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Running Head: Vascular Stem-like Cells from porcine aorta

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Abstract

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

We recently isolated and characterized a population of MSC-like cells from porcine aorta (porcine Aortic Vascular Precursor Cells pAVPCs) with MSCs and pericyte-like proprieties *in vitro*. The main aim of the present work was to investigate whether pAVPCs possess VSC-like properties assessing their differentiation potential toward the endothelial and the smooth muscle lineages. pAVPCs have been isolated as previously described by us and cultured in in a medium specified for pericyte to maintain stemness and multipotency. In order to assess if pAVPCs differentiate to smooth muscle cells, pAVPCs were grown for 21 days in high glucose – DMEM + 10% Foetal Bovine Serum (DMEM + 10% FBS) (Long Term Medium – LTM), whilst in order to assess if cells are able to differentiate toward the endothelial phenotype cells were seeded in a 24 well plate (5000 cells/cm2) and cultured for 21 days in Endothelial Differentiation Medium (EDM) (human Endothelial Serum Free Medium supplemented with 5% FBS and 50 ng/mL of human recombinant Vascular Endothelial Growth Factor [hVEGF]).

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

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

Introduction

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

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

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

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

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

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

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

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

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

Material and methods

Cells isolation and culture

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

Briefly, the cells were isolated, from the media layer of the aortas through a collagenase IA digestion. The cells were cultured overnight (15-16 h) in high glucose (hg) DMEM (GIBCO – Life Technology Corporation, Carlsbad, CA, USA) to which 10% FBS and 10X antibiotic-antimycotic (hgDMEM-10X) were added in a 5% CO_2 incubator at 38.5°C. The culture medium was then replaced with hgDMEM + 10% FBS (GIBCO) + 1X antibiotic-antimycotic (GIBCO) (hgDMEM-1X).

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

Transcriptional Characterization of pAVPCs cultured in PGM

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

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

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

Quantitative PCR was carried out using a CFX96 (Bio-Rad) thermal cycler. A master mix of the following reaction components was prepared in nuclease free water to the final concentrations indicated: 0.2 μM forward primer, 0.2 μM reverse primer, 1X iTaq Universal SYBR Green Supermix (Bio-Rad). One μl of cDNA were added to 19 μl of the master mix. All samples were analysed in duplicate. The qPCR protocol used for the transcriptional characterization was: 10 min 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 95°C (80 cycle of 0.5°C increase/cycle).

The gene expression was evaluated using the ΔCq method (reference gene Cq – gene of interest Cq). As Cq for the reference gene, the geometric mean of Cq of three different reference genes (β-actin – βAct, Hypoxanthine phosphoribosyltransferase – HPRT, Glyceraldehyde 3-phosphate dehydrogenase – GAPDH) was considered. Reference genes primers sequences and size (bp) of each product are listed in Table 1.

Phenotypical Characterization of pAVPCs cultured in PGM

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

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

Moreover, cells were analyzed by immunofluorescence experiments for the expression of PDGFβ, αSMA, NG2, nestin, CD34, CD31 and smooth muscle myosin – heavy chain (SMM-hc). The primary and secondary antibodies and their concentration used for the analysis are listed in Table 3.

Mesenchymal Trilineage Differentiation Potential

Cells at P3, grown in PGM, were assessed for the mesenchymal trilineage differentiation potential.

A StemPro® Adipogenesis Differentiation Kit, a StemPro® Osteogenesis Differentiation Kit and a StemPro® Chondrogenesis Differentiation Kit (all purchased from GIBCO, Life Technologies) were used following the manufacturer's instructions.

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

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

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

Long term culture and assessing of smooth muscle cells differentiation potential

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

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

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

Endothelial Differentiation Potential

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

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

Statistical analysis

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

Results and Discussion

Morphology and doubling time of pAVPCs cultured in PGM

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

Transcriptional Characterization of pAVPCs cultured in PGM

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

Moreover, pAVPCs do express pericytes main transcripts (NG2, Nestin, CD146, αSMA, PDGFRβ) (11) and CD34 mRNA, considered a markers of Advential Cells, Myoendothelial Cells (8) and mesangioblasts (31).

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

Phenotypical Characterization of pAVPCs cultured in PGM

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

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

283 expressed it. The αSMA 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 PPARγ 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

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

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

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

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

Long term culture and smooth muscle cells differentiation potential

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

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

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

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

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

Endothelial Differentiation Potential

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

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

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

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

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

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

Conclusion

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

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

In particular, in the present paper pAVPCs cultured in Pericytes Growth Medium has been shown to be rather pure population of cells that express the main markers of MSCs (CD105, CD90, CD73, CD44) and lack the expression of the main markers of hemopioetic stem cells (CD45 and CD34), as requested by the ISCT (13). Moreover, pAVPCs has been shown to express the main markers (PDGFRβ, NG2 and αSMA) that characterized pericytes (11). In addition, pAVPCs have been shown to be able to differentiate toward osteo-, adipo- and chondrocyte phenotype.

