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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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10 Zaniboni., C.B. and M.B. prepared figures; A.Zaniboni, C.B., and M.F. drafted manuscript; M.F.
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23
24 **Running Head:** Vascular Stem-like Cells from porcine aorta

25

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29

30

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/cm²) 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 (α SMA, CNN1, SMM-hc) and immunofluorescence
55 confirmed the presence of α 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% CO₂ 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 β [PDGFR β], CD146, α -smooth muscle myosin
145 [α 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 2×10^6 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 μ 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 μ M forward primer, 0.2 μ M reverse primer, 1X iTaq Universal SYBR Green
159 Supermix (Bio-Rad). One μ l of cDNA were added to 19 μ 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 Δ 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 (β -
165 actin – β 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 β ,
176 α 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 γ
187 [PPAR γ], 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 ΔCq method (gene of interest Cq – reference gene Cq). As reference gene
192 Cq, the geometric mean of β 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 – ΔCq
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 μ 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/cm² 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 (α SMA, calponin [CNN1], smooth muscle myosin heavy chain [SMM-hc]) and
212 immunofluorescence (α 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/cm² 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 ± 0.6 hours, between P1 and P2, to 44.4 ± 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 ± 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, α SMA, PDGFR β)
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 β , 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 ± 0.1 %), CD90 (99.5 ± 0.2 %),
272 CD44 (99.6 ± 0.3 %) and less than 2% of them expressed the hemopoietic lineage markers as CD45
273 (1.4 ± 0.4 %), CD34 (1.3 ± 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 ± 0.1 %). In
275 particular, as already described for cells cultured in DM, cells expressed CD56 (99.9 ± 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 β , 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 α SMA (Figure 4 D), while in DM (49) 100% of cells

283 expressed it. The α 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 γ 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* = 6.20×10^{-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 = 5.65×10^{-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 (α SMA, CNN1, SMM-hc [47]) analysed in
335 LTM cultured cells compared to the control one. In particular, α 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 α 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/ μ 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/ μ L VEGF
363 (34).

364

365 Mesenchymal stromal cells in which depletion of PDGFR β 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 β signaling in vascular smooth muscle cells (VSMCs) through the assembly of a
370 PDGFR β /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 β signaling. That could be the first stimulus to
374 induce the endothelial differentiation ensuring that the main receptor (PDGFR β) 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 β , NG2 and α 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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- 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 | CATAGGTGTGTC AAGGGTATCG | 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 | CACGGCATCATCACCAACTG | ACCGCCTGAATAGCCACATAC | 200 |
| CNN1 | NM_213878 | ATGTCTCTGTCTCACTTCAAC | CCTGCTGGTGGTCATACTTC | 91 |
| SMM-hc | XR_131283 | AGGAGGTGACGATGCTGAAG | TGCTTGTCTTGTCCAGGTTG | 160 |
| <i>Endothelial Cell Markers</i> | | | | |
| CD31 | NM_213907 | CTCATCGCAGTGGTTGTCATC | TGTCTTCTCATTTGTTGGAGTTCAG | 150 |
| VE-Cadherin | NM_001001649 | ACTCCTCAGCCTCTCTCTCAG | 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 | CCTTGCCTTGTCTGTCTTACC | CGTCCATGAACCTTCCACTTTC | 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 | TGGTGAAGGTTCGGAGTGAAC | TGTAGTGGAGGTCAATGAAGGG | 120 |
| HPRT | AF143818 | GGACAGGACTGAACGGCTTG | GTAATCCAGCAGGTGAGCAAAG | 115 |
| βAct | AJ312193 | GTCTCTCCTCCCTG | GTGGTCTCGTGGATGCC | 141 |

Table 1: List of primer pairs, *P. number*, *Species*, *Supplier* and *Dilution* amplicon size (bp) and genes

analyzed in the paper. *Antibody*

| <i>Primary</i> | | | | | |
|-------------------|------------|--------|-------------|--|----------------------------|
| CD45-APC | K252-1E4 | mouse | AbD Serotec | | 10µl/10 ⁶ cells |
| CD90-APC | Ab139364 | mouse | Abcam | | 10µl/10 ⁶ cells |
| CD105-FITC | Ab53318 | mouse | Abcam | | 20µl/10 ⁶ cells |
| CD56-PE | 304606 | mouse | Biolegend | | 10µl/10 ⁶ cells |
| CD44-PerPC | 103036 | rat | Biolegend | | 10µl/10 ⁶ 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.

