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In vitro differentiation of porcine aortic vascular precursor cells to endothelial and vascular smooth muscle cells

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Zaniboni A, Bernardini C, Bertocchi M, Zannoni A, Bianchi F, Avallone G, Mangano C, Sarli G, Calzà L, Bacci ML, Forni M. In vitro differentiation of porcine aortic vascular precursor cells to endothelial and smooth muscle cells. Am J Physiol Cell Physiol 309: C320–C331, 2015. First published July 1, 2015; doi:10.1152/ajpcell.00049.2015.—Recent findings suggest that progenitor and multipotent mesenchymal stromal cells (MSCs) are associated with vascular niches. Cells displaying mesenchymal properties and differentiating to whole components of a functional blood vessel, including endothelial and smooth muscle cells, can be defined as vascular stem cells (VSCs). Recently, we isolated a population of porcine aortic vascular precursor cells (pAVPCs), which have MSC- and pericyte-like properties. The aim of the present work was to investigate whether pAVPCs possess VSC-like properties and assess their differentiation potential toward endothelial and smooth muscle lineages. pAVPCs, maintained in a specific pericyte growth medium, were cultured in high-glucose DMEM + 10% FBS (long-term medium, LTM) or in human endothelial serum-free medium + 5% FBS and 50 ng/ml of hVEGF (endothelial differentiation medium, EDM). After 21 days of culture in LTM, pAVPCs showed an elongated fibroblast-like morphology, and they seem to organize in cord-like structures. qPCR analysis of smooth muscle markers [α-smooth muscle actin (α-SMA), calponin, and smooth muscle myosin (SMM) heavy chain] showed a significant increment of the transcripts, and immunofluorescence analysis confirmed the presence of α-SMA and SMM proteins. After 21 days of culture in EDM, pAVPCs displayed an endothelial cell-like morphology and revealed the upregulation of the expression of endothelial markers (CD31, vascular endothelial-cadherin, von Willebrand factor, and endothelial nitric oxide synthase) showing the CD31-typical pattern. In conclusion, pAVPCs could be defined as a VSC-like population considering that, if they are maintained in a specific pericyte medium, they express MSC 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 cell phenotypes.

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vascular stem cells; mesenchymal stromal cells; endothelial differentiation; smooth muscle differentiation; pig animal model

RECENT FINDINGS AND THEORIES have suggested that tissue-specific progenitor cells and multipotent mesenchymal stem cells (MSCs) are anatomically and functionally associated with 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 displays MSC properties and that are able to differentiate to the whole component of a functional blood vessel (including endothelial and smooth muscle cells) can be defined as 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 opportunistically stimulated with VEGF or PDGF-β, 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-positive, VEGF receptor 2 (VEGFR2, also known as Flk1)-positive, and CD34-negative vascular cells that were able to differentiate to endothelial and vascular smooth muscle cells, if cultured, respectively, with VEGF or PDGF-β, 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 MSCs, too, as reported by Pasquini and colleagues (36), who isolated from tunica media and adventitia, respectively, CD34-positive and c-kit-positive progenitor cells. Both cell populations were positive for the expression of MSC 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 the field of regenerative medicine has been reported (6, 10, 12, 25), several studies have described these cells as potentially involved in pathogenesis of different diseases (21). Using a knockout mouse model of apolipoprotein E, Hu and colleagues (22) showed that adventitial progenitor cells contributed to atherosclerotic lesions in vein grafts. Instead, in 2010, Juchem and colleagues (27) described a high prothrombotic potential of intimal pericytes in the endothelial-de-nudated vascular region, which supports the hypothesis that these cells are involved in the pathogenesis of atherosclerosis, thrombosis, and saphenous vein graft disease. Moreover, in 2012, in a well-performed work by Tang and colleagues (43), it was described that a population of stem cells resident in the blood vessel wall, named multipotent VSCs, were able to differentiate to an MSC-like phenotype and subsequently to the smooth muscle one spontaneously. In that work, it was also shown that, in response to 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 the field of regenerative medicine (10) and cardiovascular physiopathology (21) and the usefulness of pig as an 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 the fact that pAVPCs have been properly characterized for their morphological, phenotypical, and functional properties as pericyte-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 pericyte models (13, 46).