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

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

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

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

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

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

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580

581 **Tables**

Genes	*Accession Number*	*Forward (5'-3')*	*Reverse (5'-3')*	*Product size (bp)*
Mesenchymal Stromal Cell markers				
CD106	NM_213891	GAGGATGGAAGATTCTGGAATTTACG	ATCACTAGAGCAGGTCATGTTCAC	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
Pericytes Markers				
CD146	ENSSSCT00000016482	CTCATCTGTGCCTTCCTGCTAG	CCTCCCACTTCCACCTCCAG	110
NG2	ENSSSCT00000002098	ACCACCTCCTCCTACAACTC	GTCACTCAGCAGCATCTCTG	104
PDGFRβ	ENSSSCT00000015788	GCAACGAGGTGGTCAACTTC	GCAGGATAGAACGGATGTGG	111
Nestin	ENSSSCT00000027298	CAGTGGTTCCAAGGCTTCTC	CATAGGTGTGTCAAGGGTATCG	163
Hemopoietic Stem Cell Markers				
CD45	XM_003482796.1	CTCACTCGCAAGCATCTCTG	CGGTTGAAGTAGCTGTGTCTG	188
CD34	NM_214086	CAGCCTCCACACTGTCTCTC	CACCTCTGAAGTTGTAAAGTTGATG	184
Smooth Muscle Cell Markers				
αSMA	NM_001164650	CACGGCATCATCACCAACTG	ACCGCCTGAATAGCCACATAC	200
CNN1	NM_213878	ATGTCCTCTGCTCACTTCAAC	CCTGCTGGTGGTCATACTTC	91
SMM-hc	XR_131283	AGGAGGTGACGATGCTGAAG	TGCTTGTTCTTGTCCAGGTTG	160
Endothelial Cell Markers				
CD31	NM_213907	CTCATCGCAGTGGTTGTCATC	TGTCTTCTCATTGTTGGAGTTCAG	150
VE-Cadherin	NM_001001649	ACTCCTCAGCCTCTCCTCAG	GTCAACACTCAGCACAGCATAG	111
vWF	NM_001246221	GTGGAGAGTGCTGAGTGTTC	TGAAGGTGAAGTGTCTGTTGTC	193
eNOS	AY266137	GCTCTCACCTTCTTCCTG	CCACTTCCACTCCTCATAG	144
Receptor and Growth Factor				
Flt1	AY566244	TTGGACTGTTGGCACAAAGAC	GCTGTTGCTCGTCAGAATGG	141
Flk1	AJ245446	AACGAGTGGAGGTGACAGATTG	CGGGTAGAAGCACTTGTAGGC	104
VEGF	AF318502	CCTTGCCTTGCTGCTCTACC	CGTCCATGAACTTCACCACTTC	101
PDGFβ	ENSSSCT00000029224	CTCTGGCTGCTGCAACAACC	TGGCTTCTTCCGCACAATCTC	100
Adipocyte Markers				
PPARγ	AF103946	AGGAAAGACCACAGACAAATCAC	CAGGGATGTTCTTGGCATACTC	200
Adiponectin	AY135647	GCGAGAAGGGTGAGAAAGG	ACAGTGACCCGAGTCTCCAG	187
Osteocyte Markers				
ALP	XM_003361247	GCAAGCAGCACTCTCACTATATC	TCCACCAGCAAGAAGAAGCC	211
SPP1	NM_214023	AAACAAGAGACCCTGCCAAG	TCATCGGATTCATCGGAGTG	173
Chondrocyte Markers				
ACAN	ENSSSCT00000002052	CCATCATCGCCACACCAGAG	CCCGTAGCAACCTTCCCTTG	128
COL2A	ENSSSCT00000031054	CCTGGTGATGATGGTGAAGC	ACCTGGGTAACCTCTGTGAC	132
Reference Genes				
GAPDH	AF017079	TGGTGAAGGTCGGAGTGAAC	TGTAGTGGAGGTCAATGAAGGG	120
HPRT	AF143818	GGACAGGACTGAACGGCTTG	GTAATCCAGCAGGTCAGCAAAG	115
βAct	AJ312193	GTCCTCCTCCTCCCTGG	GTGGTCTCGTGGATGCC	141

