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

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Running Head: Vascular Stem-like Cells from porcine aorta

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Abstract

Recent findings suggest that progenitor and multipotent mesenchymal stromal cells (MSCs) are associated to vascular niches. Cells displaying mesenchymal proprieties and differentiate to whole components of a functional blood vessel, including endothelial and smooth muscle cells, can be defined Vascular Stem Cells (VSCs).

Recently, we isolated a population of porcine Aortic Vascular Precursor Cells (pAVPCs) which own MSCs and pericyte-like proprieties. The aim of the present work was to investigate whether pAVPCs possess VSC-like properties assessing their differentiation potential toward endothelial and smooth muscle lineages. pAVPCs, maintained in a specific Pericyte Growth Medium (PGM), were cultured in high glucose DMEM + 10% FBS (LTM) or in human Endothelial Serum Free Medium + 5% FBS and 50 ng/mL of hVEGF (EDM).

After 21 days of culture in LTM, pAVPCs showed an elongated fibroblast-like morphology and it seems to organize in cord-like structures. qPCR analysis of smooth muscle markers (αSMA, CNN1, SMM-hc) showed a significant increment of the transcripts and immunofluorescence analysis confirmed the presence of αSMA and SMM proteins. After 21 days of culture in EDM, pAVPCs displayed an endothelial cell-like morphology and revealed the up-regulation of the expression of endothelial markers (CD31, VE-Cadherin, vWF, eNOS) showing the CD31 typical pattern.

In conclusion, pAVPCs could be defined a VSC-like population considering that, if maintained in a specific pericytes medium, they express MSCs markers and they have, in addition to the classical mesenchymal trilineage differentiation potential, the capacity to differentiate *in vitro* toward the smooth muscle and the 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, 39, 41-44, 54, 55). According to these theories, blood vessels distributed in the whole body, could be considered a systemic reservoir of multipotent stem/progenitor cells (5, 45). In a recent review, Lin and Lue (30) 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) (30).

The presence of VSCs has been demonstrated in different types of vessels including embryonic, fetal and adult aorta. Undifferentiated mesenchymal cells from human fetal aorta have been isolated by Invernici and colleagues (23, 24). 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 (15) 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 (14, 23, 24).

Adult aorta is a good source of vascular mesenchymal stromal cells, too, as reported by Pasquinelli and colleagues (36) 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 structures in an *in vitro* angiogenesis assay (36).

Although the high potential of MSCs and precursor cells from vasculature in field of regenerative medicine (6, 10, 12, 25), several papers described these cells as potentially involved in pathogenesis of different diseases (21). Using a Knock Out (KO) mouse model of Apolipoprotein E (ApoE), Hu and colleagues (22) showed that adventitial progenitor cells contributed to atherosclerotic lesions in vein grafts. 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 (27). 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 (43).

Considering the potential of perivascular cells in field of regenerative medicine (10) and cardiovascular physio-pathology (21) and the usefulness of pig as animal model in these fields (16, 18, 28, 40, 48, 52, 53), 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* (51).

Despite pAVPCs have been properly characterized for their morphological, phenotypical and functional properties as pericytes-like cells (51), we observed (unpublished data) that they tend to lose their trilineage multipotency and to undergo senescence during *in vitro* culture, as demonstrated in other perycite-models (13, 46).

Therefore in the present work we decided to modify the previous protocol (51) and we cultured pAVPCs in a pericytes specific medium (Pericyte Growth Medium-PGM), that it was successfully used by other authors (3). Indeed, we verified with this new culture conditions MSC-like and pericyte-like properties of pAVPCs.

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 as Vascular Stem Cells (VSCs).

Material and methods

Cells isolation and culture

pAVPCs have been isolated as previously described by us (51) 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 Pericyte 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 (51).

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, CD44), pericytes (neural/glial antigen 2 [NG2], Nestin, platelet derived growth factor (PDGF) receptor β [PDGFR β], CD146, α-smooth muscle myosin [αSMA]), hemopoietic (CD45) marker 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 (51).

In particular, P3 cells were analyzed through flow cytometry for the expression of CD105, CD90, CD56, CD44, CD45, CD34, CD31. The antibodies and their 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 were cultured with the StemPro® Adipogenesis Differentiation Kit or the StemPro® Osteogenesis Differentiation Kit or the StemPro® Chondrogenesis Differentiation Kit (all purchased from GIBCO, Life Technologies) or in PGM (undifferentiated control cells) for 21 days.

At the end of each treatments, differentiated and undifferentiated (control cells cultured in PGM) cells were collected and 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. For the osteogenic differentiation, as suggested by the respective kit, cells were collected also at 7 days. 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 (44) 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) or in PGM (undifferentiated control cells). After 21 days, treated and control 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) or in PGM (undifferentiated control cells). After 21 days of culture, treated and respective control 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 and vWF) 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.

