR  $\beta$ ], CD146,  $\alpha$ -smooth muscle myosin  
145 [ $\alpha$ SMA]), hemopoietic (CD45, CD34) markers and for the gene expression of receptor and growth  
146 factor as vascular endothelial growth factor (VEGF) receptor 1 (VEGFR1, also known as Flt1),  
147 VEGFR2 (also known as Flk1), VEGF and PDGFB.

148 Total RNA was extracted from  $2 \times 10^6$  cells using a NucleoSpin RNA kit (Macherey Nagel, Düren,  
149 Germany) following the manufacturer's instructions. The extracted RNA was quantified using a  
150 GeneQuant 1300 (GE Healthcare, Pittsburgh, PA) spectrophotometer, and an A260/A280 ratio was  
151 used to evaluate RNA extraction quality. One microgram of RNA was retrotranscribed using an  
152 iScript cDNA synthesis kit (Bio-Rad Laboratories Inc., California, USA), following the  
153 manufacturer's instructions, in a 20 $\mu$ l final volume to obtain cDNA.

154 Primers were designed using Beacon Designer 2.07 Software (Premier Biosoft International, Palo  
155 Alto, CA). Primers sequences and size (bp) of each product are listed in Table 1.

156 Quantitative PCR was carried out using a CFX96 (Bio-Rad) thermal cycler. A master mix of the  
157 following reaction components was prepared in nuclease free water to the final concentrations  
158 indicated: 0.2  $\mu$ M forward primer, 0.2  $\mu$ M reverse primer, 1X iTaq Universal SYBR Green  
159 Supermix (Bio-Rad). One  $\mu$ l of cDNA were added to 19  $\mu$ l of the master mix. All samples were  
160 analysed in duplicate. The qPCR protocol used for the transcriptional characterization was: 10 min  
161 at 95°C, 40 cycles at 95°C for 15 s and at 61°C for 30 s, followed by a melting step from 55°C to  
162 95°C (80 cycle of 0.5°C increase/cycle).

163 The gene expression was evaluated using the  $\Delta$ Cq method (reference gene Cq – gene of interest  
164 Cq). As Cq for the reference gene, the geometric mean of Cq of three different reference genes ( $\beta$ -  
165 actin –  $\beta$ Act, Hypoxanthine phosphoribosyltransferase – HPRT, Glyceraldehyde 3-phosphate  
166 dehydrogenase – GAPDH) was considered. Reference genes primers sequences and size (bp) of  
167 each product are listed in Table 1.

168

#### 169 *Phenotypical Characterization of pAVPCs cultured in PGM*

170 Phenotypical characterization of P3 cells was carried out through immunocytochemistry and flow  
171 cytometry following the same protocols described by us (49).

172 In particular, P3 cells were analyzed through flow cytometry for the expression of CD105, CD90,  
173 CD56, CD44, CD45, CD34, CD31. The antibodies and the concentration used for the analysis are  
174 listed in Table 2.

175 Moreover, cells were analyzed by immunofluorescence experiments for the expression of PDGF $\beta$ ,  
176  $\alpha$ SMA, NG2, nestin, CD34, CD31 and smooth muscle myosin – heavy chain (SMM-hc). The  
177 primary and secondary antibodies and their concentration used for the analysis are listed in Table 3.

178

179 *Mesenchymal Trilineage Differentiation Potential*

180 Cells at P3, grown in PGM, were assessed for the mesenchymal trilineage differentiation potential.  
181 A StemPro® Adipogenesis Differentiation Kit, a StemPro® Osteogenesis Differentiation Kit and a  
182 StemPro® Chondrogenesis Differentiation Kit (all purchased from GIBCO, Life Technologies)  
183 were used following the manufacturer's instructions.

184 After 7 and 21 days for osteogenic differentiation and after 21 days for adipogenic and  
185 chondrogenic differentiation the expression of typical genes of differentiated osteocytes (Alkaline  
186 Phosphatase [ALP], Osteopontin [SSP1]), adipocytes (Peroxisome proliferator-activated receptor  $\gamma$   
187 [PPAR $\gamma$ ], Adiponectin) and chondrocytes (Aggrecan [ACAN], Collagen type II alpha 1 [COL2A1])  
188 was evaluated through qPCR. Total RNA extraction, retrotranscription and qPCR were performed  
189 as described above. Primers for the analysis were designed with Beacon Designer 2.07 Software.  
190 Primers sequences and size (bp) of each product are listed in Table 1. The expression of these genes  
191 was calculated using the  $\Delta Cq$  method (gene of interest Cq – reference gene Cq). As reference gene  
192 Cq, the geometric mean of  $\beta$ Act, HPRT and GAPDH Cq value was considered. Relative expression  
193 was calculated as fold of increase with the  $2^{-\Delta\Delta Cq}$  method ( $\Delta\Delta Cq = \Delta Cq$  differentiated cells –  $\Delta Cq$   
194 control cells).

195 After 21 days, assessment of differentiation was carried out through classical histological staining,  
196 too, (OilRedO, Alizarin Red and Alcian Blue [all purchased by Sigma-Aldrich Co.]) following the  
197 manufacturer's protocol.

198 Briefly, for chondrogenic differentiation assessment, pellets were fixed in 10% buffered formalin,  
199 routinely processed and embedded in paraffin blocks. Sections of 3  $\mu$ m thickness were obtained and  
200 mounted on polarized slide. Sections were deparaffinized with xylene, rehydrated through passages  
201 into an increasing concentration alcoholic ladder. Slides were stained with Alcian blue solution  
202 (pH2.5) for 30 minutes, washed in water and counterstained with Harris hematoxylin (Merk).

203

204 *Long term culture and assessing of smooth muscle cells differentiation potential*

205 Tang and colleagues (42) reported that long term cultured vessel derived multipotent cells  
206 spontaneously differentiate toward the smooth muscle cells phenotype. In order to verify the effect  
207 of the long term culture on pAVPCs, 1500 cells/cm<sup>2</sup> cells at P3 were seeded in a 24 well plate and  
208 cultured in DMEM + 10 % FBS + 1X antibiotic-antimycotic (Long Term Medium – LTM). Porcine  
209 Aortic Precursor Cells grown in PGM has been used as control.

210 After 21 days, cells were analyzed for the expression of differentiated smooth muscle cells markers  
211 both with qPCR ( $\alpha$ SMA, calponin [CNN1], smooth muscle myosin heavy chain [SMM-hc]) and  
212 immunofluorescence ( $\alpha$ SMA, SMM-hc).

213 Quantitative PCR analysis and immunofluorescence staining were carried out as described above.  
214 Primers for the analysis were designed with Beacon Designer 2.07 Software. Primers sequences and  
215 size (bp) of each product are listed in Table 1. Relative expression was calculated as described  
216 above. The primary and secondary antibodies and their concentration used for the analysis are listed  
217 in Table 3.

218

219 *Endothelial Differentiation Potential*

220 Cells at P3 were seeded in a 24 well plate at density of 5000 cells/cm<sup>2</sup> and cultured in human  
221 endothelial Serum Free Medium (GIBCO) supplemented with 5% FBS, 1X antibiotic-antimycotic  
222 and 50 ng/mL of human recombinant Vascular Endothelial Growth Factor (hVEGF) (Endothelial  
223 Differentiation Medium - EDM). Porcine Aortic Precursor Cells grown in PGM has been used as  
224 control. After 21 days of culture, cells were analyzed for the expression of differentiated endothelial  
225 cells markers both with qPCR (CD31, vascular endothelial [VE]-Cadherin, vWF, endothelial nitric

226 oxide synthase [eNOS]) and immunofluorescence (CD31) as described above. Primers for the  
227 analysis were designed with Beacon Designer 2.07 Software. Primers sequences and size (bp) of  
228 each product are listed in Table 1. Relative expression was calculated as described above. The  
229 primary and secondary antibody and their concentration used for the analysis are listed in Table 3.