Therefore, in the present work, we decided to modify the previous protocol (51), and we cultured pAVPCs in a pericyte-specific medium (pericyte growth medium, PGM), which was successfully used by other authors (3). Indeed, we verified with these 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 PGM possess VSC-like properties, assessing their differentiation potential toward the endothelial and the smooth muscle lineages to define these cells as VSCs.

**MATERIALS AND METHODS**

**Cell isolation and culture.** pAVPCs were 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, Carlsbad, CA), to which 10% FBS and 10× antibiotic-antimycotic (hgDMEM-10×) were added in a 5% CO2 incubator at 38.5°C. The culture medium was then replaced with hgDMEM + 10% FBS (GIBCO) + 1× antibiotic-antimycotic (GIBCO) (hgDMEM-1×).

After 3 days of culture with hgDMEM-1× medium, the cells were serum starved overnight (24 h) with hgDMEM + 1× antibiotic-antimycotic. After serum starvation, the cells were cultured in hgDMEM-199 (GIBCO) (1:1), to which 10% FBS and 1× antibiotic-antimycotic (DM medium) were added until 60–65% confluency was reached. The cells were trypsinized and cultured to passage (P) 6 in 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 MSCs (CD105, CD90, CD73, CD56, CD106, and CD44), pericytes (neural/glial antigen 2 (NG2), nestin, PDGF receptor-β (PDGFR-β), CD146, and α-smooth muscle actin (α-SMA)), hemopoietic (CD45) marker, and for the gene expression of VEGFR1 (also known as Flt1), VEGFR2 (also known as Flk1), VEGF, and PDGF-β.

Total RNA was extracted from 2×10⁶ 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, Hercules, CA), following the manufacturer’s instructions, in a 20-µl final volume to obtain cDNA.

Primers were designed using Beacon Designer 2.07 Software (Premier Biosoft International, Palo Alto, CA). Primer 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, and 1× iTaq Universal SYBR Green Supermix (Bio-Rad). One microliter of cDNA was added to 19 µl of the master mix. All samples were analyzed in duplicate. The qPCR protocol used for the transcriptional characterization was as follows: 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 cycles of 0.5°C increase/cycle).

The gene expression was evaluated using the ΔCq method (refer-ence gene Cq — gene of interest Cq). As Cq for the reference gene, the geometric mean of Cq of three different reference genes [β-actin, hypoxanthine phosphoribosyltransferase (HPRT), and GAPDH] was considered. Reference gene primer 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, and CD31. The antibodies and their concentrations used for the analysis are listed in Table 2.

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

**Mesenchymal trilineage differentiation potential.** Cells at P3 were cultured with the StemPro Adipogenesis Differentiation Kit, the StemPro Osteogenesis Differentiation Kit, the StemPro Chondrogenesis Differentiation Kit (all purchased from GIBCO-Life Technologies), or with PGM (undifferentiated control cells) for 21 days.
At the end of each treatment, differentiated and undifferentiated (control cells cultured in PGM) cells were collected, and the expression of typical genes of differentiated osteocytes [alkaline phosphatase (ALP) and osteopontin], adipocytes [peroxisome proliferator-activated receptor-β (PPAR-β) or adiponectin], and chondrocytes [aggrecan (ACAN) and collagen type II (COL2A1)] was evaluated through qPCR. For the osteogenic differentiation, as suggested by the respective kit, cells were collected also at 7 days.

Table 1. List of primer pairs, amplicon size, and genes analyzed

<table>
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<tr>
<th>Genes Accession Number</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
<th>Product Size, bp</th>
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<td>CD106 NM_213891</td>
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<td>ATCACTAGACAGGCTCATGTTTCAC</td>
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<tr>
<td>CD1105 NM_214031.1</td>
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<td>CD90 NM_001146129.1</td>
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<td>CD44 EU041925</td>
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<td>GGAATACAGGCTGAGGAGGTC</td>
<td>TGCCTGGCAAAGTCAGGTAG</td>
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</table>

NG2, neural/glial antigen 2; PDGFR-β, PDGF receptor-β; α-SMA, α-smooth muscle actin; CNN1, calponin 1; SMM-hc, smooth muscle myosin heavy chain; VE-cadherin, vascular endothelial cadherin; vWF, von Willebrand factor; eNOS, endothelial nitric oxide synthase; PPAR-γ, peroxisome proliferator-activated receptor-γ; ALP, alkaline phosphatase; SPP1, secreted phosphoprotein 1; ACAN, aggrecan; COL2A, collagen type II α1; HPRT, hypoxanthine phosphoribosyltransferase.