In vitro Angiogenesis assay

After 21 days of culture in EDM, pAVPCs were detached and an *in vitro* angiogenesis assay was performed using undiluited Geltrex™ LDEV-Free Reduced Growth Factor Basement Membrane Matrix (Gibco – catalog number A1413201) according to the manufacturer's protocol. 30000 cells/well were seeded on an eight-well chamber slide (BD Falcon) pretreated with 100ul/well of extracellular matrix. After 6 hours, cells were washed twice in PBS, fixed in 4% paraformaldehyde and immunofluorescence of CD31 and vWF was peformed.

Statistical analysis

Statistical analysis was carried out with R software (37). 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 controlcells, 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 a medium specific for pericytes- 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 (51) 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 (51). 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 (51) 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 And Phenotypical Characterization of pAVPCs cultured in PGM

The transcriptional profile of pAVPCs was reported in fig 2 A. Porcine Aortic Precursor Cells cultured in PGM express the main transcripts of MSCs (CD105, CD90, CD73, CD56, CD106, CD44), while do not express CD45, a marker of the hemopoietic lineage (data not shown). Moreover, pAVPCs do express pericytes main transcripts (NG2, Nestin, CD146, αSMA, PDGFRβ). 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 (Fig 2A).

264 Considering the vascular origin of pAVPCs, VEGF expression could be an important factor for the
265 cross-talk with endothelial cells in physiological and pathological angiogenic processes that involve
266 vascular precursor cells (17).

267 Passage 3 pAVPCs cultured in PGM have been characterized for MSC and pericytes markers
268 expression through flow cytometry analysis. Flow cytometry analysis (Fig. 2B) revealed that
269 pAVPCs expressed MSC markers as CD105 (85,97 ± 0.5 %), CD90 (99.5 ± 0.2 %), CD44 (99.6 ±
270 0.3 %) and less than 2% of them expressed the hemopoietic lineage markers as CD45 (1.4 ± 0.4 %),
271 CD34 (1.3 ± 0.1 %), as requested by the International Society of Cell Therapy (ISCT) guide lines
272 (14). Moreover, cells were negative for the expression of CD31(1.5 ± 0.1 %). In particular, as
273 already described for cells cultured in DM (51), cells expressed CD56 (99.9 ± 0.1 %) that is
274 considered a marker of a subsets of MSCs (2, 4, 38). The FACS analysis revealed the presence of a
275 more uniform population of cells when the *in vitro* culture is performed in PGM compared to the
276 culture in DM (51).

277 Immunocytochemical analysis revealed the expression of cell markers typical of pericytes (8, 11,
278 20, 27) as PDGFRβ, NG2 and Nestin (respectively Figure 3 A, B, C) as already described for the
279 culture in DM (51). A clear difference observed between cells cultured in different media was that
280 in PGM less than 2% of cells expressed αSMA (Figure 3 D), while in DM (51) 100% of cells
281 expressed it. The αSMA protein is considered a functional marker of differentiated pericytes (10)
282 and so it is expressed in pre-committed cells to the fully differentiated pericytes lineage, as also
283 showed by Tigges and colleagues (46). The observation that the cells cultured in PGM lost the
284 expression of that marker could indicate that the culture medium was able to maintain cells more
285 undifferentiated than DM.

286 Moreover, CD34 and CD31 (Fig. 3 E and F) was not expressed, too, so both these data allowed us
287 to exclude contamination of hemopoietic and endothelial cells (10).

Taken together, these data suggested that pAVPCss in PGM could be phenotypically considered pericyte-like cells, as they displayed several MCS and pericytes markers (8) confirming what it has been observed in our previous work (51)

Mesenchymal Trilineage Differentiation Potential

pAVPCs (P3) has been cultured in adipo-, osteo-, chondrogenesis induction media in order to investigate the classical trilineage differentiation potential (Fig. 4) requested for MSCs characterization (14) as already shown for pAVPCs cultured in DM (51).

Quantitative PCR analysis revealed the up-regulation of the expression of transcripts typical of each one of the three lineages described above compared with undifferentiated control cells cultured for the same time in PGM.

For adipogenic differentiation PPARγ and Adiponectin has been used as markers of differentiation (31). Transcripts of both genes have been detected significantly increased in differentiated cells compared to control cells respectively about 525 times (p *t-test* = 6.20×10^{-5}) and about 45000 times (p *t-test* = 0.003973) (Fig. 4 A).

For osteogenic differentiation ALPL and SPP1 have been used as markers of differentiation (29). In Fig. 4 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 have been used as markers of differentiation (50). Transcripts of both genes have 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. 4 G).

Moreover, cells cultured with adipogenic medium for 21 days grew as a monolayer showing the accumulation of lipid droplets stained with OilRedO (Fig. 4 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. 4 E, F) and cells, cultured as pellets in a chondrogenic medium for 21 days, positively stained for AlcianBlue confirming the presence of proteoglicans (Fig. 4 H, I).