230

### 231 *Statistical analysis*

232 Statistical analysis was carried out with R software (36). Data obtained from gene expression  
233 analysis of adipogenic, chondrogenic, smooth muscle and endothelial differentiation were analyzed  
234 using the Student-*t* test comparing differentiated and undifferentiated (control) cells, while data  
235 obtained from gene expression analysis of osteogenic differentiation were analysed using one-way  
236 ANOVA, followed by the Tuckey post-hoc comparison, to detect differences among control cells  
237 and cells differentiated for 7 and 21 days.

238

239 **Results and Discussion**

240

241 *Morphology and doubling time of pAVPCs cultured in PGM*

242 Porcine Aortic Precursor Cells *in vitro* cultured using the commercial medium Pericytes Growth  
243 Medium (Promocell) showed a small cell body with little thin and elongated arms (Fig. 1 A and B).  
244 This morphology has been already described for perivascular stem progenitor cells (8). The same  
245 cells cultured in the unspecific medium DM showed a spindle shape/fibroblast-like morphology  
246 (49) unlike if cultured in PGM. Moreover, it was not observed the growth of spheroidal structures in  
247 PGM, while in the first passages of the culture in DM it was possible observing these kind of  
248 structures (49). Doubling time has been calculated for cells cultured between P1 and P6 and  
249 increase from  $27.5 \pm 0.6$  hours, between P1 and P2, to  $44.4 \pm 10.9$  hours, between P5 and P6 (Fig. 1  
250 C). Interestingly, the growth curve obtained for cells cultured in PGM is parallel to the one obtained  
251 for the culture in DM (49) with a downward shift of about 10 hours, so cells cultured in PGM grew  
252 more rapidly than cultured in DM. Cells at P6 reached a cumulative cell doubling number of  $11.1 \pm$   
253  $1.3$ , comparable to the value ( $10.7 \pm 0.9$ ) obtained for culture in DM (49).

254

255 *Transcriptional Characterization of pAVPCs cultured in PGM*

256 Porcine Aortic Precursor Cells cultured in PGM express the main transcripts of MSCs (CD105,  
257 CD90, CD73, CD56, CD106), while do not express CD45, a marker of the hemopoietic lineage (13)  
258 (Fig 2).

259 Moreover, pAVPCs do express pericytes main transcripts (NG2, Nestin, CD146,  $\alpha$ SMA, PDGFR $\beta$ )  
260 (11) and CD34 mRNA, considered a markers of Advential Cells, Myoendothelial Cells (8) and  
261 mesangioblasts (31).

262 Transcripts of the growth factor VEGF and its main receptors (Flt-1 and Flk1) has been detected as  
263 well as for the growth factor PDGF $\beta$ , although at a lower level for the latter. Considering the  
264 vascular origin of pAVPCs, VEGF expression could be an important factor for the cross-talk with  
265 endothelial cells in physiological and pathological angiogenic processes that involve vascular  
266 precursor cells (16).

267

#### 268 *Phenotypical Characterization of pAVPCs cultured in PGM*

269 Passage 3 pAVPCs cultured in PGM has been characterized for MSC and pericytes markers  
270 expression through immunocytochemistry and flow cytometry. Flow cytometry analysis (Fig. 3)  
271 revealed that pAVPCs expressed MSC markers as CD105 ( $66.8 \pm 0.1$  %), CD90 ( $99.5 \pm 0.2$  %),  
272 CD44 ( $99.6 \pm 0.3$  %) and less than 2% of them expressed the hemopoietic lineage markers as CD45  
273 ( $1.4 \pm 0.4$  %), CD34 ( $1.3 \pm 0.1$  %), as requested by the International Society of Cell Therapy (ISCT)  
274 guide lines (13). Moreover, cells were negative for the expression of CD31( $1.5 \pm 0.1$  %). In  
275 particular, as already described for cells cultured in DM, cells expressed CD56 ( $99.9 \pm 0.1$  %) that  
276 is considered a marker of subsets of MSCs (2, 4, 37). The FACS analysis revealed the presence of a  
277 more uniform population of cells when the *in vitro* culture is performed in PGM compared to the  
278 culture in DM (49).

279 Immunocytochemical analysis revealed the expression of cell markers typical of pericytes (8, 11,  
280 19, 26) as PDGFR $\beta$ , NG2 and Nestin (respectively Figure 4 A, B, C) as already described for the  
281 culture in DM (49). A clear difference observed among cells cultured in different media was that in  
282 PGM less than 2% of cells expressed  $\alpha$ SMA (Figure 4 D), while in DM (49) 100% of cells

283 expressed it. The  $\alpha$ SMA protein is considered a functional marker of differentiated pericytes (10)  
284 and so it is presumably expressed in pre-committed cells to the fully differentiated pericytes  
285 lineage. The observation that the cells cultured in PGM lost the expression of that marker could  
286 indicate that the culture medium was able to maintain cells more undifferentiated than DM.

287 Despite the expression of CD34 mRNA, cells did not express that marker at the protein level (Fig. 4  
288 E). Moreover, CD31 (Fig. 4 F) was not expressed, too, so both these data allowed us to exclude  
289 contamination of hemopoietic and endothelial cells (10).

290 Taken together, these data suggested that pAVPCs in PGM could be phenotypically considered  
291 pericyte-like cells, as they displayed several MCS and pericytes markers (8) confirming what it has  
292 been observed in our previous work (49)

293

#### 294 *Mesenchymal Trilineage Differentiation Potential*

295 Passage 3 cells, cultured in PGM, has been cultured in adipo-, osteo-, chondrogenesis induction  
296 media in order to investigate the classical trilineage differentiation potential (Fig. 5) requested for  
297 MSCs characterization (13) as already shown for culture in DM (49).

298 Quantitative PCR analysis revealed the over-expression of transcripts typical of each one of the  
299 three lineages described above.

300 For adipogenic differentiation PPAR $\gamma$  and Adiponectin has been used as markers of differentiation  
301 (30). Transcripts of both genes has been detected significantly increased in differentiated cells  
302 compared to control cells respectively about 525 times (p *t-test* =  $6.20 \times 10^{-5}$ ) and about 45000  
303 times (p *t-test* = 0.003973) (Fig. 5 A).

304 For osteogenic differentiation ALPL and SPP1 has been used as markers of differentiation (28). In  
305 Fig. 5 D the expression of both markers is represented at 7 and 21 days of cell culture in osteogenic

306 differentiation medium. The expression of ALPL was significantly increased in differentiated cells  
307 (about 1300 times) compared to control only after 21 days of culture in osteogenic medium (p  
308 ANOVA =  $5.65 \times 10^{-5}$ ), while the expression of SPP1 was significantly increased in differentiated  
309 cells (about 17 times) compared to control after just 7 days of culture in osteogenic medium (p  
310 ANOVA = 0.00188).

311 For chondrogenic differentiation ACAN and COL2A1 has been used as markers of differentiation  
312 (48). Transcripts of both genes has been detected significantly increased in differentiated cells,  
313 compared to control cells respectively, about 585 times (p *t-test* = 0.001668) and about 11 times (p  
314 *t-test* = 0.03973) (Fig. 5 G).

315 Moreover, cells cultured with adipogenic medium for 21 days grew as monolayer showing the  
316 accumulation of lipidic droplets stained with OilRedO (Fig. 5 B, C), cells cultured with osteogenic  
317 medium for 21 days grew as aggregates and they positively stained for AlizarinRed confirming the  
318 presence of calcium deposits (Fig. 5 E, F) and cells, cultured as pellets in a chondrogenic medium  
319 for 21 days, positively stained for AlcianBlue confirming the presence of proteoglicans (Fig. 5 H,  
320 I).

321 All these data suggested that PGM was able to maintain pAVPCs undifferentiated as them  
322 displayed the classical trilineage differentiation potential that is requested for MSCs  
323 characterization (13) and that has been already showed by us for pAVPCs cultured using DM (49).  
324 Moreover, these data confirm that pAVPCs could be considered MSC-like cells.

325

326 *Long term culture and smooth muscle cells differentiation potential*

327

328 In order to evaluate whether pAVPCs spontaneously differentiate towards smooth muscle  
329 phenotype cells has been cultured in LTM and then analyzed through qPCR and ICC.