At the end of each treatment, differentiated and undifferentiated (control cells cultured in PGM) cells were collected, and the expression of typical genes of differentiated osteocytes [alkaline phosphatase (ALP) and osteopontin], adipocytes [peroxisome proliferator-activated receptor-γ (PPAR-γ) or adiponectin], and chondrocytes [aggrecan (ACAN) and collagen type II α1 (COL2A1)] was evaluated through qPCR. For the osteogenic differentiation, as suggested by the respective kit, cells were collected also at 7 days. Total RNA extrac-

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

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<th>Supplier</th>
<th>Dilution</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<tr>
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<td>Abcam</td>
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<td>CD31 unconjugated</td>
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<td>Mouse</td>
<td>AbD Serotec</td>
<td>1:100</td>
</tr>
<tr>
<td>Secondary</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-rabbit-PE</td>
<td>Ab97070</td>
<td>Goat</td>
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<td>Anti-mouse-FTTC</td>
<td>420-120-050</td>
<td>Sheep</td>
<td>BioFX</td>
<td>1:100</td>
</tr>
</tbody>
</table>

APC, allophycocyanin.
tion, retrotranscription, and qPCR were performed as described above. Primers for the analysis were designed with Beacon Designer 2.07 software. Primer sequences and size (bp) of each product are listed in Table 1. The expression of these genes was calculated using the \( \Delta \Delta C_q \) method (gene of interest \( C_q \) – reference gene \( C_q \)). As reference gene \( C_q \), the geometric mean of \( \beta \)-actin, HPRT, and GAPDH \( C_q \) value was considered. Relative expression was calculated as the fold of increase with the \( 2^{-\Delta\Delta C_q} \) method (\( \Delta \Delta C_q = \Delta C_q \) differentiated cells – \( \Delta C_q \) control cells).

After 21 days, assessment of differentiation was carried out through classical histological staining, too [Oil Red O, Alizarin red, and Alcian blue (all purchased from Sigma-Aldrich, St. Louis, MO)] 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-\( \mu \)m thickness were obtained and mounted on polarized slides. Sections were deparaffinized with xylene and rehydrated through passages into an increasing-concentration alcoholic ladder. Slides were stained with Alcian blue solution and rehydrated through passages into an increasing-concentration alcoholic ladder. Slides were stained with Alcian blue solution (pH 2.5) for 30 min, washed in water, and counterstained with Harris hematoxylin (Merck, Nottingham, Nottinghamshire, United Kingdom).

Long-term culture and assessment of smooth muscle cell differentiation potential. Tang and colleagues (43) reported that long-term culture on pAVPCs, 1,500 cells/cm\(^2\) at P3 were seeded in a 24-well plate and cultured in DMEM + 10% FBS + 1× 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 cell markers both with qPCR (α-SMA, calponin (CNN1), and SMM-hc) and immunofluorescence (α-SMA and 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. Relative expression was calculated as described above. The primary and secondary antibodies and their concentrations used for the analysis are listed in Table 3.

**Endothelial differentiation potential.** Cells at P3 were seeded in a 24-well plate at a density of 5,000 cells/cm\(^2\) and cultured in human endothelial serum-free medium (GIBCO) supplemented with 5% FBS, 1× antibiotic-antimycotic, and 50 ng/ml of hVEGF EDM or with PGM (undifferentiated control cells). After 21 days of culture, treated and control cells were analyzed for the expression of differentiated endothelial cell markers both with qPCR [CD31, vascular endothelial (VE)-cadherin, vWF, and 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. Relative expression was calculated as described above (see Tables 1 and 3).

**In vitro angiogenesis assay.** After 21 days of culture in EDM, pAVPCs were detached, and an in vitro angiogenesis assay was performed using undiluted Gelretx LDEV-Free Reduced Growth Factor Basement Membrane Matrix (GIBCO, catalog no. A1413201).