All these data suggested that PGM was able to maintain pAVPCs undifferentiated as they displayed the classical trilineage differentiation potential that is requested for MSCs characterization (14) and that has been already showed by us for pAVPCs cultured using DM (51). 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 spontaneously differentiation towards smooth muscle phenotype cells has been cultured in LTM and then analyzed through qPCR and ICC.

pAVPCs cultured in LTM showed an elongated fibroblast-like shape (Fig 5 B, E, H) while pAVPCs cultured in PGM for the same time grew as a multilayer maintaining their classical shape with little thin and elongated arms (Fig. 5 A, D, G).

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 [49]) 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 (43) 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 in EDM cultured cells displayed an endothelial cell-like morphology growing as a monolayer upon which few spheroidal structures could be observed (Fig. 5C, F, I).

Gene expression analysis (Fig. 7 A) revealed the up-regulation of the 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 EDM cultured cells compared to undifferentiated control cells. CD31 and vWF expression has been

355 investigated at the protein level, too, and both endothelial markers have been detected only in
356 differentiated cells showing their typical distribution pattern(Fig. 7 C and 7 E). Control cells did not
357 express CD31 or vWF protein (Fig. 7 B and D).

358 *In vitro* angiogenesis assay

359 pAVPCs cultured in EDM were able to develop a complex tube networks with elongated branch
360 points (Fig 7G, I, L) when cultured on an extracellular matrix, while control cells failed to create a
361 capillary-like network even if they tried to organize in cord-like structure(Fig 8F) as indicated by
362 our previous report (51). Endothelial differentiated cells that formed the network expressed both
363 CD31 and vWF proteins (Fig 7 H and 7 M).

364 All these data confirm that pAVPCs cultured in a specific endothelial growth medium
365 supplemented with Vascular Endothelial Growth Factor differentiate to the endothelial phenotype,
366 in agreement with results obtained for multipotent cells derived from embryonic, fetal and human
367 aorta (15, 23, 24, 32-34) and by Pankajakshan et al (2013) that reported the differentiation of
368 porcine bone marrow derived-MSCs to endothelial cells after stimulation with 50 ng/μL VEGF
369 (35).

370

371 Mesenchymal stromal cells in which depletion of PDFGRβ signaling occurs have been reported to
372 have an high angiogenic potential as they produced pro-angiogenic growth factor and expressed
373 endothelial cells marker *in vitro*, while *in vivo* they potently stimulate neo-vascularization (1).
374 Greenberg and colleagues (19) described that VEGF, activating its receptor VEGFR2, is able to
375 suppress PDGFRβ signaling in vascular smooth muscle cells (VSMCs) through the assembly of a
376 PDGFRβ/VEGFR2 complex.

Considering that pAVPCs have been defined as MSC-like cells (51), 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. This 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* (20) 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 (51) 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 Pericyte Growth Medium have been shown to be a 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 (14). Moreover, pAVPCs have 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 (51), leads 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 pAVPCscould 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 (30).

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 (21, 22, 27) 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 (43). Moreover, in the present paper we presented data that support the ability of pAVCPs to differentiate to endothelial phenotype after 21 days of culture in a specific endothelial growth medium supplemented with VEGF, infact they expressed markers of endothelial cells and they were able to develop a complex capillary network in an *in vitro* angiogenesis assay, demonstrating their *in vitro* functional endothelial properties. 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, 25).

Indeed, it is important to remember that cells, like the multipotent pericytes with which pAVPCss 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, 45).

So, considering the recent definition of Vascular Stem Cells (VSCs) by Lin and Lue (30) 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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601 **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
CD44	EU041925	CAGGTACGGATTCAAATATCATCTCAGC	ACTGGGGTGTTTGTCTCTTTCATCTTC	80
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
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

602

603 **Table 1:** List of primer pairs, amplicon size (bp) and genes analyzed in the paper

Antibody	*P. number*	*Species*	*Supplier*	*Dilution*
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

604

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

606

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
vWF	MCA4677G	Mouse	Serotec	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

607

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

Figure Captions

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 pAVPCss cultured in PGM is represented in the picture. Cells displayed an increasing replication time between P1 and P6. Scale bar A = 200 μm. Scale bar B = 100 μm.

Figure 2

A: Transcriptional characterization of pAVPCs. The graph represent the mRNA quantification, through qPCR, of the main markers of MSCs –*CD90 (Thy-1), CD73 (5'-nucleotidase [5'-NT], CD105 (endoglin)also known as ecto-5'-nucleotidase), CD106 (vascular cell adhesion molecule 1 [VCAM-1]) CD56 (NCAM – neural cell adhesion molecule), CD44* – 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 derived growth factor β)*. Data are expressed as ΔCq calculated as Cq value obtained from the geometric mean of the reference genes minus Cq value of the gene of interest.