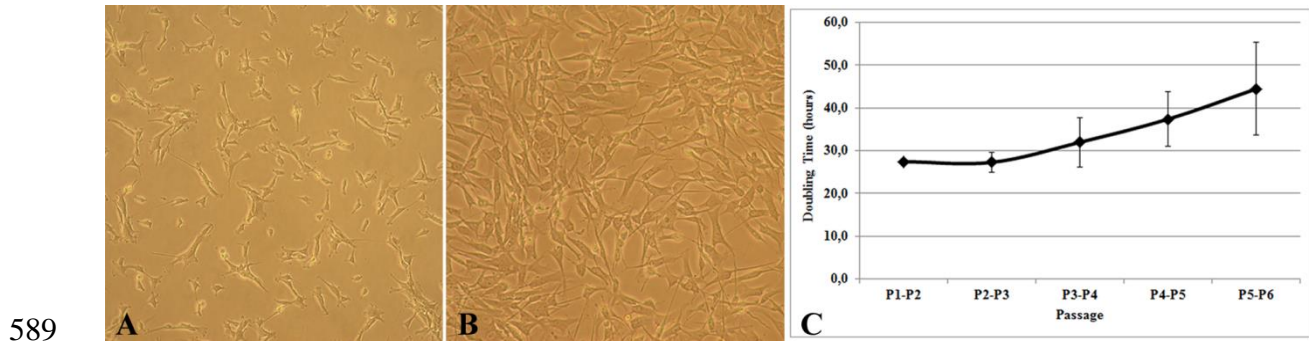
| <i>Antibody</i> | <i>P. number</i> | <i>Species</i> | <i>Supplier</i> | <i>Dilution</i> |
|-------------------------------------|------------------|----------------|------------------------|-----------------|
| <i>Primary</i> | | | | |
| NG2 | AB-11160 | rabbit | Immunological Sciences | 1:200 |
| Smooth Muscle Actin (α 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**