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**Table 3. Immunocytochemistry antibodies list used for cell characterization**

<table>
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<tr>
<th>Antibody</th>
<th>Product Number</th>
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<th>Dilution</th>
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<td>Santa Cruz Biotechnology</td>
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*PECAM, platelet endothelial cell adhesion molecule; RRX, Rhodamine Red X.*

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**Fig. 1.** Porcine aortic vascular precursor cell (pAVPC) morphology and doubling time during culture in pericyte growth medium (PGM). A and B: cells cultured in PGM, after isolation and starvation step, displayed a small cell body and elongated thin arms at their ends. In A a microphotograph (×10 magnification) of a low-confluence culture of pAVPCs is shown, whereas in B a microphotograph (×10 magnification) of a 60% confluence culture of pAVPCs is shown. C: growth curve of pAVPCs cultured in PGM is represented in the picture. Cells displayed an increasing replication time between passage 1 (P1) and P6. Scale bar in A = 200 \( \mu \)m. Scale bar in B = 100 \( \mu \)m.
according to the manufacturer’s protocol. Cells (30,000 cells/well) were seeded on an eight-well chamber slide (BD Falcon, Franklin Lakes, NJ) pretreated with 100 μl/well of extracellular matrix. After 6 h, cells were washed twice in PBS and fixed in 4% paraformaldehyde, and immunofluorescence of CD31 and vWF was performed.

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 Student’s-t test comparing differentiated and undifferentiated control cells, whereas data obtained from gene expression analysis of osteogenic differentiation were analyzed using one-way ANOVA, followed by Tukey’s post hoc comparison, to detect differences among control cells and cells differentiated for 7 and 21 days.

RESULTS

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

Fig. 2. A: transcriptional characterization of pAVPCs. The graph represents the mRNA quantification, through qPCR, of the main markers of mesenchymal stromal cells (MSCs), CD90 (Thy-1), CD73 [5’-nucleotidase (5’-NT)], CD105 (endoglin, also known as ecto-5’-nucleotidase), CD106 (vascular cell adhesion molecule 1), CD56 (neural cell adhesion molecule), and CD44; markers of pericytes, neuron-glial antigen 2 (NG2, also known as chondroitin sulfate proteoglycan 4), nestin, CD146 (melanoma cell adhesion molecule), α-smooth muscle actin (α-SMA), and platelet-derived growth factor receptor-β (PDGFRβ); and markers of angiogenesis-related growth factors and receptors, fms-related tyrosine kinase 1 (Flt1, also known as vascular endothelial growth factor receptor 1 (VEGFR1)), fetal liver kinase 1 (Flk1, also known as VEGFR2), VEGF, and PDGF-β. Data are expressed as ΔCq calculated as Cq values obtained from the geometric means of the reference genes minus Cq values of the gene of interest. B: immunophenotyping pAVPCs cultured in PGM. Flow cytometry analysis of P3 pAVPCs cultured in PGM shows that they stained positively for CD105, CD90, CD56, and CD44, whereas <2% of cells were positive for CD45 (protein tyrosine phosphatase, receptor type C) and CD34, and they were negative for the expression of CD31 (platelet endothelial cell adhesion molecule). Red histograms, stained cells; blue histograms, control cells.
showed a spindle-shaped/fibroblast-like morphology (51), unlike if cultured in PGM. Moreover, the growth of spheroidal structures in PGM was not observed, whereas, in the first passages of the culture in DM, it was possible to observe these kind of structures (51). Doubling time was calculated for cells cultured between P1 and P6 and increased from 27.5 ± 0.6 h, between P1 and P2, to 44.4 ± 10.9 h, between P5 and P6 (Fig. 1C). Interestingly, the growth curve obtained for cells cultured in PGM was parallel to the one obtained for the culture in DM (51) with a downward shift of about 10 h, so cells cultured in PGM grew more rapidly than those cultured in DM. Cells at P6 reached a cumulative cell-doubling number of 11.1 ± 1.3 h, comparable with the value (10.7 ± 0.9 h) obtained for culture in DM (49).

Transcriptional and phenotypical characterization of pAVPCs cultured in PGM. The transcriptional profile of pAVPCs is shown in Fig. 2A. Porcine aortic precursor cells cultured in PGM express the main transcripts of MSCs (CD105, CD90, CD73, CD56, CD106, and CD44) but do not express CD45, a marker of the hemopoietic lineage (data not shown). Moreover, pAVPCs do express pericyte main transcripts (NG2, nestin, CD146, α-SMA, and PDGFR-β). Transcripts of the growth factor VEGF and its main receptors (Flt-1 and Flk1) were detected as well as the growth factor PDGF-β, although at a lower level for the latter (Fig. 2A).