B: Immunophenotyping pAVPCs cultured in PGM. Flow cytometry analysis of P3 pAVPCscultured 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 3

Immunocytochemical characterization of pAVPCs cultured in PGM. In each microphotograph 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. Scale bar = 20 μm.

Figure 4

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 transcripts, 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 differentiated cells with undifferentiated control cells) 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

669 phenotype, was present in differentiated pellet (**I**) and absent in controls (**H**). Samples has been

670 counterstained with hematoxylin. Scale bar = 100 μm.

671

672 **Figure 5**

673

674 **Muscle and endothelial lineage differentiation potential of pAVPCs – morphology. A**, **D**, **G :**
675 pAVPCs grew in Pericyte Growth Medium (undifferentiated control cells) at 7, 14 and 21 days,
676 respectively; pAVPCs cultured in PGM grew as multilayer maintaining their classical shape with
677 little thin and elongated arms. **B**, **E**, **H** pAVPCs grew in Long Term Medium at 7, 14 and 21 days,
678 respectively; pAVPCs cultured in LTM showed an elongated fibroblast-like shape and seems to be
679 organized in elongated superstructures. Magnification: 10X. **C, F, I** pAVPCs grew in Endothelial
680 Differentiation Medium at 7, 14 and 21 days, respectively; pAVPCs stimulated with EDM
681 displayed an endothelial cell-like morphology growing in cluster and forming an endothelial cell-
682 like monolayer upon which some cells organized in few spheroidal structures. Scale bar = 100 μm.

683

684

685

Figure 6

Smooth muscle lineage differentiation potential of pAVPCss. 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 pAVPCss. In the Y-axis in the graph is represented the relative expression of each transcripts analyzed in pAVPCs cultured in LTM for 21 days compared to control cells cultured for the same period in PGM ($2^{-\Delta\Delta Cq}$ method). 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. Nuclei has been stained with Hoechst 33258 (blue). Scale bar = 50 μm.

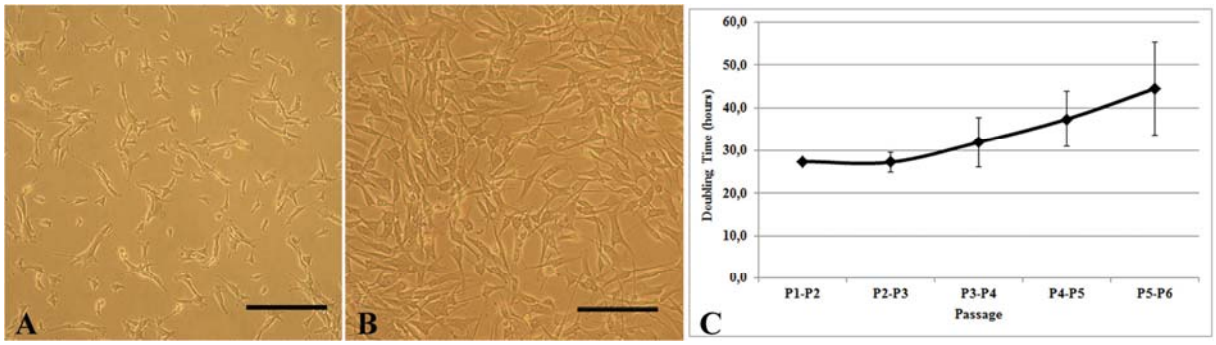
Figure 7

Endothelial lineage differentiation potential of pAVPCs. Porcine Aortic Precursor Cells are able to differentiate toward the endothelial cell phenotype if *in vitro* cultured in an endothelial specific growth 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 pAVPCss. In the Y-axis in the graph is represented the relative expression of each transcripts analyzed in pAVPCs cultured in EDM for 21 days compared to undifferentiated control cells cultured for the same period in PGM ($2^{-\Delta\Delta Cq}$ 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, D: CD31 and vWF immunostaining respectively of undifferentiated control cells. **C, E**: CD31 and vWF immunostaining respectively of differentiated cells in EDM. Nuclei has been stained with Hoechst 33258 (blue). Scale bar = 50 μm

F-M In vitro Angiogenesis assay. pAVPCs developed a complex tube networks with elongated branch points (Fig 8 G, I, L) when cultured on an extracellular matrix after endothelial differentiation protocol, while control cells tried to form only a cord like structure (Fig 8 F). Differentiated cells that formed the network expressed both CD31 and vWF protein (Fig 8 H and 8 M). Scale bar F, I, L = 100 μm; Scale bar G = 150 μm; Scale bar H, M = 50 μm.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Passage