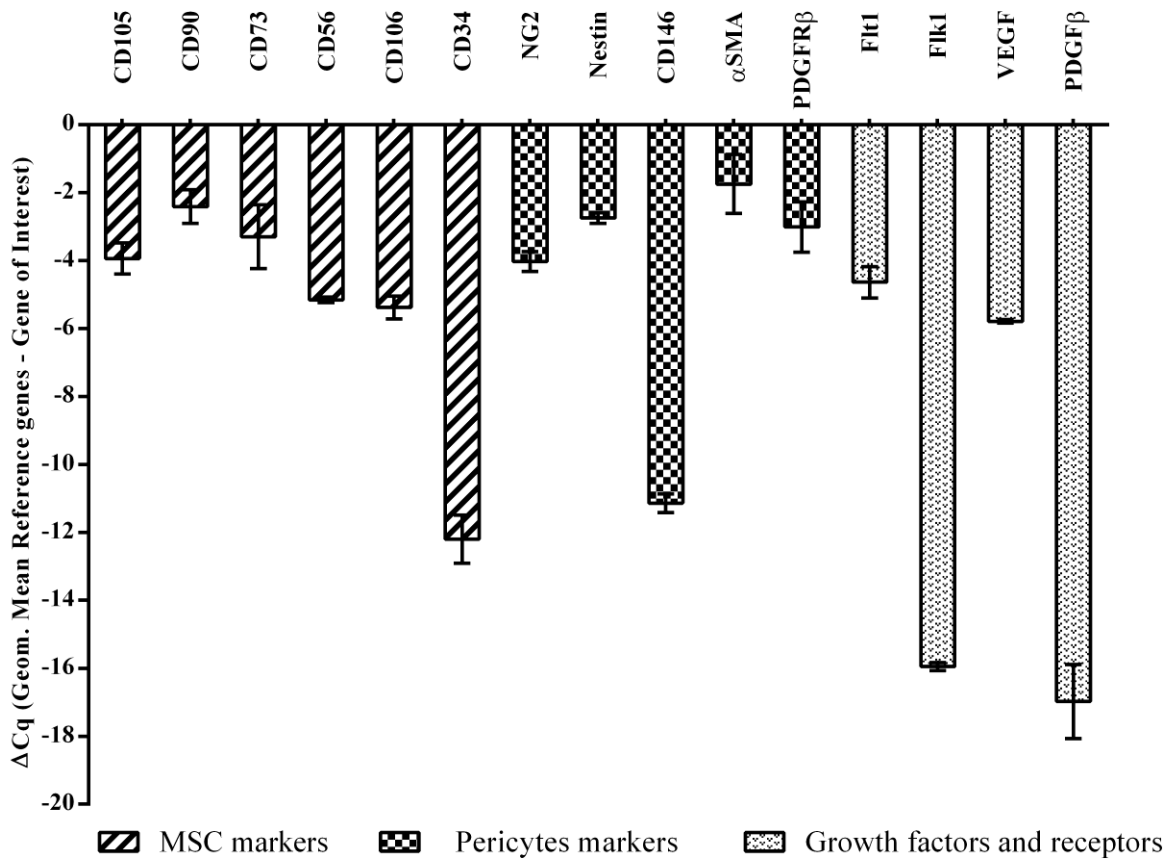
587

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**



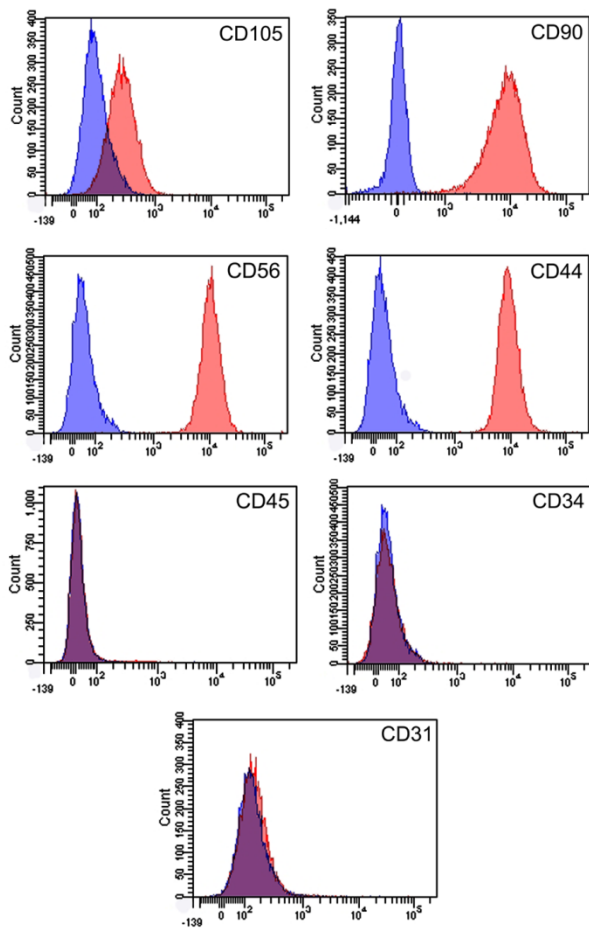
598

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-gial 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 β*). Data are expressed as ΔCq 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**