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

P3 pAVPCs cultured in PGM have been characterized for MSC and pericycle marker expression through flow cytometry analysis. Flow cytometry analysis (Fig. 2B) revealed that pAVPCs expressed MSC markers as CD105 (85.97 ± 0.5%), CD90 (99.5 ± 0.2%), and CD44 (99.6 ± 0.3%), and less than 2% of them expressed the hemopoietic lineage markers as CD45 (1.4 ± 0.4%) and CD34 (1.3 ± 0.1%), as requested by the International Society of Cell Therapy (ISCT) guidelines (14). Moreover, cells were negative for the expression of CD31 (1.5 ± 0.1%). In particular, as already described for cells cultured in DM (51), cells expressed CD56 (99.9 ± 0.1%), which is considered a marker of subsets of MSCs (2, 4, 38). The fluorescence-activated cell sorting analysis revealed the presence of a more uniform population of cells when the in vitro culture was performed in PGM compared with the culture in DM (51).

Immunocytochemical analysis revealed the expression of cell markers typical of pericytes (8, 11, 20, 27) as PDGFR-β, NG2, and nestin (Fig. 3, A–C, respectively), as already described for the culture in DM (51). A clear difference observed between cells cultured in different media was that, in PGM, <2% of cells expressed α-SMA (Fig. 3D), whereas, in DM (51), 100% of cells expressed it. The α-SMA protein is considered a functional marker of differentiated pericytes (10), and it is expressed in precommitted cells to the fully differentiated pericyte lineage, as also shown by Tigges and colleagues (46). The observation that the cells cultured in PGM lost the expression of that marker could indicate that the culture medium was able to maintain cells more undifferentiated than DM.

Moreover, CD34 and CD31 (Fig. 3, E and F) were not expressed either, so these data allowed us to exclude contamination of hemopoietic and endothelial cells (10).

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

Mesenchymal trilineage differentiation potential. pAVPCs (P3) were cultured in adipogenic, osteogenic, and chondrogenic induction media to investigate the classical trilineage differentiation potential (Fig. 4) requested for MSC characterization (14), as already shown for pAVPCs cultured in DM (51).

Quantitative PCR analysis revealed the upregulation 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, PPAR-γ and adiponectin have been used as markers of differentiation (31). Transcripts of both genes were detected to significantly increase in differentiated cells compared with control cells, respectively, about 525 times (P = 6.20 × 10⁻⁵) and about 45,000 times (P = 0.003973) (Fig. 4A).

For osteogenic differentiation, liver alkaline phosphatase (ALPL) and secreted phosphoprotein I (SPP1) have been used as markers of differentiation (29). In Fig. 4D, 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 1,300 times) compared with control only after 21 days of culture in osteogenic medium (P = 5.65 × 10⁻⁵), whereas the expression of SPP1 was significantly increased in differentiated cells.
Fig. 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, and G: gene expression analysis of transcripts, respectively, of adipocytes [peroxisome proliferator-activated receptor-γ (PPAR-γ) and adiponectin], osteocytes [liver alkaline phosphatase (ALPL) and secreted phosphoprotein 1 (SPP1)], and chondrocytes [aggrecan (ACAN) and collagen type II α1 (COL2A1)] in differentiated pAVPCs. In the y-axis in each graph is the relative expression of each transcript analyzed in differentiated pAVPCs compared with the control ($2^{-\Delta\DeltaCT}$ method). For adipogenic differentiation (A) and chondrogenic differentiation (G), gene expression was evaluated after 21 days of culture in differentiation media, whereas, for osteogenic differentiation (D), gene expression was evaluated after 7 and 21 days of culture in differentiation medium. Data obtained for every single gene have been statistically analyzed (comparing differentiated cells with undifferentiated control cells) for adipogenic differentiation (A) and chondrogenic differentiation (G) through Student’s t-test (*P < 0.05; **P < 0.01; ***P < 0.001) or for osteogenic differentiation (D) through 1-way ANOVA followed by Tukey’s post hoc comparison (different capital and lowercase letters correspond to statistically different samples). B and C: Oil Red O staining ($\times 40$ magnification) showed the presence of lipid droplets (red) in the cytoplasm of pAVPCs cultured for 21 days in adipogenesis induction medium (C), whereas no lipid droplets were observed in control cells (B). E and F: Alizarin Red staining ($\times 10$ magnification) showed calcium-rich deposits (red) in pAVPCs cultured for 21 days in osteogenesis induction medium that grew as spheroidal aggregates (F), whereas no calcium deposits were observed in control cells (E). H and I: Alcian blue staining of cross sections of pAVPC pellets. Blue staining of the extracellular matrix, indicating presence of proteoglycans and suggesting differentiation toward the chondrocyte phenotype, was present in differentiated pellets (I) and absent in controls (H). Samples were counterstained with hematoxylin. Scale bar = 100 μm.