330 After 21 days of culture in LTM, pAVPCs showed an elongated fibroblast-like shape and seems to  
331 organize in cord-like structures, while pAVPCs cultured in PGM grew as multilayer maintaining  
332 their classical shape (Suppl. 1).

333 Quantification of smooth muscle cells markers through qPCR (Fig. 6 A) showed a sensible and  
334 significant increment of each of the three transcripts ( $\alpha$ SMA, CNN1, SMM-hc [47]) analysed in  
335 LTM cultured cells compared to the control one. In particular,  $\alpha$ SMA showed a significant (p *t-test*  
336 = 0.01623) increment of about 12 times, while CNN1 showed a significant (p *t-test* = 0.0314)  
337 increment of about 41 times and SMM-hc a significant (p *t-test* = 0.0002068) increment of about  
338 966 times.

339 The presence of  $\alpha$ SMA and SMM proteins has been investigated in long term cultured cells and the  
340 results obtained confirmed the lack of expression of both protein in control cells (Fig. 6 B, D) and  
341 the expression of both protein in LTM cultured cells (Fig. 6 C, E).

342 All these data confirm that cells cultured for long time in the same support with a standard culture  
343 medium spontaneously differentiate to the smooth muscle phenotype, without growth factor  
344 stimulation, as reported by Tang and colleagues (42) for other vascular derived multipotent cells.

345

#### 346 *Endothelial Differentiation Potential*

347 In order to assess whether pAVPCs were able to differentiate to endothelial cells, a stimulation with  
348 50 ng/ $\mu$ L VEGF has been performed in a culture medium specific for endothelial cells culture. After  
349 21 days of treatment, VEGF cultured cells displayed an endothelial cell-like morphology growing  
350 as a monolayer upon which few spheroidal structures could be observed (Suppl. 2).

351 Gene expression analysis (Fig. 7 A) revealed the over-expression of the following typical markers  
352 of endothelial cells: CD31, VE-Cadherin, vWF and eNOS. Respectively, a significant increase of  
353 these transcripts of about 22 times ( $p$  *t-test* = 0.01756), 33 times ( $p$  *t-test* = 0.007046), 7 times ( $p$  *t-*  
354 *test* = 0.02367) and 20 times ( $p$  *t-test* = 0.0111) has been detected in VEGF cultured cells compared  
355 to control cells. CD31 expression has been investigated at the protein level, too, and the marker has  
356 been detected just in differentiated cells showing its typical distribution pattern, mainly along the  
357 cell membrane (Fig. 7 C). Control cells did not express the CD31 protein (Fig. 7 B).

358 All these data confirm that pAVPCs cultured in human endothelial Serum Free Medium (GIBCO)  
359 supplemented with Vascular Endothelial Growth Factor differentiate to the endothelial phenotype,  
360 in agreement with results obtained for multipotent cells derived from embryonic, fetal and human  
361 aorta (14, 22, 23, 31, 35) and by Pankajakshan et al (2013) that reported the differentiation of  
362 porcine bone marrow derived-MSCs to endothelial cells after stimulation with 50 ng/ $\mu$ L VEGF  
363 (34).

364

365 Mesenchymal stromal cells in which depletion of PDGFR $\beta$  signaling occur have been reported to  
366 have an high angiogenic potential as they produced pro-angiogenic growth factor and expressed  
367 endothelial cells marker *in vitro*, while *in vivo* they potently stimulate neo-vascularization (1).  
368 Greenberg and colleagues (18) described that VEGF, activating its receptor VEGFR2, is able to  
369 suppress PDGFR $\beta$  signaling in vascular smooth muscle cells (VSMCs) through the assembly of a  
370 PDGFR $\beta$ /VEGFR2 complex.

371 Considering that pAVPCs have been defined as MSC-like cells (49), the expression on  
372 endothelial/angiogenic markers in these cells, after the VEGF stimulation, could be explained  
373 through the VEGF-mediated inhibition of the PDGFR $\beta$  signaling. That could be the first stimulus to  
374 induce the endothelial differentiation ensuring that the main receptor (PDGFR $\beta$ ) involved in

375 pericytes/VSMCs regulation and differentiation *in vivo* (18) is blocked as the pathway that  
376 underwent its activation. Indeed, further investigations are necessary to confirm this hypothesis.

377

## 378 **Conclusion**

379 In the present paper we described the ability of porcine Aortic Vascular Precursor Cells (pAVPCs)  
380 to differentiate toward the smooth muscle and the endothelial cell phenotypes. In our previous paper  
381 (49) we described a method to isolate these multipotent cells from the tunica media of pig aorta.

382 In this paper we modified the previous protocol culturing cells in a specific culture medium able to  
383 maintain pericytes multipotency (3).

384 In particular, in the present paper pAVPCs cultured in Pericytes Growth Medium has been shown to  
385 be rather pure population of cells that express the main markers of MSCs (CD105, CD90, CD73,  
386 CD44) and lack the expression of the main markers of hemopoietic stem cells (CD45 and CD34), as  
387 requested by the ISCT (13). Moreover, pAVPCs has been shown to express the main markers  
388 (PDGFR $\beta$ , NG2 and  $\alpha$ SMA) that characterized pericytes (11). In addition, pAVPCs have been  
389 shown to be able to differentiate toward osteo-, adipo- and chondrocyte phenotype.

390 All the data obtained from PGM cultured pAVPCs characterization, associated to their already  
391 proved capability to form capillary-like network if co-cultured with Human Umbilical Vein  
392 Endothelial Cells (HUVEC) on extracellular matrix (49), lead us to reinforce the definition of them  
393 as MSC/pericyte-like cells.

394 Based on the recent definition of Vascular Stem Cells (VSCs) by Lin and Lue we wanted, then, to  
395 assess whether pAVPCs could be considered a population of VSC-like cells, in particular, whether  
396 they were be able to differentiate toward the smooth muscle and the endothelial phenotypes (29).

397 The data we presented in this paper showed that pAVPCs are able to differentiate spontaneously to  
398 the smooth muscle phenotype if long term cultured in an unspecific culture medium. This  
399 spontaneous differentiation process could lead to think about these cells in a pathological fashion as  
400 it has been reported that some populations of vascular wall resident cells are responsible for several  
401 vascular pathologies (20, 21, 26) and for some of these cells the shift to the smooth muscle  
402 phenotype is a requirement for their involvement in vascular remodeling and neointimal hyperplasia  
403 (42). Moreover, in the present paper we presented data that support the endothelial differentiation of  
404 pAVCPs as after 21 days of culture in a medium supplemented with VEGF they expressed markers  
405 of endothelial cells. The endothelial differentiation of these cells, instead, could lead to think about  
406 pAVPCs in a regenerative medicine fashion. In fact, for regenerative medicine purposes it is of  
407 considerable importance the improvement of the vascular network that could be damaged in the  
408 organ that has to be regenerated (6, 12, 24).

409 Indeed, it is important to remember that cells, like the multipotent pericytes with which pAVPCs  
410 share multiple features, have been recently identified as blood vessel wall resident cells that  
411 physiologically make the vasculature a dynamic reservoir of stem/progenitor cells (5, 10, 44).

412 So, considering the recent definition of Vascular Stem Cells (VSCs) by Lin and Lue (29) and all the  
413 data we presented on pAVPCs in this paper, we conclude that they can be defined as a population of  
414 VSC-like cells considering that they express markers of MSCs, display the classical MSC trilineage  
415 differentiation and differentiate, *in vitro*, toward smooth muscle and endothelial cells phenotypes.

416 Indeed, both smooth muscle and endothelial differentiations require to be further investigated in *in*  
417 *vivo* animal models of pathologies in order to confirm the involvement of pAVPCs in vascular  
418 diseases development and/or the possible usefulness of these cells for regenerative medicine studies  
419 in porcine animal model.

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424 dell'Emilia (BO), Italy.