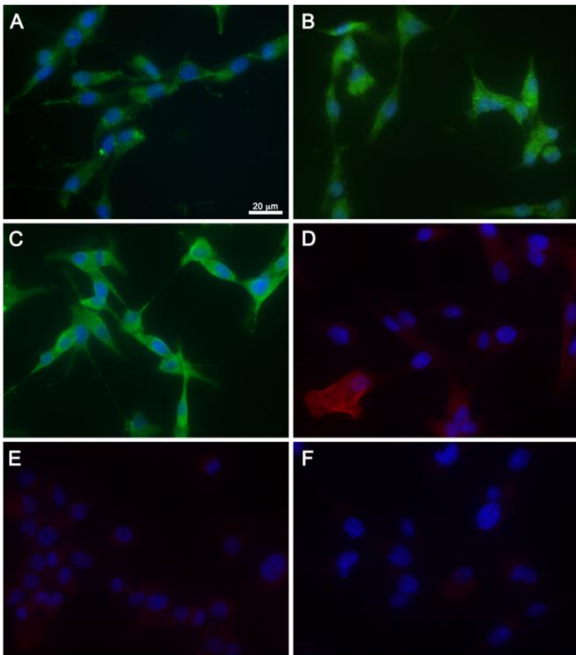


613

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

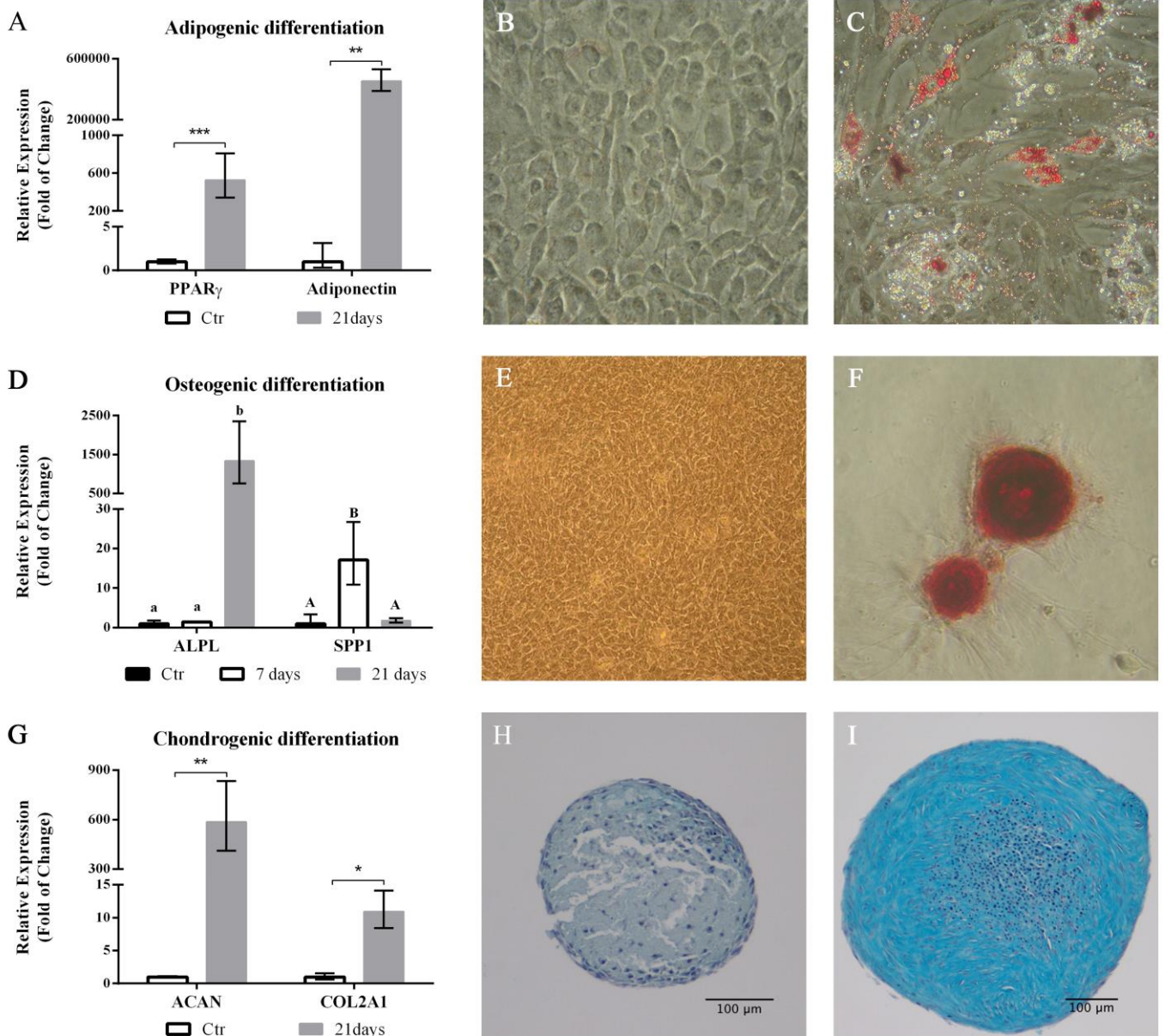
620 **Figure 4**



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:** PDGFR β ; **B:** NG2; **C:** Nestin; **D:** α SMA; **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 γ , 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).