(about 17 times) compared with control after just 7 days of culture in osteogenic medium ($P = 0.00188$).

For chondrogenic differentiation, ACAN and COL2A1 have been used as markers of differentiation (50). Transcripts of both genes were detected to significantly increase in differentiated cells compared with control cells, respectively, about 585 times ($P = 0.001668$) and about 11 times ($P = 0.03973$) (Fig. 4G).

Moreover, cells cultured with adipogenic medium for 21 days grew as a monolayer showing the accumulation of lipid
droplets stained with Oil Red O (Fig. 4, B and C); cells cultured with osteogenic medium for 21 days grew as aggregates, and they positively stained for Alizarin Red, confirming the presence of calcium deposits (Fig. 4, E and F). Cells, cultured as pellets in a chondrogenic medium for 21 days, positively stained for Alcian Blue, confirming the presence of proteoglycans (Fig. 4, H and I).

All these data suggested that PGM was able to maintain undifferentiated pAVPCs as they displayed the classical trilineage differentiation potential that is requested for MSC characterization (14) and that has already been shown 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 cell differentiation potential.** To evaluate whether pAVPCs spontaneously differentiate, smooth muscle phenotype cells were cultured in LTM and then analyzed through qPCR and immunocytochemistry. pAVPCs cultured in LTM showed an elongated fibroblast-like shape (Fig. 5, B, E, and H), whereas 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, and G).

Quantification of smooth muscle cell markers through qPCR (Fig. 6A) showed a sensible and significant increment of each of the three transcripts (α-SMA, CNN1, and SMM-hc) (49) analyzed in LTM cultured cells compared with the control. In particular, α-SMA showed a significant (P = 0.01623) increment of about 12 times, whereas CNN1 showed a significant (P = 0.0314) increment of about 41 times and SMM-hc a significant (P = 0.0002068) increment of about 966 times.

The presence of α-SMA and SMM proteins was investigated in long-term cultured cells, and the results obtained confirmed the lack of expression of both proteins in control cells (Fig. 6, B and D) and the expression of both proteins in LTM-cultured cells (Fig. 6, C and E).

All these data confirm that cells cultured for a 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.

**Fig. 5. Muscle and endothelial lineage differentiation potential of pAVPCs.** A, D, and G: pAVPCs grew in PGM (undifferentiated control cells) at 7, 14, and 21 days, respectively; pAVPCs cultured in PGM grew as multilayers maintaining their classical shape with little, thin, and elongated arms. B, E, and H: pAVPCs grew in long-term medium (LTM) at 7, 14, and 21 days, respectively; pAVPCs cultured in LTM showed an elongated fibroblast-like shape and seem to be organized in elongated superstructures. Magnification: ×10. C, F, and I: pAVPCs grew in endothelial differentiation medium (EDM) at 7, 14, and 21 days, respectively; pAVPCs stimulated with EDM displayed an endothelial cell-like morphology, growing in clusters and forming an endothelial cell-like monolayer upon which some cells organized in a few spheroidal structures. Scale bar = 100 μm.
Endothelial differentiation potential. To assess whether pAVPCs were able to differentiate to endothelial cells, a stimulation with 50 ng/μl VEGF was performed in a culture medium specific for endothelial cell culture. After 21 days of treatment in EDM, cultured cells displayed an endothelial cell-like morphology, growing as a monolayer on which few spheroidal structures could be observed (Fig. 5, C, F, and I).

Gene expression analysis (Fig. 7A) revealed the upregulation 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 = 0.01756), 33 times (P = 0.00704), 7 times (P = 0.02367), and 20 times (P = 0.0111) was detected in EDM-cultured cells compared with undifferentiated control cells. CD31 and vWF expression was investigated at the protein level, too, and both endothelial markers were detected only in differentiated cells showing their typical distribution pattern (Fig. 7, C and E). Control cells did not express CD31 or vWF protein (Fig. 7, B and D).