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580

<i>Genes</i>	<i>Accession Number</i>	<i>Forward (5'-3')</i>	<i>Reverse (5'-3')</i>	<i>Product size (bp)</i>
<i>Mesenchymal Stromal Cell markers</i>				
CD106	NM_213891	GAGGATGGAAGATTCTGGAATTTACG	ATCACTAGAGCAGGTCATGTTTAC	172
CD105	NM_214031.1	ATACAAAGGGCTCCATCATC	TGAGTGTGAGACTTCCATTC	151
CD90	NM_001146129.1	GACTGCCGCCATGAGAATAC	GGTAGTGAAGCCTGATAAGTAGAG	180
CD73	XM_003353250.1	AACTCATCGCTCAGAAGGTG	ATCGGAGGTGACTATGAATGG	131
CD56	ENSSSCT00000016411	GGAAATCAGCGTTGGAGAGTC	TGTTGGCATTGTAGATGGTGAG	172
<i>Pericytes Markers</i>				
CD146	ENSSSCT00000016482	CTCATCTGTGCCTTCTGTCTAG	CCTCCCACTTCCACCTCCAG	110
NG2	ENSSSCT00000002098	ACCACCTCCTCTACAACCTC	GTCACCTAGCAGCATCTCTG	104
PDGFRβ	ENSSSCT00000015788	GCAACGAGGTGGTCAACTTC	GCAGGATAGAACGGATGTGG	111
Nestin	ENSSSCT00000027298	CAGTGGTTCCAAGGCTTCTC	CATAGGTGTGTCAAGGGTATCG	163
<i>Hemopoietic Stem Cell Markers</i>				
CD45	XM_003482796.1	CTCACTCGCAAGCATCTCTG	CGGTTGAAGTAGCTGTGTCTG	188
CD34	NM_214086	CAGCCTCCACACTGTCTCTC	CACCTCTGAAGTTGTAAAGTTGATG	184
<i>Smooth Muscle Cell Markers</i>				
αSMA	NM_001164650	CACGGCATCATCACCACCTG	ACCGCCTGAATAGCCACATAC	200
CNN1	NM_213878	ATGTCTCTGTCTCACTTCAAC	CCTGCTGGTGGTCATACTTC	91
SMM-hc	XR_131283	AGGAGGTGACGATGCTGAAG	TGCTTGTCTTGTCCAGGTTG	160
<i>Endothelial Cell Markers</i>				
CD31	NM_213907	CTCATCGCAGTGGTTGTCATC	TGTCTTCTCATTGTGGAGTTCAG	150
VE-Cadherin	NM_001001649	ACTCCTCAGCCTCTCCTCAG	GTCAACACTCAGCACAGCATAG	111
vWF	NM_001246221	GTGGAGAGTGTCTGAGTGTTC	TGAAGGTGAAGTGTCTGTGTGTC	193
eNOS	AY266137	GCTCTCACCTTCTTCTCTG	CCACTTCCACTCCTCATAG	144
<i>Receptor and Growth Factor</i>				
Flt1	AY566244	TTGGAAGTGGTGGCACAAGAC	GCTGTTGCTCGTCAGAATGG	141
Flk1	AJ245446	AACGAGTGGAGGTGACAGATTG	CGGGTAGAAGCACTTGTAGGC	104
VEGF	AF318502	CCTTGCCTTGTCTGCTTACC	CGTCCATGAACCTTCCACTTC	101
PDGFβ	ENSSSCT00000029224	CTCTGGCTGTGCAACAACC	TGGCTTCTCCGCACAATCTC	100
<i>Adipocyte Markers</i>				
PPARγ	AF103946	AGGAAAGACCACAGACAAATCAC	CAGGGATGTTCTTGGCATACTC	200
Adiponectin	AY135647	GCGAGAAGGGTGAGAAAGG	ACAGTGACCCGAGTCTCCAG	187
<i>Osteocyte Markers</i>				
ALP	XM_003361247	GCAAGCAGCACTCTCACTATATC	TCCACCAGCAAGAAGAAGCC	211
SPP1	NM_214023	AAACAAGAGACCCTGCCAAG	TCATCGGATTCATCGGAGTG	173
<i>Chondrocyte Markers</i>				
ACAN	ENSSSCT00000002052	CCATCATCGCCACACCAGAG	CCCGTAGCAACCTTCCCTTG	128
COL2A	ENSSSCT00000031054	CCTGGTGATGATGGTGAAGC	ACCTGGGTAACCTCTGTGAC	132
<i>Reference Genes</i>				
GAPDH	AF017079	TGGTGAAGGTCGGAGTGAAC	TGTAGTGGAGGTCAATGAAGGG	120
HPRT	AF143818	GGACAGGACTGAACGGCTTG	GTAATCCAGCAGGTGAGCAAAG	115
βAct	AJ312193	GTCTCTCCTCCCTGG	GTGGTCTCGTGGATGCC	141

**Table 1:** List of primer pairs, *P. number*, *Species*, *Supplier*, *Dilution* and genes

analyzed in the paper. *Antibody*

<i>Primary</i>					
CD45-APC	K252-1E4	mouse	AbD Serotec		10µl/10 <sup>6</sup> cells
CD90-APC	Ab139364	mouse	Abcam		10µl/10 <sup>6</sup> cells
CD105-FITC	Ab53318	mouse	Abcam		20µl/10 <sup>6</sup> cells
CD56-PE	304606	mouse	Biolegend		10µl/10 <sup>6</sup> cells
CD44-PerPC	103036	rat	Biolegend		10µl/10 <sup>6</sup> cells
CD34 unconjugated	Ab81289	rabbit	Abcam		1:60
CD31 unconjugated	MCA1746	mouse	AbD Serotec		1:100
<i>Secondary</i>					
Anti Rabbit-PE	Ab97070	goat	Abcam		1:200
Anti Mouse-FITC	420-120-05	sheep	BioFX		1:100

583

584 **Table 2:** Flow cytometry antibodies list used for the immunophenotyping of cells.

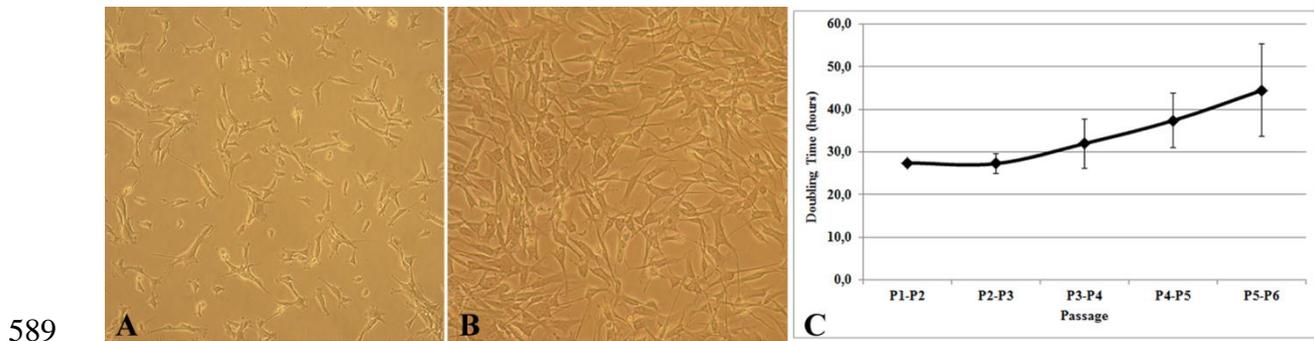
<i>Antibody</i>	<i>P. number</i>	<i>Species</i>	<i>Supplier</i>	<i>Dilution</i>
<i>Primary</i>				
NG2	AB-11160	rabbit	Immunological Sciences	1:200
Smooth Muscle Actin ( $\alpha$ SMA)	1A4	mouse	Cell Marque	1:500
Nestin	AB5922	rabbit	Chemicon	1:150
PDGFR-b	#3169	rabbit	Santa Cruz	1:100
PECAM-1	sc-1506	goat	Santa Cruz	1:150
CD34 unconjugated	Ab81289	rabbit	Abcam	1:60
SMM-hc	MAB3570	mouse	Chemicon	1:100
<i>Secondary</i>				
Anti mouse RRX	715-295-151	Donkey	Jackson ImmunoResearch	1:100
Anti goat RRX	705-295-147	Donkey	Jackson ImmunoResearch	1:100
Anti rabbit Alexa Fluor 488	A21206	Donkey	Molecular Probes	1:600
Anti mouse FITC	F4143	Goat	Sigma Aldrich	1:800

585 **Table 3:** Immunocytochemistry antibodies list used for the cells characterization.