582

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

analyzed in the paper.*Antibody*				
Primary				
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
Secondary				
Anti Rabbit-PE	Ab97070	goat	Abcam	1:200
Anti Mouse-FITC	420-120-05	sheep	BioFX	1:100

583

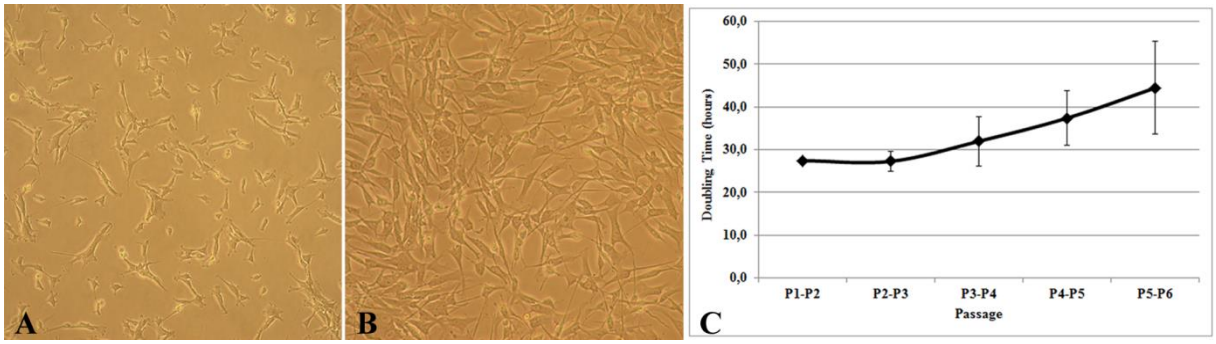
584 **Table 2:** Flow cytometry antibodies list used for the immunophenotyping of cells.

Antibody	*P. number*	*Species*	*Supplier*	*Dilution*
Primary				
NG2	AB-11160	rabbit	Immunological Sciences	1:200
Smooth Muscle Actin (αSMA)	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
Secondary				
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.