In vitro angiogenesis assay. pAVPCs cultured in EDM were able to develop a complex tube network with elongated branch points (Fig. 7, G, I, and J) when cultured on an extracellular matrix, whereas control cells failed to create a capillary-like network even if they tried to organize in cord-like structures, as indicated by our previous report (51). Endothelial differentiated cells that formed the network expressed both CD31 and vWF proteins (Fig. 7, H and K).

DISCUSSION

All these data confirm that pAVPCs cultured in a specific endothelial growth medium supplemented with VEGF differentiate to the endothelial phenotype, in agreement with results obtained for multipotent cells derived from embryonic, fetal, and human aorta (15, 23, 24, 32–34) 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 (35).

MSCs in which depletion of PDGFR-β signaling occurs have been reported to have a high angiogenic potential, as they produced proangiogenic growth factor and expressed endothelial cell marker in vitro, whereas in vivo they potentially stimulate neovascularization (1). Greenberg and colleagues (19) described that VEGF, activating its receptor VEGFR2, is able to suppress PDGFR-β signaling in vascular smooth muscle cells through the assembly of a PDGFR-β/VEGFR2 complex.

With consideration 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 PDGFR-β signaling. This could be the first stimulus to induce the endothelial differentiation ensuring that the main receptor (PDGFR-β) involved in pericyte/vascular smooth muscle cell 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 study, we described the ability of pAVPCs to differentiate toward the smooth muscle and the endothelial cell phenotypes. In our previous work (51), we described a method to isolate these multipotent cells from the tunica media of pig aorta.

In this study, we modified the previous protocol culturing cells in a specific culture medium able to maintain pericyte multipotency (3).
In particular, in the present study, pAVPCs cultured in PGM were shown to be a pure population of cells that express the main markers of MSCs (CD105, CD90, CD73, and CD44) and lack the expression of the main markers of hemopoietic stem cells (CD45 and CD34), as requested by the ISCT (14). Moreover, pAVPCs were shown to express the main markers (PDGFR-β, NG2, and α-SMA) that characterize pericytes (11). In addition, pAVPCs were shown to be able to differentiate toward osteo-, adipo-, and chondrocyte phenotypes.

All the data obtained from PGM-cultured pAVPCs characterization, associated with their already proven capability to form a capillary-like network if cocultured with human umbilical vein endothelial cells on extracellular matrix (51), lead us to reinforce the definition of them as MSC/pericyte-like cells.

On the basis of the recent definition of VSCs by Lin and Lue (30), we wanted, then, to assess whether pAVPCs could be considered a population of VSC-like cells, in particular,
whether they were able to differentiate toward the smooth muscle and the endothelial phenotypes.

The data we present in this study show that pAVPCs are able to differentiate spontaneously to the smooth muscle phenotype if cultured long term in an unspecific culture medium. This spontaneous differentiation process could lead us 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). 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 study, we present data that support the ability of pAVPCs to differentiate to endothelial phenotype after 21 days of culture in a specific endothelial growth medium supplemented with VEGF; in fact, they expressed markers of endothelial cells and 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 us to think about pAVPCs in a regenerative medicine fashion. In fact, for regenerative medicine purposes, it is of considerable importance that the improvement of the vascular network 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 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, 45).

Considering the recent definition of VSCs by Lin and Lue (30) and all the data we presented on pAVPCs in this study, 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 cell phenotypes. Indeed, both smooth muscle and endothelial differentiation require further investigation in in vivo animal models of pathologies to confirm the involvement of pAVPCs in vascular disease development and/or the possible usefulness of these cells for regenerative medicine studies in the porcine animal model.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A. Zaniboni, C.B., and M.F. conception and design of research; A. Zaniboni, C.B., M.B., A. Zannoni, F.B., G.A., and C.M. performed experiments; A. Zaniboni, C.B., G.S., L.C., and M.L.B. analyzed data; A. Zaniboni, C.B., and M.F. interpreted results of experiments; A. Zaniboni, C.B., and M.F. drafted manuscript; A. Zaniboni and C.B. edited and revised manuscript; M.F. approved final version of manuscript.

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