586 **Figures and Figures Legend**

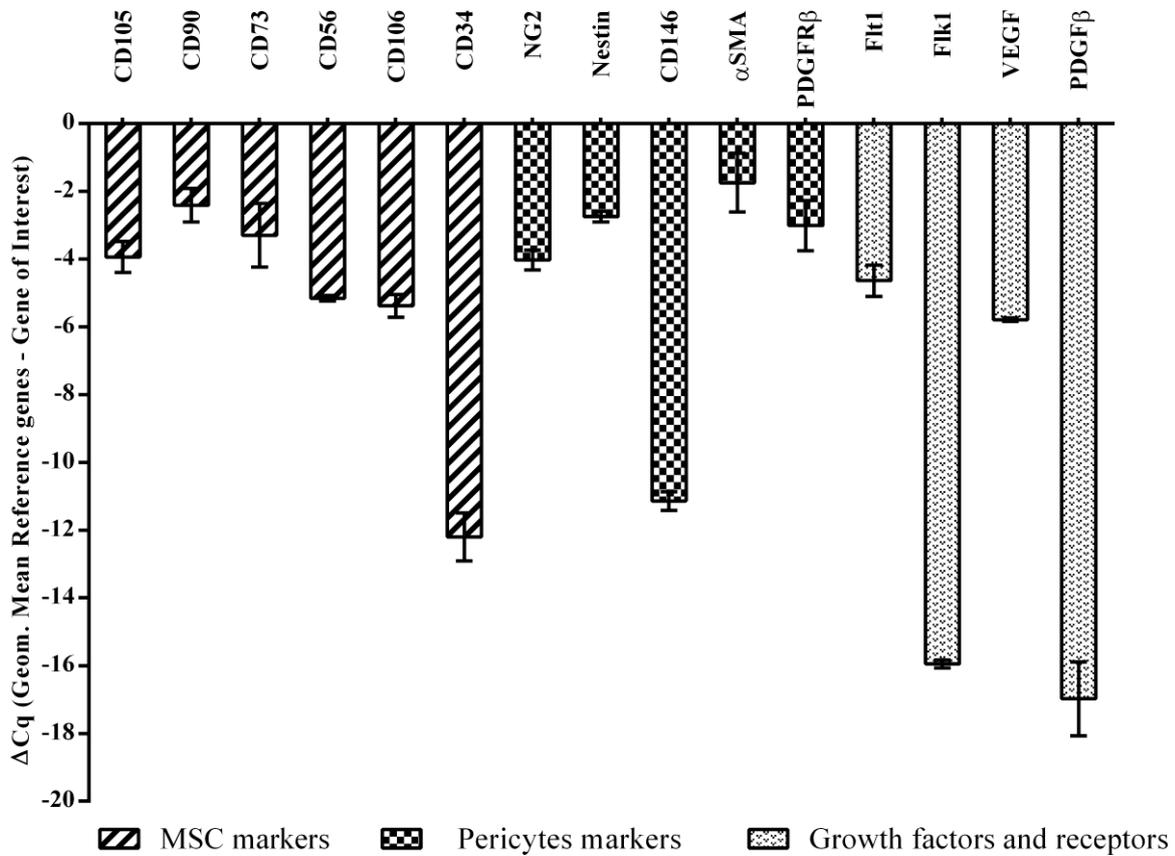
587

588 **Figure 1**



590 **Porcine Aortic Vascular Precursor Cells (pAVPCs) morphology and doubling time during**  
591 **culture in Pericytes Growth Medium (PGM). A, B:** Cells cultured in PGM, after isolation and  
592 starvation step, displayed a small cell body and elongated thin arms at their ends. In **A** it is shown a  
593 microphotograph (10X magnification) of a low confluence culture of pAVPCs, while in **B** it is  
594 shown a microphotograph (10X magnification) of a 60% confluence culture of pAVPCs. **C:** The  
595 growth curve of pAVPCs cultured in PGM is represented in the picture. Cells displayed an  
596 increasing replication time between P1 and P6.

597 **Figure 2**



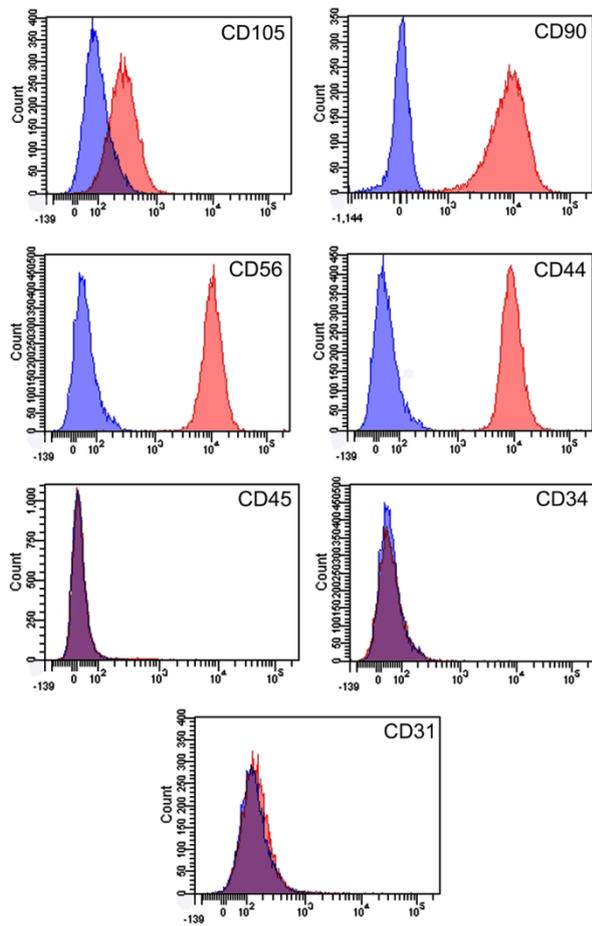
598

599

600 **Transcriptional characterization of pAVPC.** The graph represent the mRNA quantification,  
 601 through qPCR, of the main markers of MSCs – *CD105* (*endoglin*), *CD90* (*Thy-1*), *CD73* (*5'*-  
 602 *nucleotidase [5'-NT]*, also known as *ecto-5'-nucleotidase*), *CD56* (*NCAM – neural cell adhesion*  
 603 *molecule*), *CD106* (*vascular cell adhesion molecule 1 [VCAM-1]*), *CD34* – of pericytes – *NG2*  
 604 (*neuron-glia antigen 2*, also known as *chondroitin sulfate proteoglycan 4 – CSPG4*), *nestin*,  
 605 *CD146* (*melanoma cell adhesion molecule [MCAM]*), *α-SMA* (*α-smooth muscle actin*), *PDGFRβ*  
 606 (*platelet derived growth factor receptor β*) – and of angiogenesis related growth factors and  
 607 receptors – *Flt1* (*fms-related tyrosine kinase 1 also known as vascular endothelial growth factor*  
 608 *receptor 1 [VEGFR1]*), *Flk1* (*Fetal Liver Kinase 1, also known as vascular endothelial growth*  
 609 *factor receptor 2 [VEGFR2]*), *VEGF* (*vascular endothelial growth factor*), *PDGFβ* (*platelet*

610 *derived growth factor  $\beta$* ). Data are expressed as  $\Delta Cq$  calculated as Cq value obtained from the  
611 geometric mean of the reference genes minus Cq value of the gene of interest.