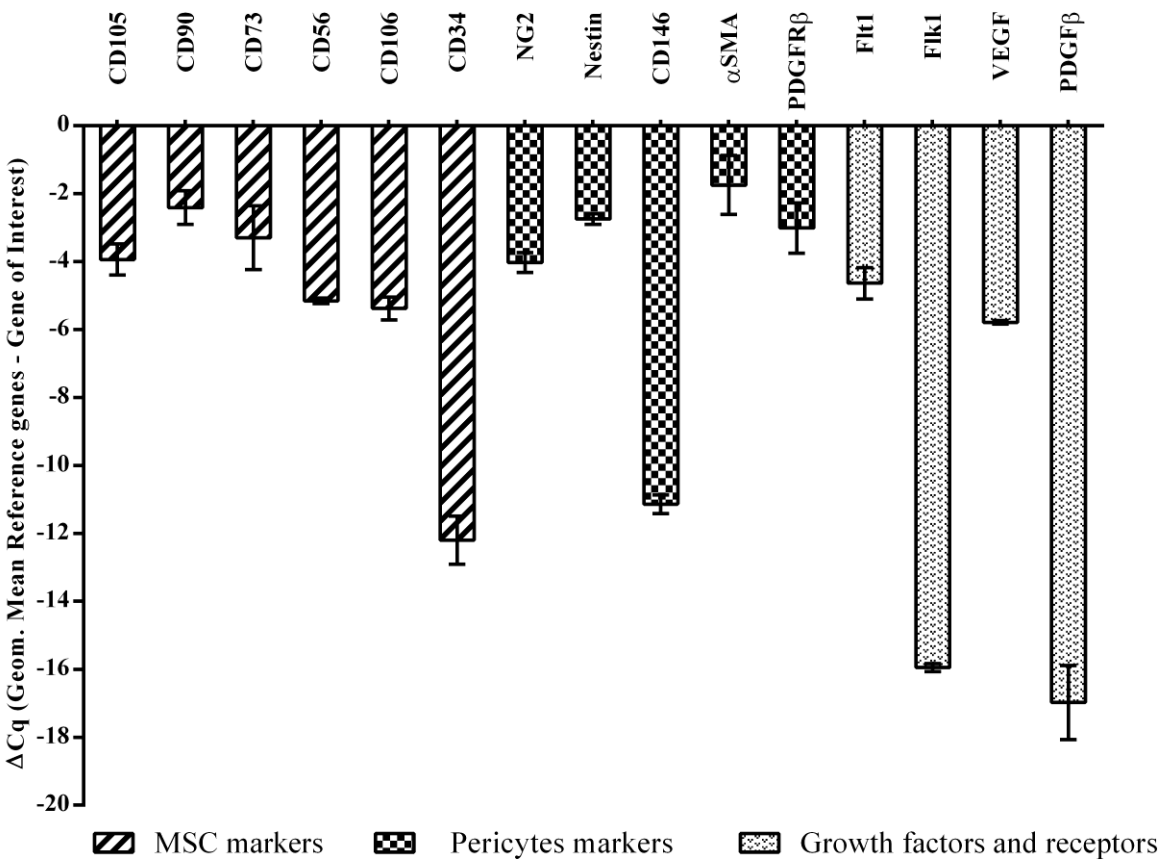
Figures and Figures Legend

Figure 1



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

Figure 2

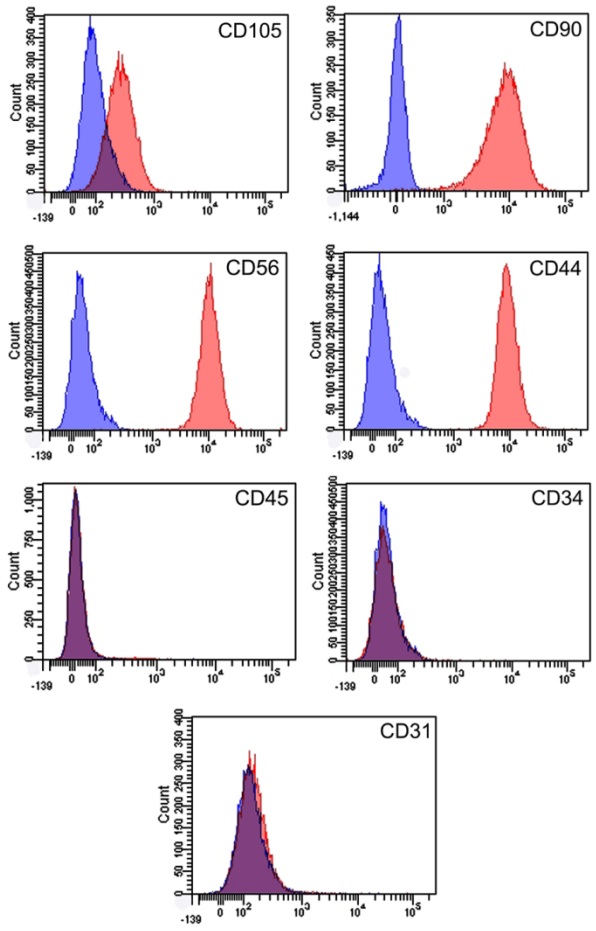


Transcriptional characterization of pAVPC. The graph represent the mRNA quantification, through qPCR, of the main markers of MSCs – *CD105 (endoglin), CD90 (Thy-1), CD73 (5'-nucleotidase [5'-NT], also known as ecto-5'-nucleotidase), CD56 (NCAM – neural cell adhesion molecule), CD106 (vascular cell adhesion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

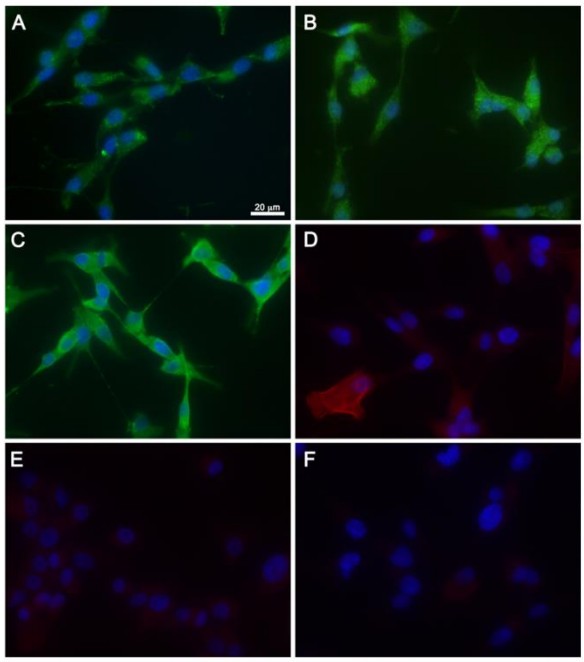
611 geometric mean of the reference genes minus Cq value of the gene of interest.