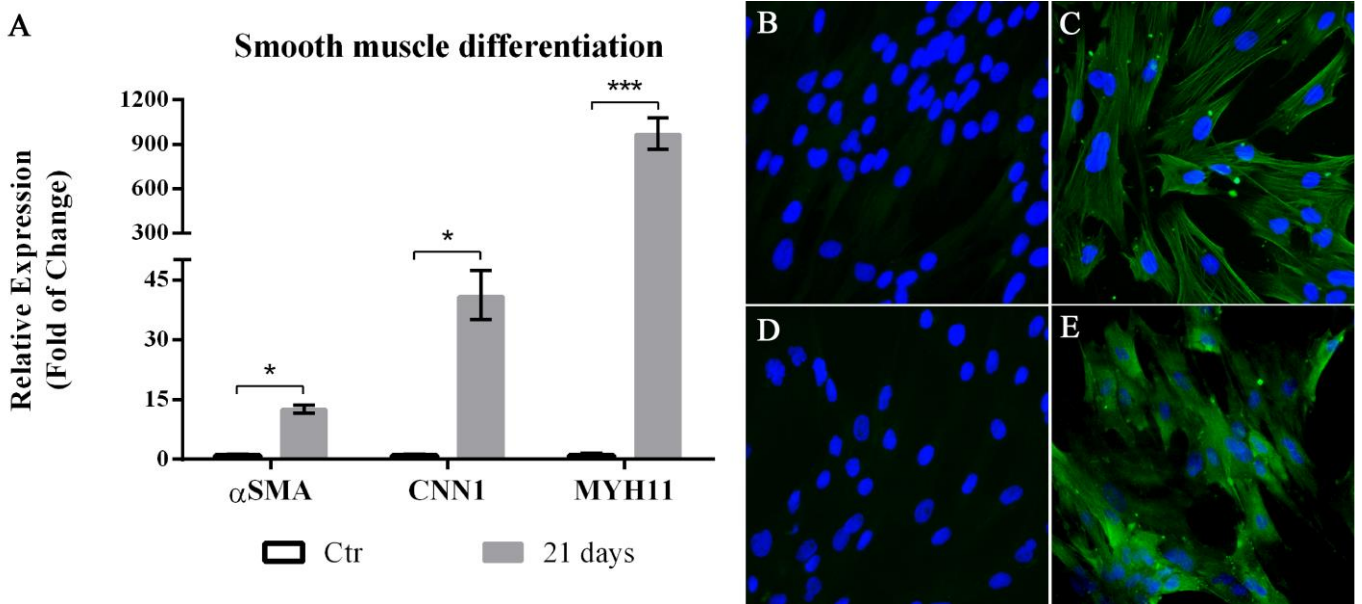
641 **B, C:** OilRedO staining (40X magnification) showed the presence of lipid droplets (red) in the
642 cytoplasm of pAVPCs cultured for 21 days in adipogenesis induction medium (**C**), while no lipid
643 droplets has been observed in control cells (**B**).

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 μm .

651

652 **Figure 6**



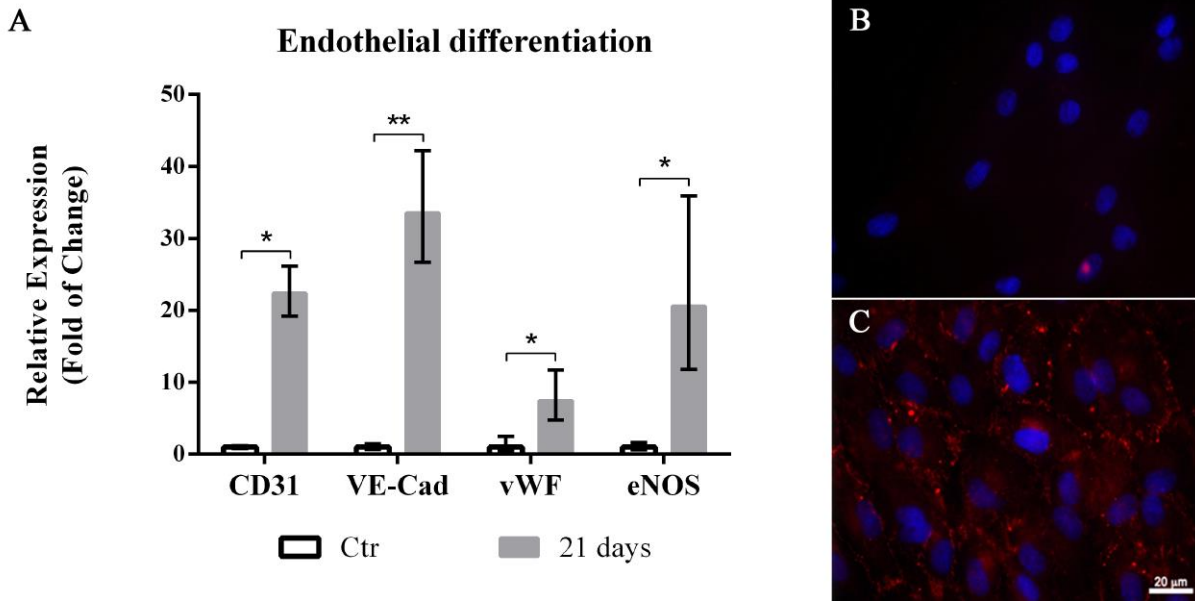
653

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 (α 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:** α 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).