612 **Figure 3**

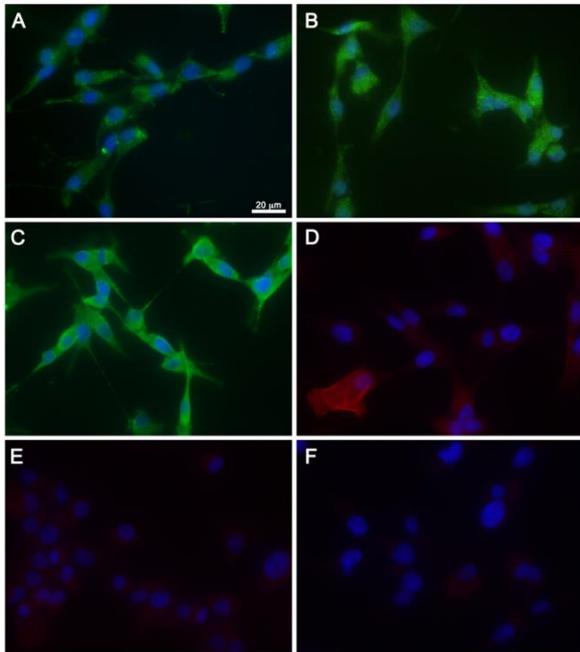


613

614 **Immunophenotyping pAVPCs cultured in PGM.** Flow cytometry analysis of P3 pAVPCs  
615 cultured in PGM showing that they stained positively for CD105, CD90, CD56, CD44, whereas less  
616 than 2% of cells were positive for CD45 (PTPRC – protein tyrosine phosphatase, receptor type, C)  
617 and CD34 and they were negative for the expression of CD31 (platelet endothelial cell adhesion  
618 molecule – PECAM). Red histograms: stained cells; blue histograms: control cells.

619

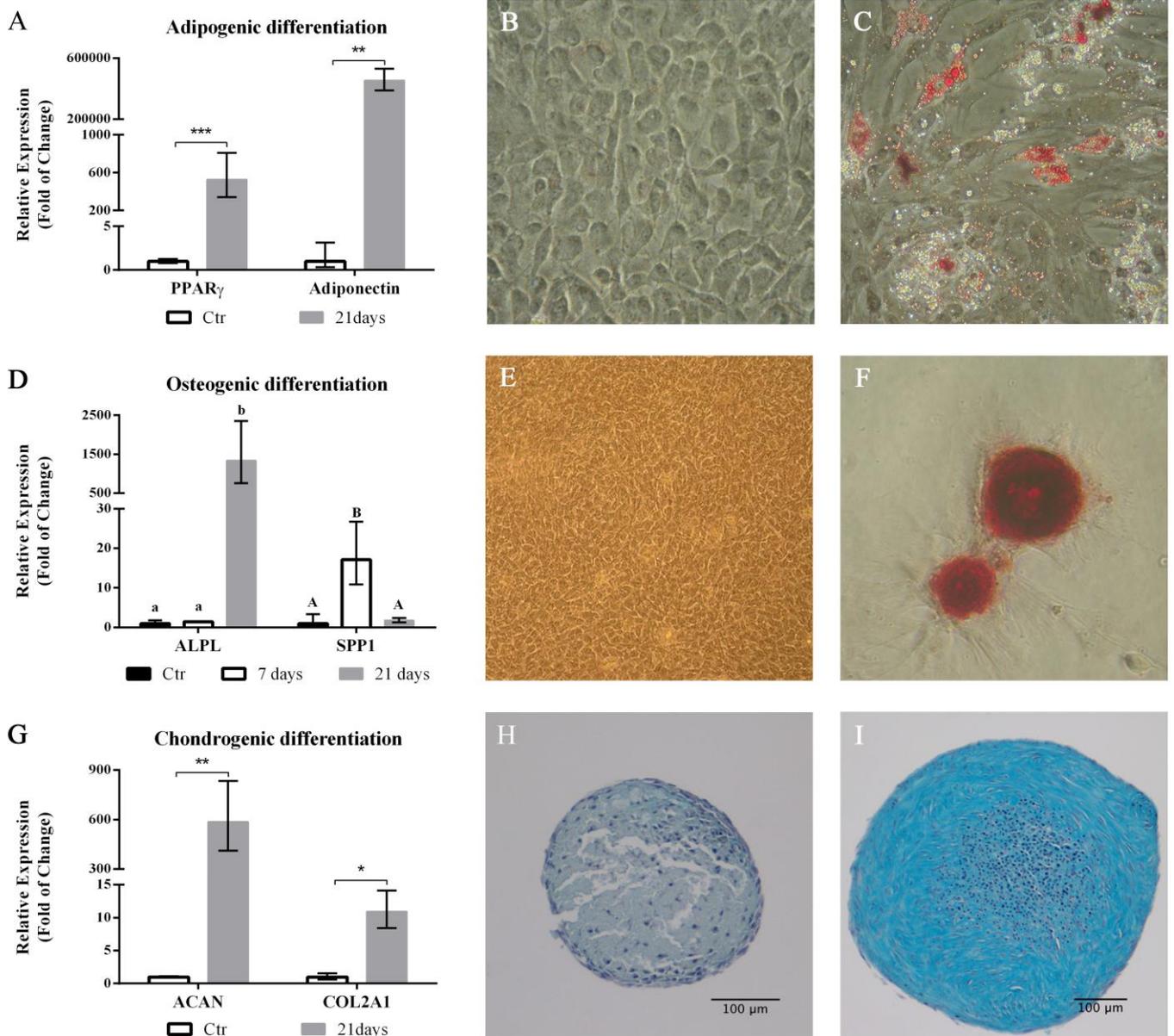
620 **Figure 4**



621

622 **Immunocytochemical characterization of pAVPC cultured in PGM.** In each microphotograph  
623 (scale bar 20 micron), P3 pAVPCs cultured in PGM were stained with different antisera; nuclei  
624 were always stained with Hoechst 33258 (blue). **A:** PDGFRβ; **B:** NG2; **C:** Nestin; **D:** αSMA; **E:**  
625 CD34; **F:** CD31.

626 **Figure 5**



627

628 **Trilineage differentiation potential of pAVPCs.** Porcine Aortic Precursor Cells are able to  
 629 differentiate toward adipo-, osteo- and chondrocyte phenotype if opportunely stimulated *in vitro*. **A**,  
 630 **D**, **G**: gene expression analysis of genes, respectively, of adipocytes (PPAR $\gamma$ , Adiponectin),  
 631 osteocytes (ALPL, SPP1) and chondrocytes (ACAN, COL2A1) in differentiated pAVPCs. In the Y-  
 632 axis in each graph is represented the relative expression of each transcripts analysed in  
 633 differentiated pAVPCs compared to the control ( $2^{-\Delta\Delta Cq}$  method). For adipogenic differentiation (**A**)  
 634 and chondrogenic differentiation (**G**) gene expression has been evaluated after 21 days of culture in

635 differentiation media while for osteogenic differentiation (**D**) gene expression has been evaluated  
636 after 7 and 21 days of culture in differentiation medium. Data obtained for every single gene has  
637 been statistically analyzed (comparing control and differentiated samples) for adipogenic  
638 differentiation (**A**) and chondrogenic differentiation (**G**) through Student-t test (\*:  $p < 0.05$ ; \*\*:  
639  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ), while for osteogenic differentiation (**D**) through one-way ANOVA followed  
640 by Tuckey post-hoc comparison (different letter correspond to statistically different sample).