Figure 3



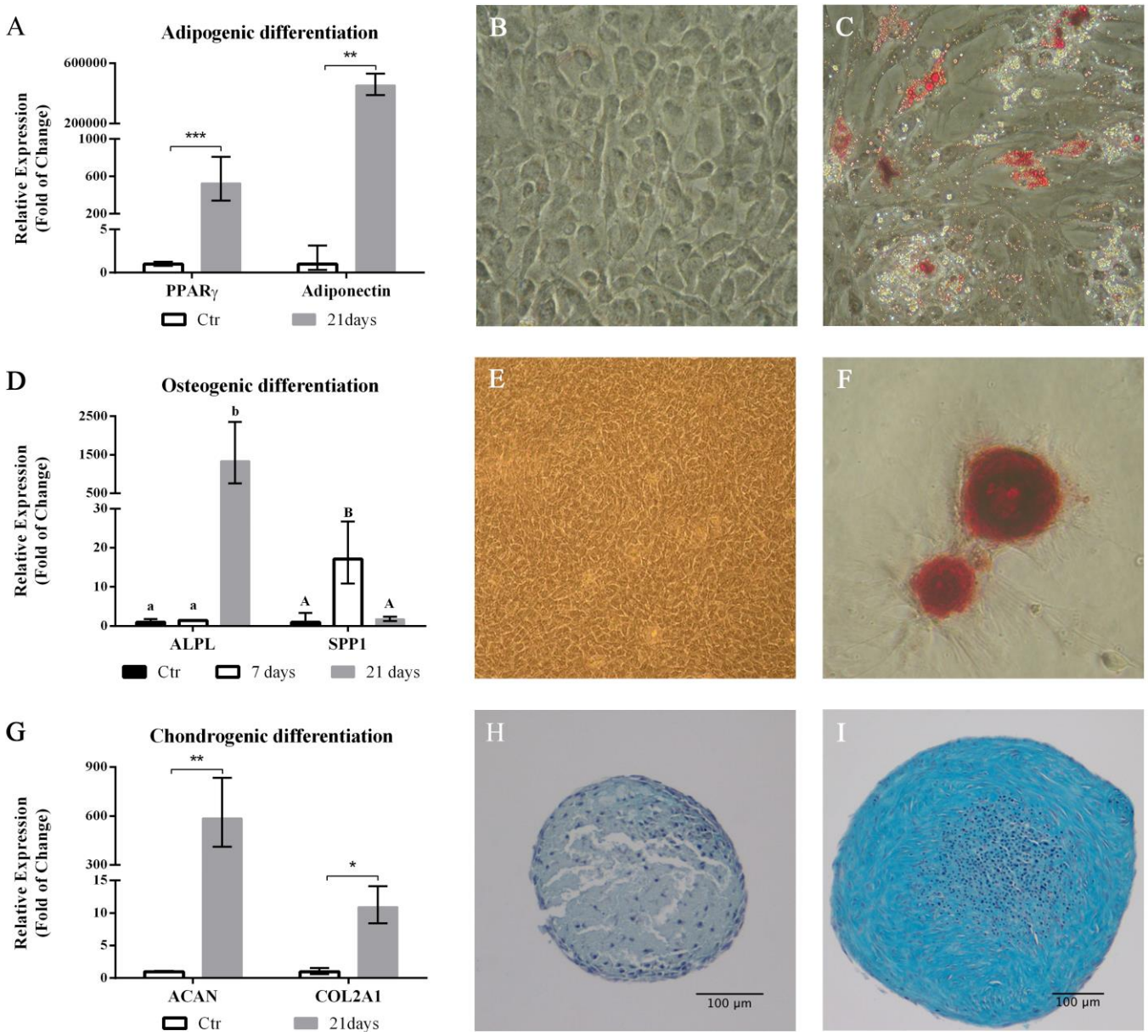
Immunophenotyping pAVPCs cultured in PGM. Flow cytometry analysis of P3 pAVPCs cultured in PGM showing that they stained positively for CD105, CD90, CD56, CD44, whereas less than 2% of cells were positive for CD45 (PTPRC – protein tyrosine phosphatase, receptor type, C) and CD34 and they were negative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