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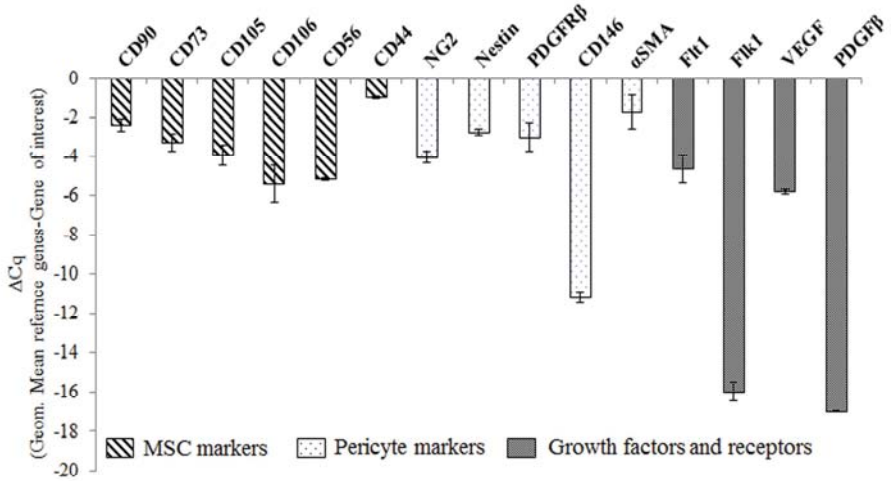
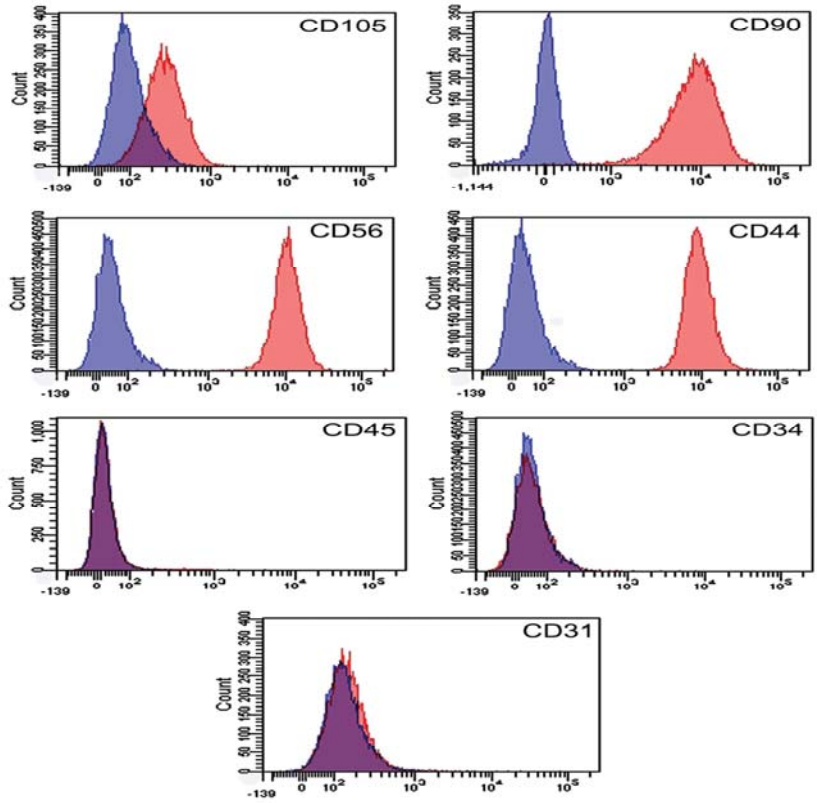