667 **Figure 7**



668

669

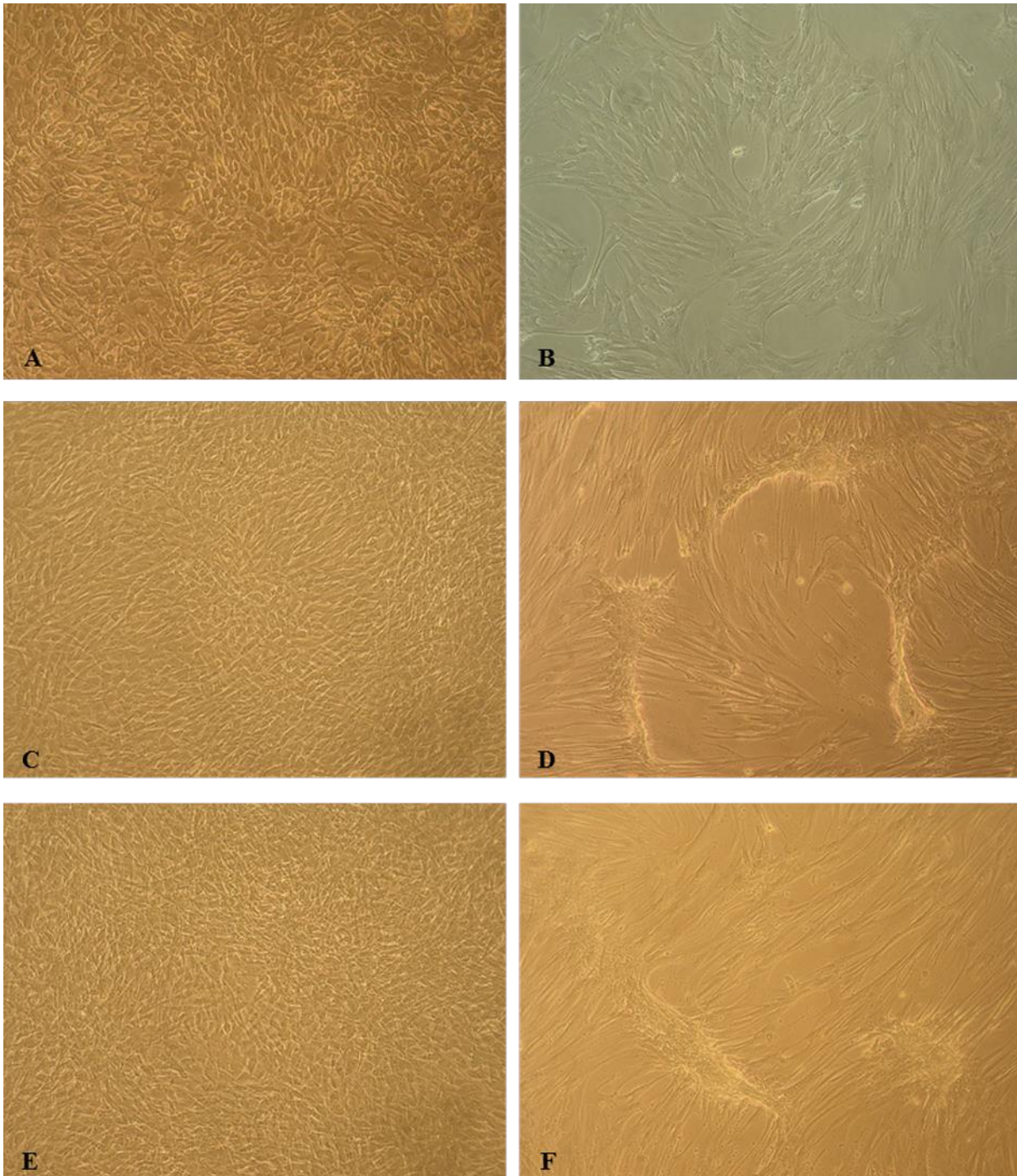
670 **Endothelial lineage differentiation potential of pAVPC.** Porcine Aortic Precursor Cells are able
671 to differentiate toward the endothelial cell phenotype if *in vitro* cultured in an endothelial cell
672 medium supplemented with 50 ng/ μ L VEGF. **A:** gene expression analysis of endothelial 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,