Figure 4



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

Figure 5



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

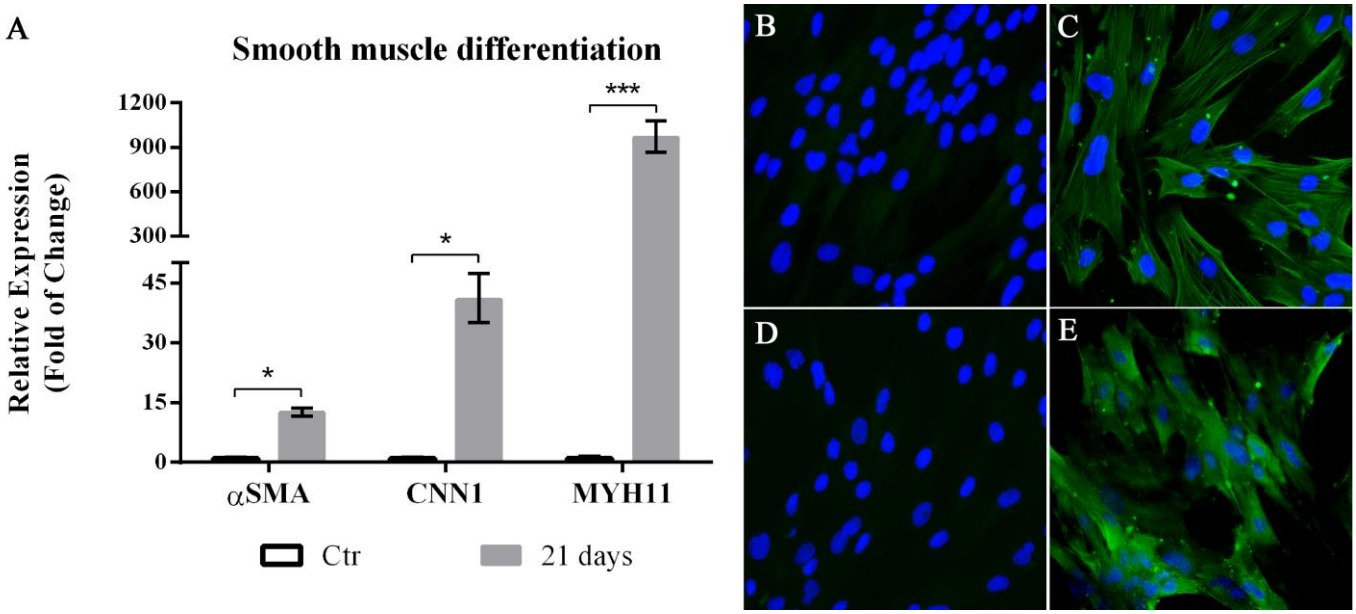
differentiation media while for osteogenic differentiation (**D**) gene expression has been evaluated after 7 and 21 days of culture in differentiation medium. Data obtained for every single gene has been statistically analyzed (comparing control and differentiated samples) for adipogenic differentiation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

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

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

H, I: Alcian blue staining of cross sections of pAVPCs pellet. Blue staining of the extracellular matrix, indicating presence of proteoglycans and suggesting differentiation toward the chondrocyte phenotype, was present in differentiated pellet (**I**) and absent in controls (**H**). Samples has been counterstained with hematoxylin. Scale bar = 100 μm.