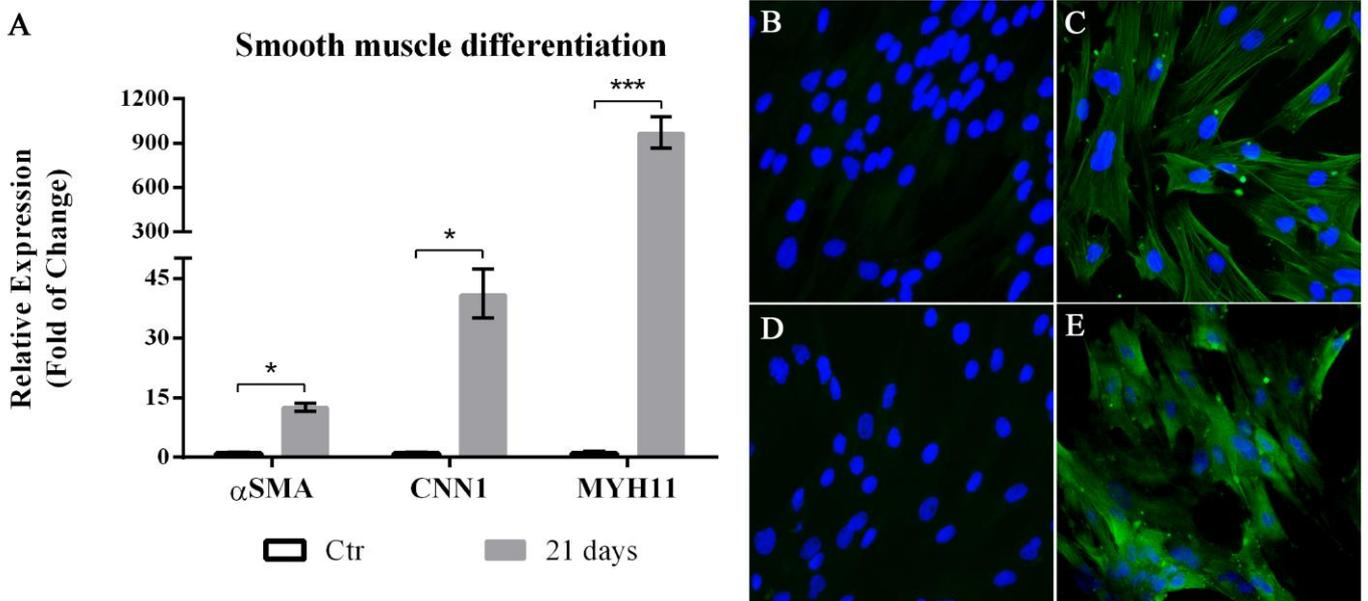
641 **B, C:** OilRedO staining (40X magnification) showed the presence of lipid droplets (red) in the  
642 cytoplasm of pAVPCs cultured for 21 days in adipogenesis induction medium (**C**), while no lipid  
643 droplets has been observed in control cells (**B**).

644 **E, F:** AlizarinRed staining (10X magnification) showed calcium rich-deposits (red) in pAVPCs  
645 cultured for 21 days in osteogenesis induction medium that grew as spheroidal aggregates (**F**),  
646 while no calcium deposits has been observed in control cells (**E**).

647 **H, I:** Alcian blue staining of cross sections of pAVPCs pellet. Blue staining of the extracellular  
648 matrix, indicating presence of proteoglycans and suggesting differentiation toward the chondrocyte  
649 phenotype, was present in differentiated pellet (**I**) and absent in controls (**H**). Samples has been  
650 counterstained with hematoxylin. Scale bar = 100  $\mu\text{m}$ .

651

652 **Figure 6**

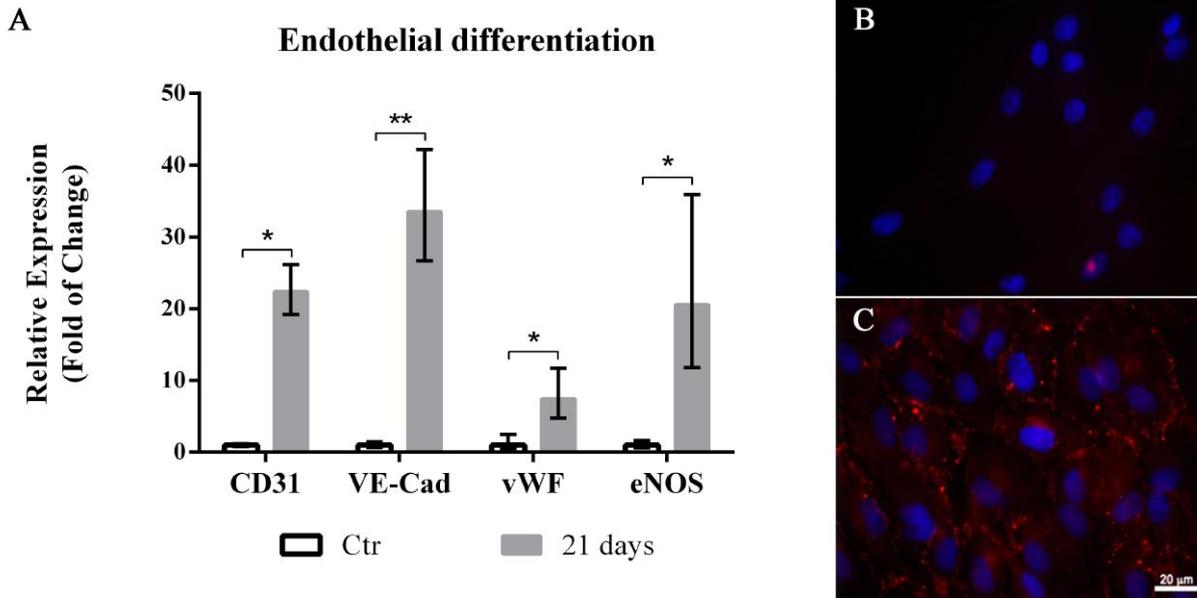


653

654

655 **Smooth muscle lineage differentiation potential of pAVPCs.** Porcine Aortic Precursor Cells are  
656 able to differentiate toward smooth muscle cell phenotype if long term cultured *in vitro*. **A:** gene  
657 expression analysis of smooth muscle cell genes ( $\alpha$ SMA, calponin [CNN1] and smooth muscle  
658 myosin heavy chain [SMM-hc]) in long term cultured pAVPCs. In the Y-axis in the graph is  
659 represented the relative expression of each transcripts analyzed in pAVPCs cultured in LTM  
660 compared to control cells ( $2^{-\Delta\Delta Cq}$  method). Gene expression has been evaluated after 21 days of long  
661 term culture in LTM and data obtained for every single gene has been statistically analyzed  
662 (comparing control and differentiated samples) through Student-t test (\*:  $p < 0.05$ ; \*\*\*:  $p < 0.001$ )

663 **B, C:**  $\alpha$ SMA immunostaining, respectively of control and long term cultured cells (40 X  
664 magnification). **D, E:** smooth muscle myosin-heavy chain (SMM-hc) immunostaining, respectively  
665 of control and long term cultured cells (40 X magnification). Nuclei has been stained with Hoechst  
666 33258 (blue).