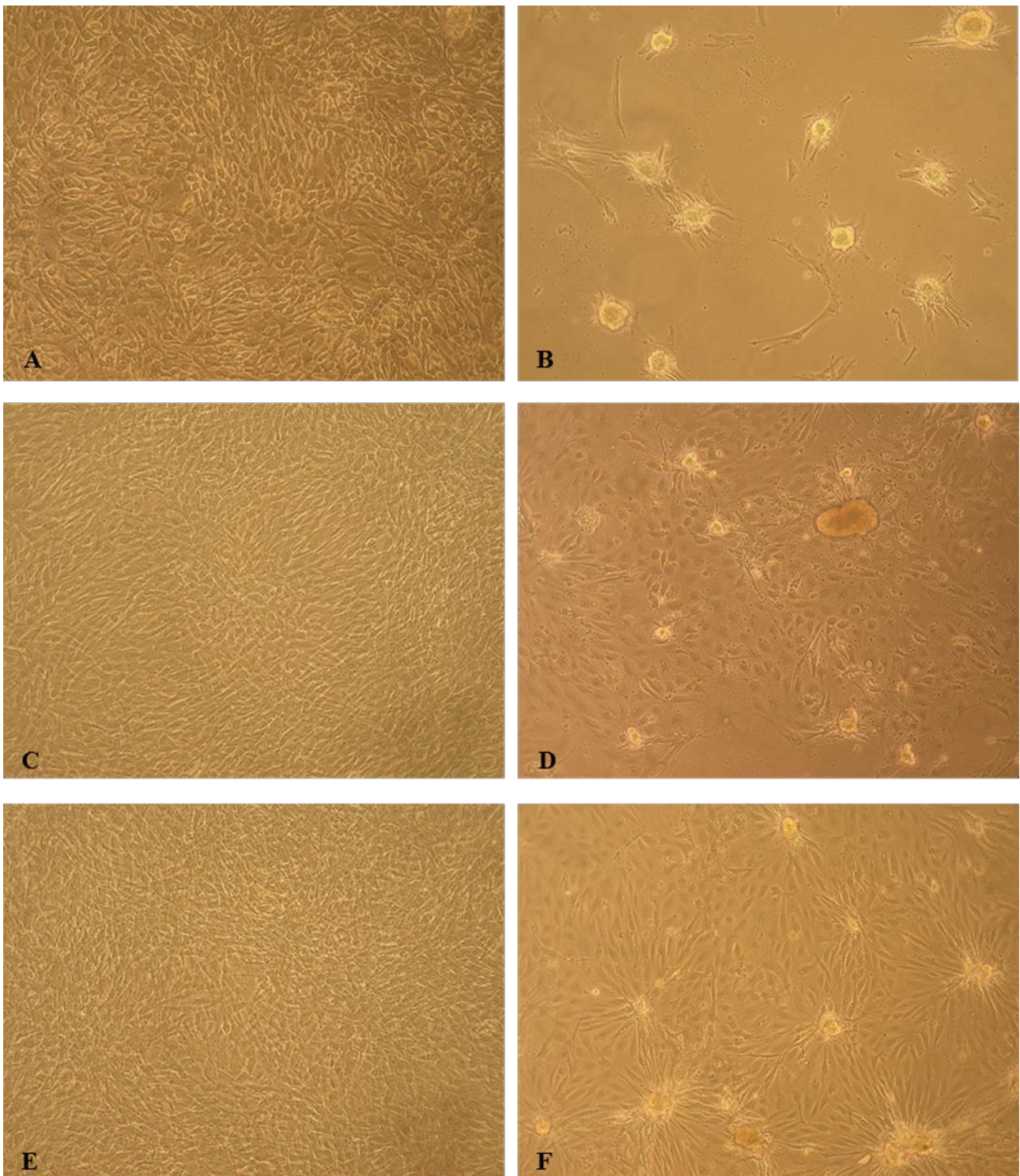
686 respectively; pAVPCs cultured in PGM grew as multilayer maintaining their classical shape with

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.