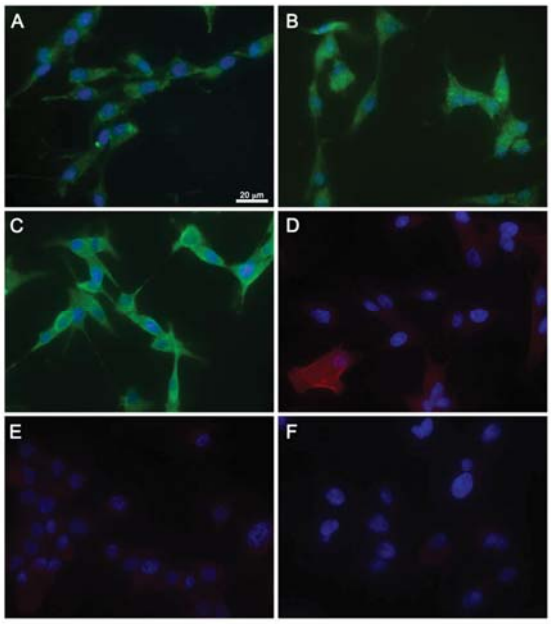
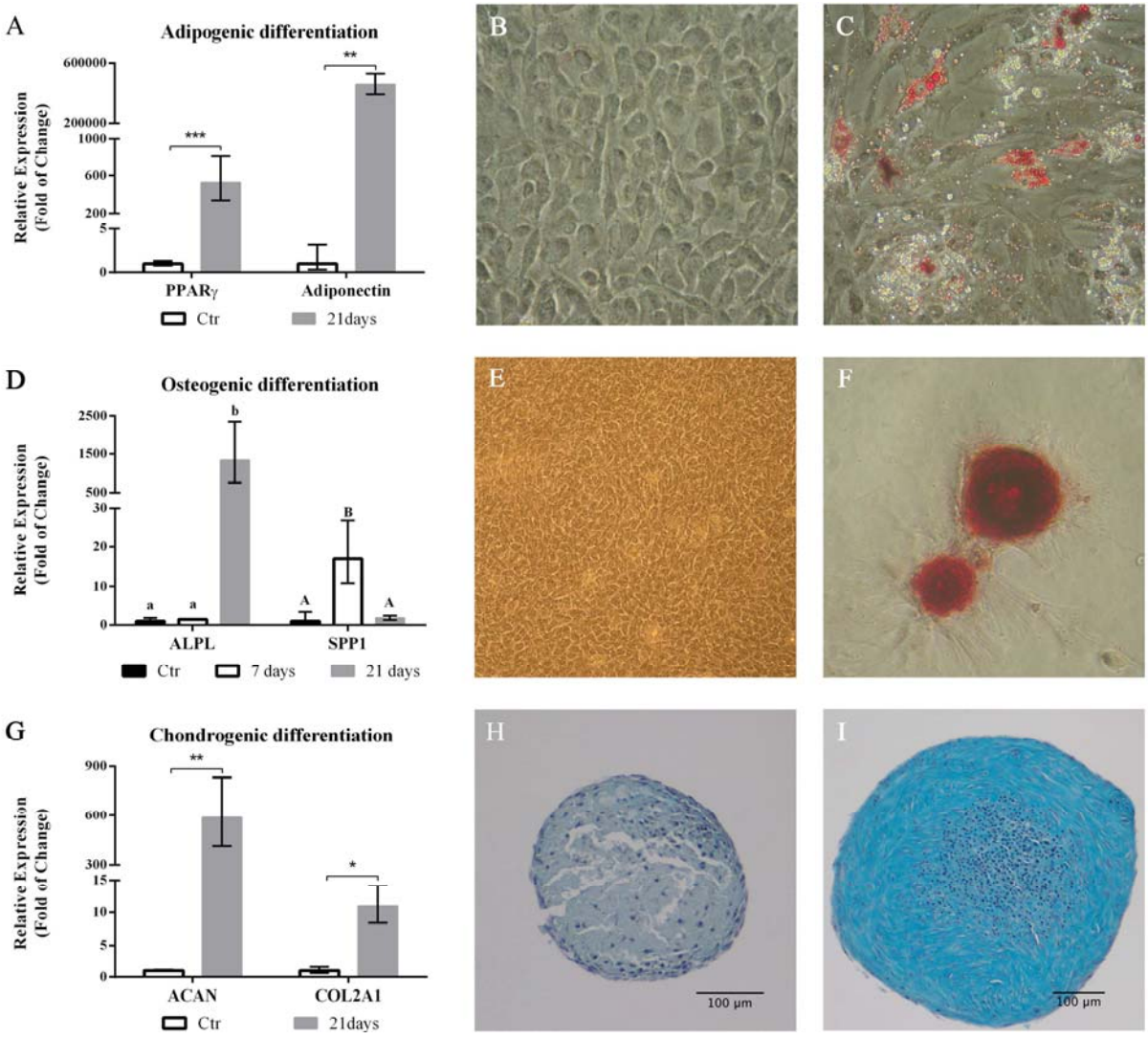
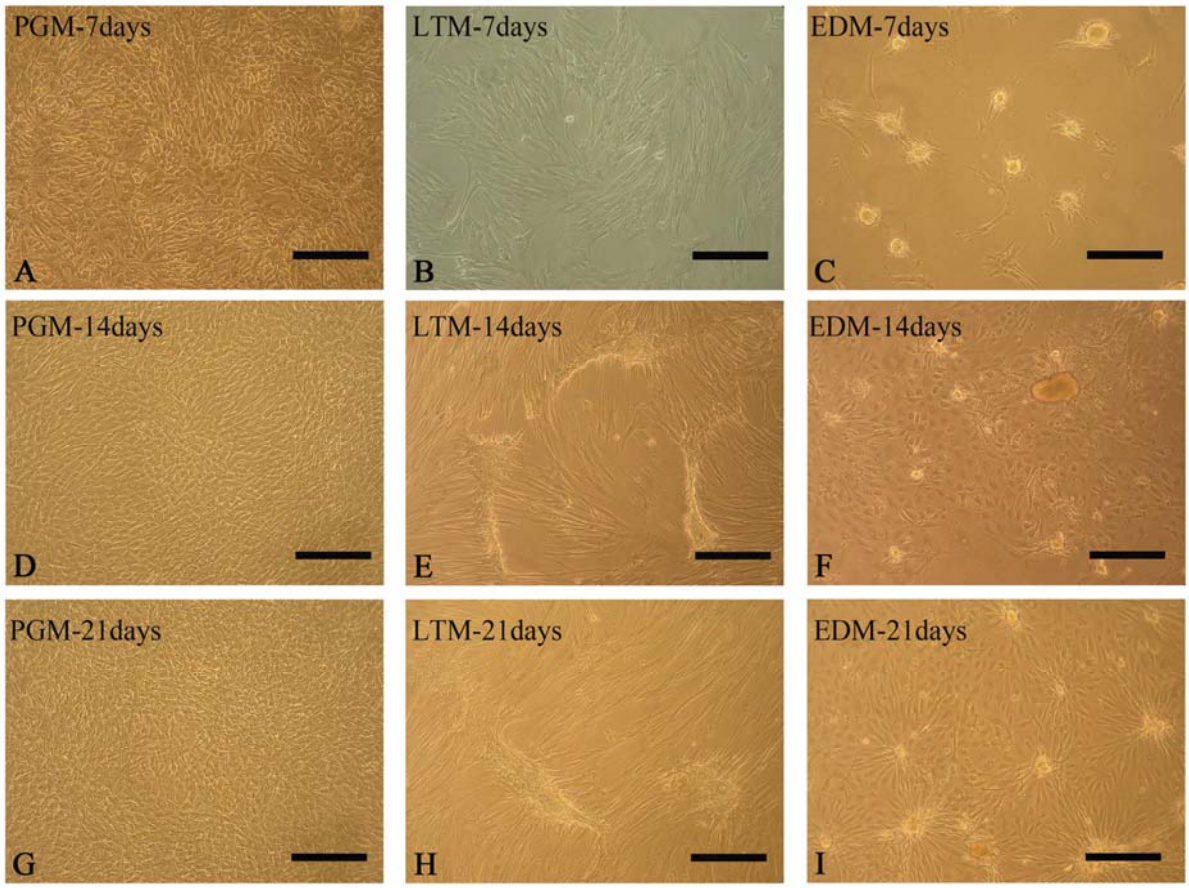