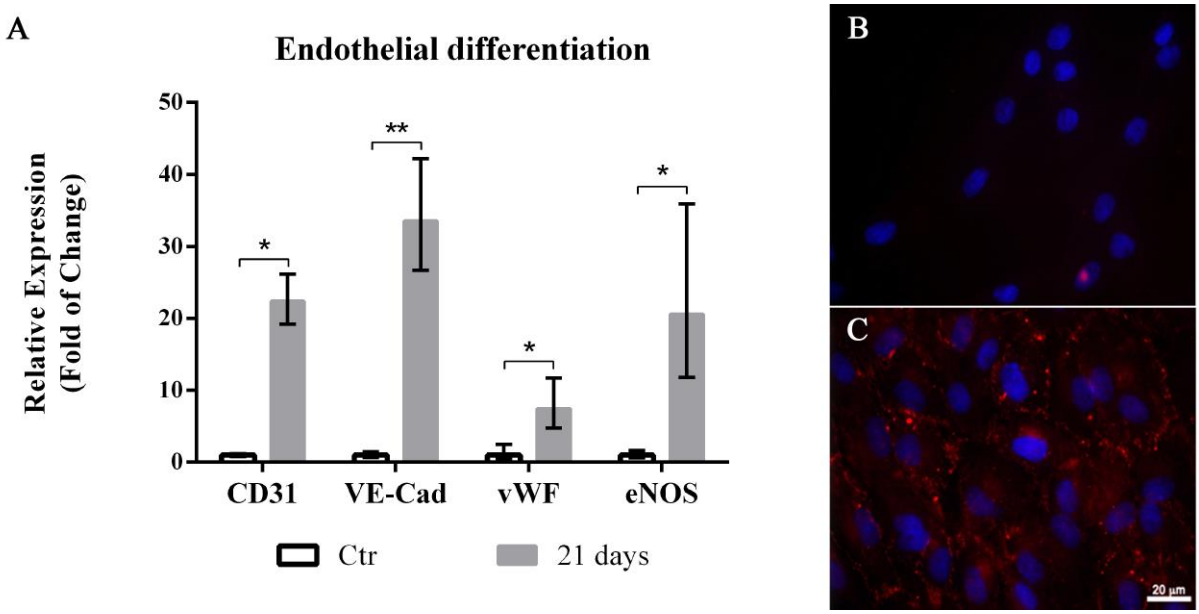
Figure 6



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

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

Figure 7

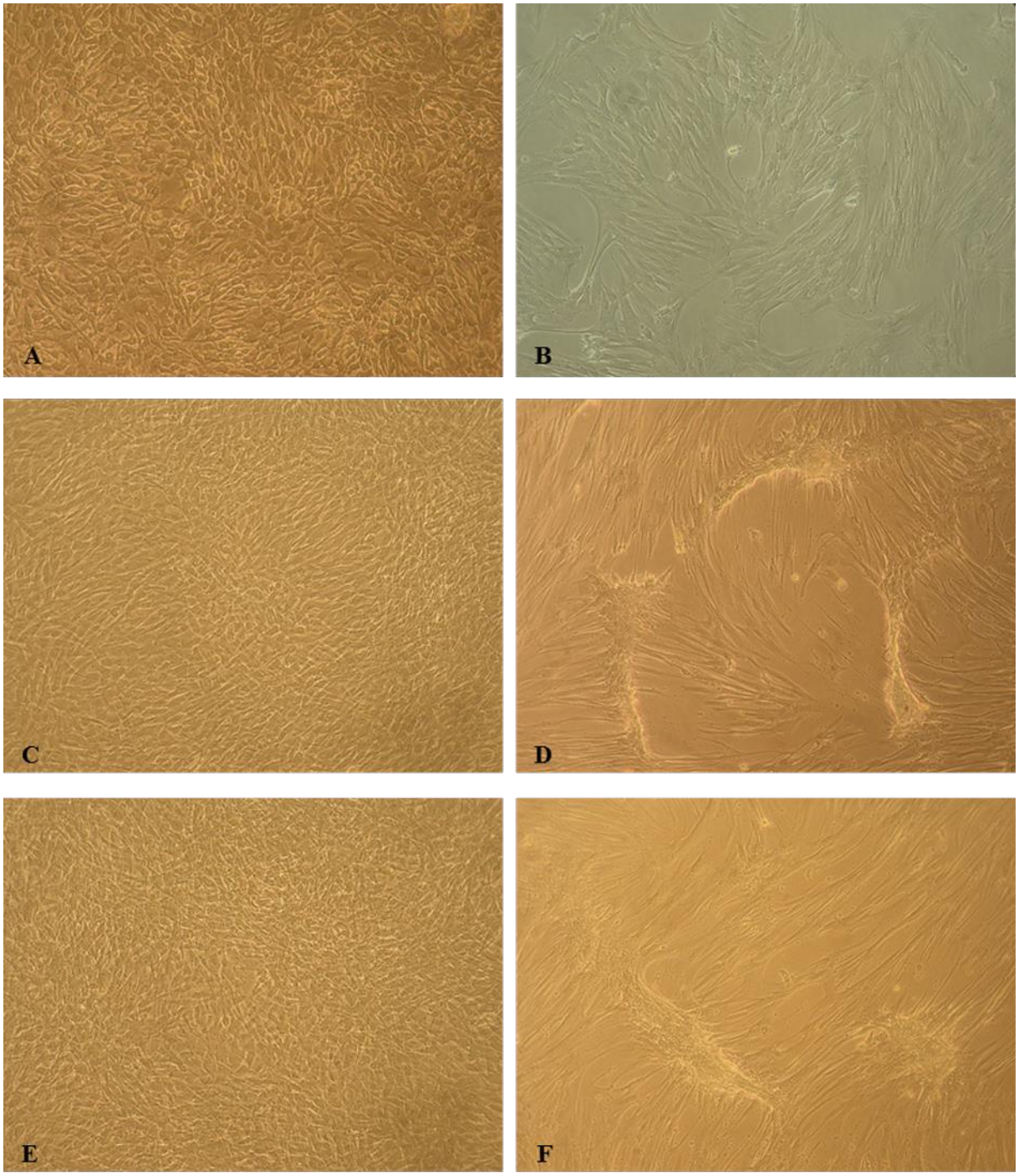


Endothelial lineage differentiation potential of pAVPC. Porcine Aortic Precursor Cells are able to differentiate toward the endothelial cell phenotype if *in vitro* cultured in an endothelial cell medium supplemented with 50 ng/µL VEGF. **A**: gene expression analysis of endothelial cell genes (CD31,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