668

669

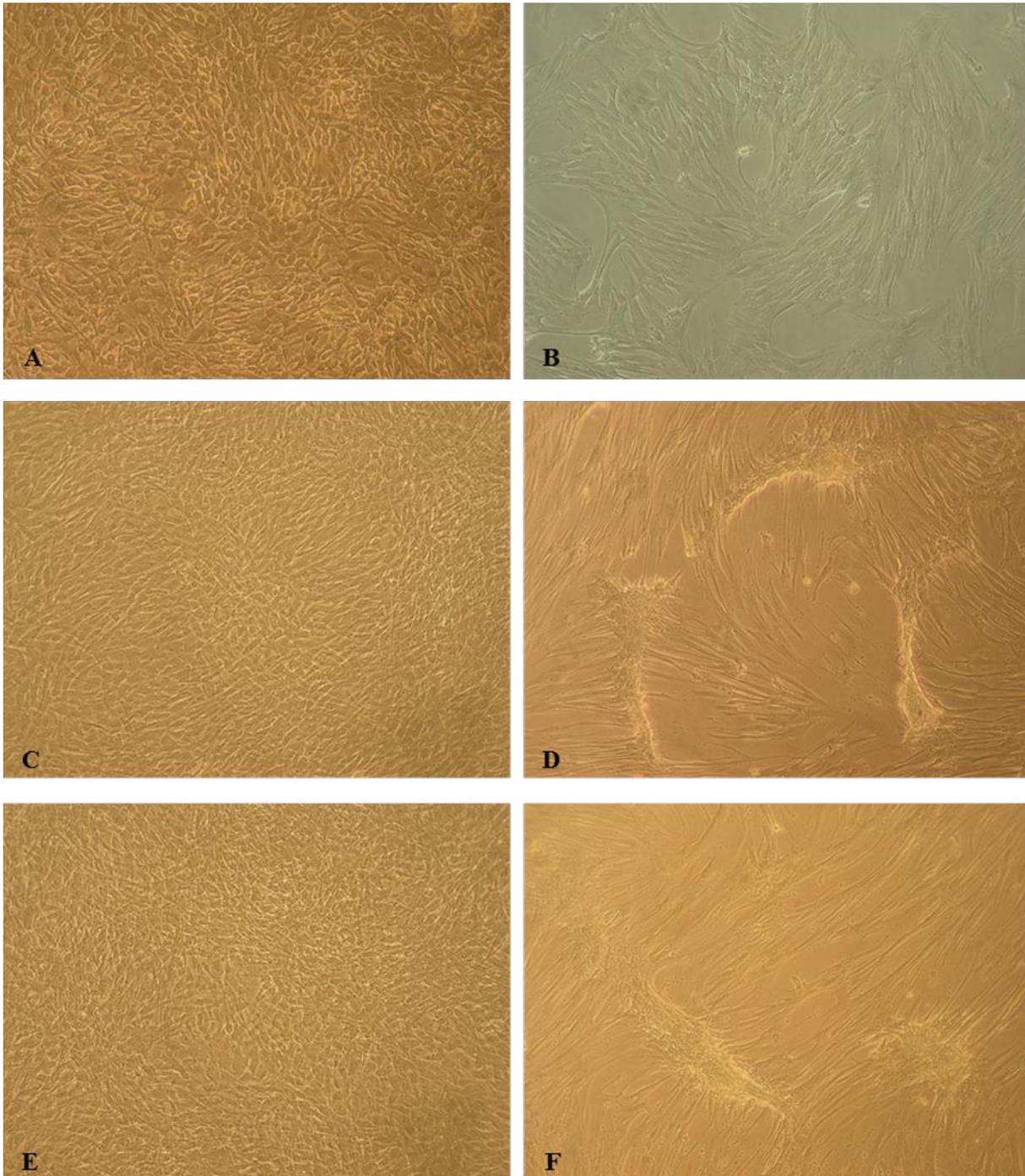
670 **Endothelial lineage differentiation potential of pAVPC.** Porcine Aortic Precursor Cells are able  
 671 to differentiate toward the endothelial cell phenotype if *in vitro* cultured in an endothelial cell  
 672 medium supplemented with 50 ng/ $\mu$ L VEGF. **A:** gene expression analysis of endothelial cell genes  
 673 (CD31, VE-Cadherin, von Willebrand Factor [vWF] and endothelial Nitric Oxide Synthase  
 674 [eNOS]) in differentiated pAVPCs. In the Y-axis in the graph is represented the relative expression  
 675 of each transcripts analyzed in pAVPCs cultured in

676 EDM compared to control cells ( $2^{-\Delta\Delta Cq}$  method). Gene expression has been evaluated after 21 days  
 677 of culture in EDM and data obtained for every single gene has been statistically analyzed  
 678 (comparing control and differentiated samples) through Student-t test (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ )

679 **B, C:** CD31 immunostaining, respectively of control and EDM cultured cells (scale bar 20 micron).  
 680 Nuclei has been stained with Hoechst 33258 (blue).

681

682 **Supplementary Figure 1**



683

684 **Long term culture and smooth muscle lineage differentiation potential of pAVPC –**

685 **morphology. A, C, E:** pAVPC grew in Pericyte Growth Medium (Control) at 7, 14 and 21 days,

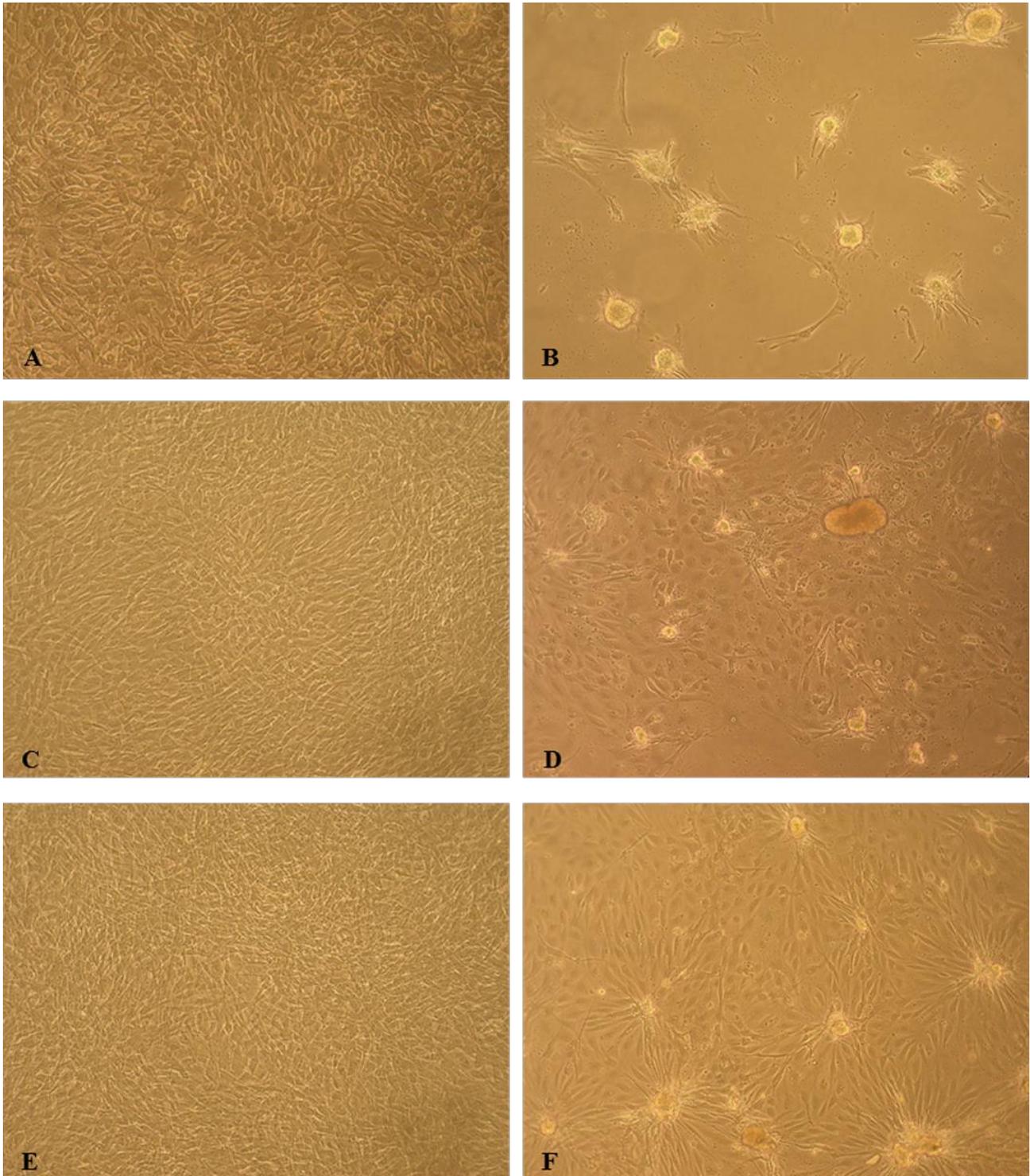
686 respectively; pAVPCs cultured in PGM grew as multilayer maintaining their classical shape with

687 little thin and elongated arms. **B, D, F;** pAVPC grew in Long Term Medium at 7, 14 and 21 days,

688 respectively; pAVPC cultured in LTM showed an elongated fibroblast-like shape and seems to be  
689 organized in cord-like structure. Magnification: 10X.

690

691 **Supplementary Figure 2**



692

693 **Endothelial Differentiation Potential of pAVPC - morphology.** A, C, E: pAVPC grew in  
694 Pericyte Growth Medium (Control) at 7, 14 and 21 days, respectively; pAVPCs cultured in PGM  
695 grew as multilayer maintaining their classical shape. B, D, F: pAVPC grew in Endothelial  
696 Differentiation Medium at 7, 14 and 21 days, respectively; pAVPC stimulated with EDM displayed

697 an endothelial cell-like morphology growing in cluster and forming an endothelial cell-like  
698 monolayer upon which some cells organized in few spheroidal structures. Magnification: 10X.