CD90
CD73
CD105
CD106
CD56
CD44
NG2
Nestin
PDGFRβ
CD146
αSMA
Flt1
Flk1
VEGF
PDGFβ
ΔCq
(Geom. Mean refernce genes-Gene of interest)
0
-2
-4
-6
-8
-10
-12
-14
-16
-18
-20
MSC markers
Pericyte markers
Growth factors and receptors

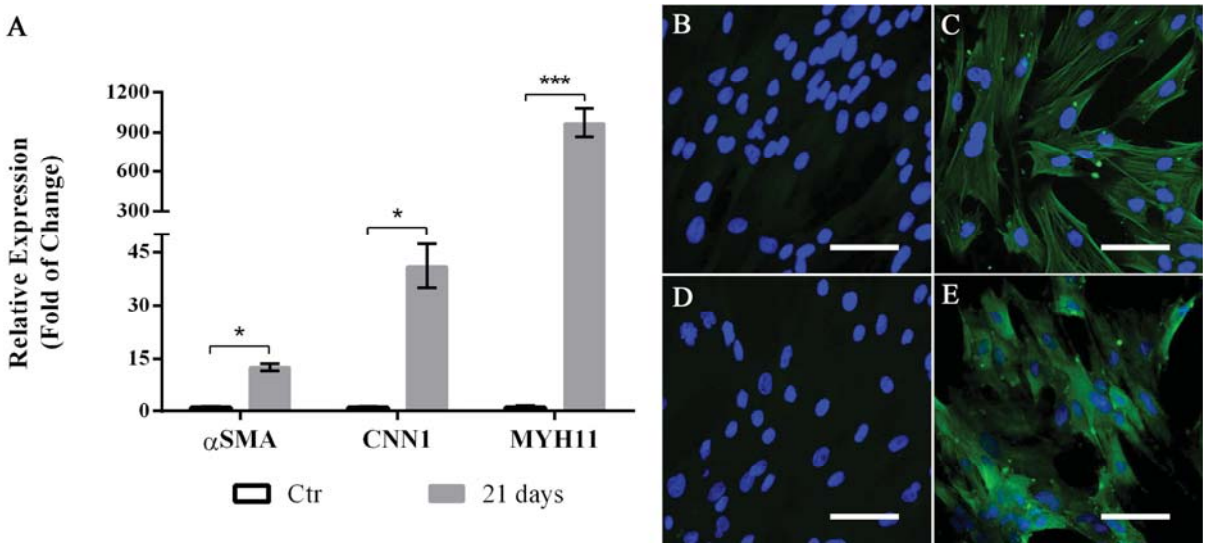
B

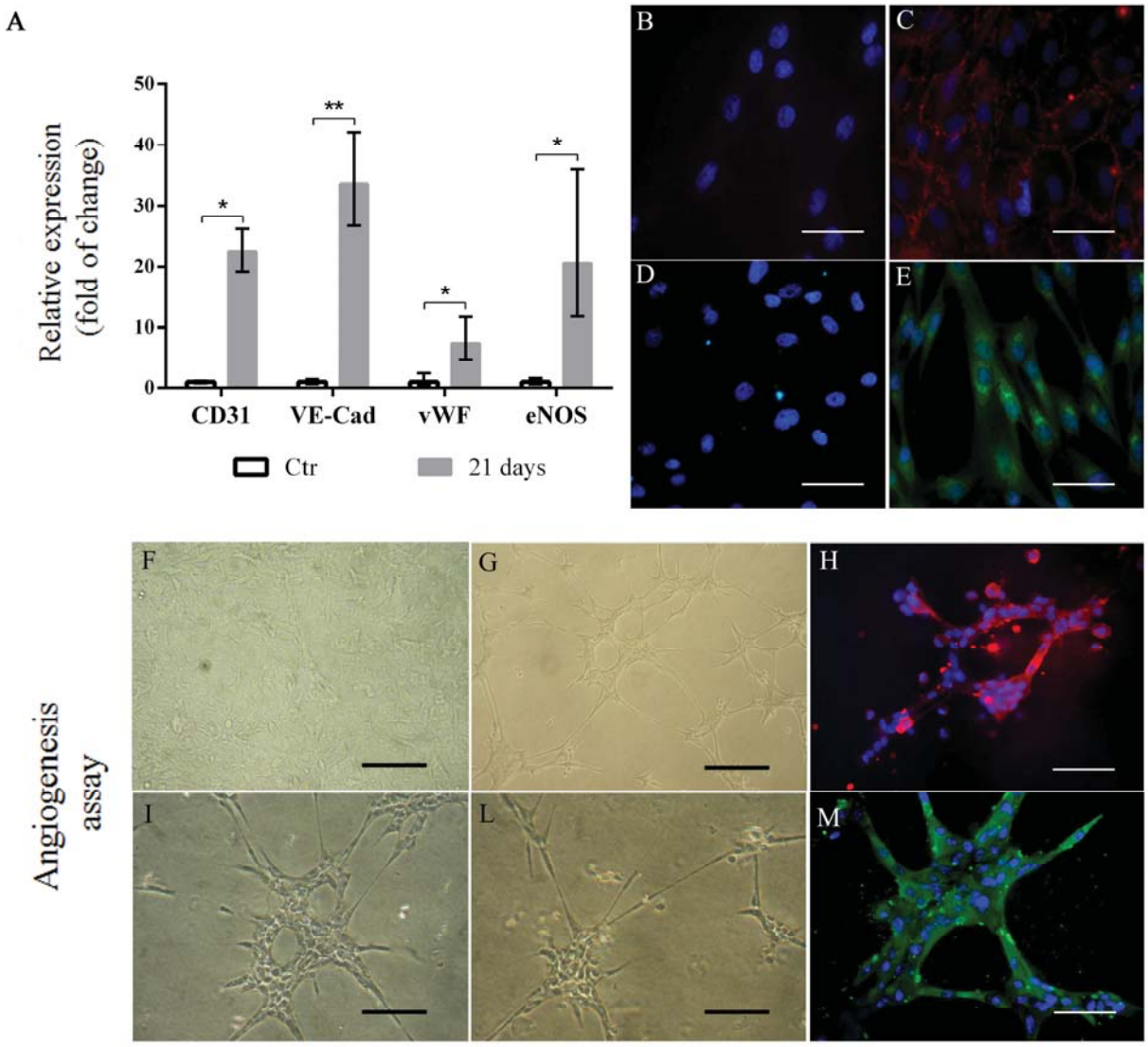

CD105
CD90
CD56
CD44
CD45
CD34
CD31
Count


A
B
20 µm
C
D
E
F


A
Adipogenic differentiation
Relative Expression (Fold of Change)
600000
200000
1000
600
200
5
0
***
**
PPARγ
Adiponectin
Ctr
21days
B
C
D
Osteogenic differentiation
Relative Expression (Fold of Change)
2500
1500
500
30
20
10
0
a
a
b
A
B
A
ALPL
SPP1
Ctr
7 days
21 days
E
F
G
Chondrogenic differentiation
Relative Expression (Fold of Change)
900
600
300
15
10
5
0
**
*
ACAN
COL2A1
Ctr
21days
H
100 µm
I
100 µm


PGM-7days
A
LTM-7days
B
EDM-7days
C
PGM-14days
D
LTM-14days
E
EDM-14days
F
PGM-21days
G
LTM-21days
H
EDM-21days
I


A
Relative Expression
(Fold of Change)
1200
900
600
300
45
30
15
0
*
*
***
αSMA
CNN1
MYH11
Ctr
21 days
B
C
D
E


A
Relative expression (fold of change)
50
40
30
20
10
0
*
**
*
*
CD31
VE-Cad
vWF
eNOS
Ctr
21 days
B
C
D
E
Angiogenesis assay
F
G
H
I
L
M