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

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

Supplementary Figure 1

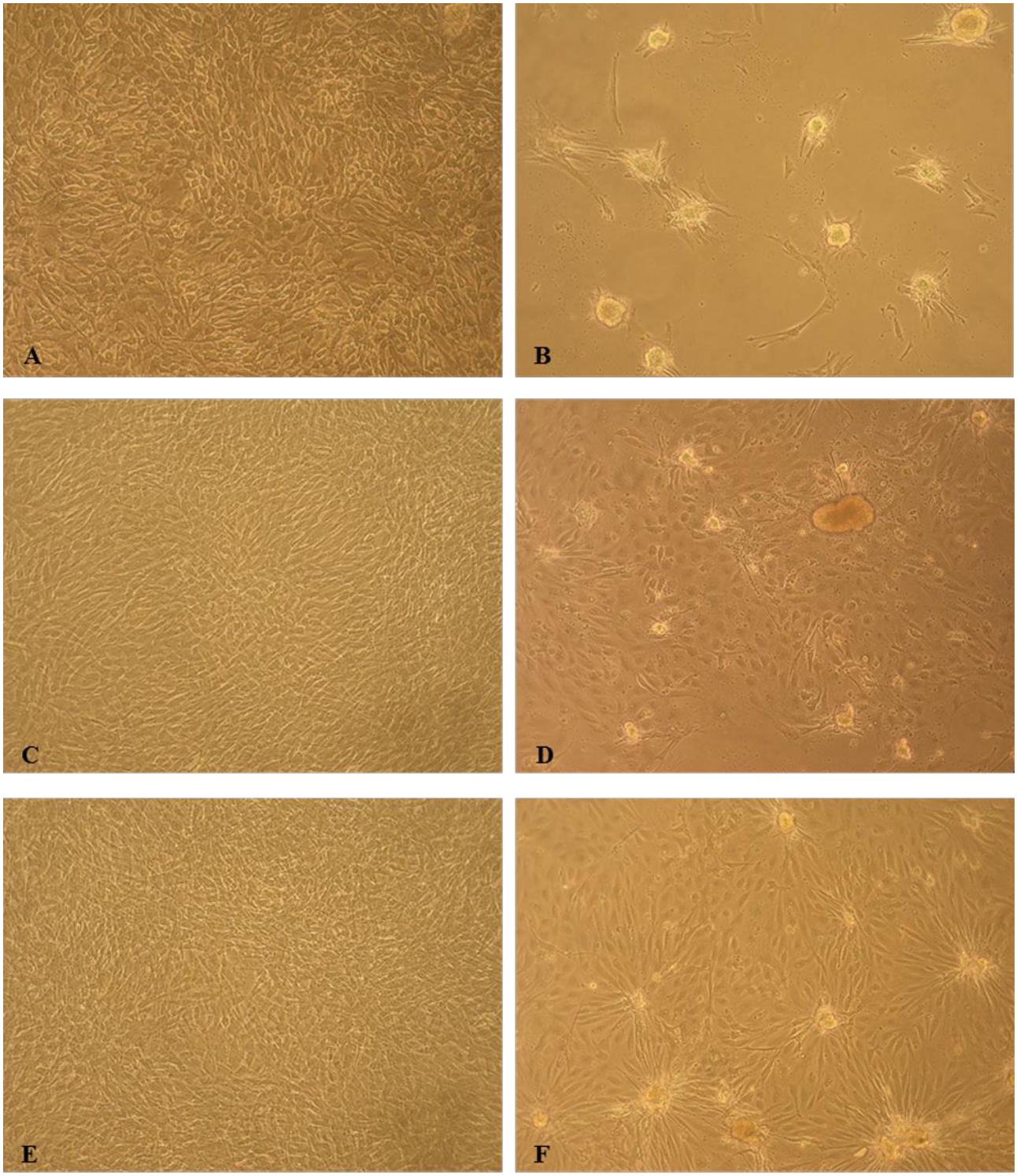


Long term culture and smooth muscle lineage differentiation potential of pAVPC – morphology. A, **C**, **E:** pAVPC grew in Pericyte Growth Medium (Control) at 7, 14 and 21 days, respectively; pAVPCs cultured in PGM grew as multilayer maintaining their classical shape with little thin and elongated arms. **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

Supplementary Figure 2



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

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