Cells derived from porcine aorta tunica media show mesenchymal stromal-like cell properties in in vitro culture

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IT IS KNOWN AND ACCEPTED THAT the vessel wall contains different types of precursor cells, including myoendothelial cells (MECs), pericytes, and adventitial cells (ACs) (10, 13, 44, 47, 58). The MECs were identified at a very low frequency in the skeletal muscle vasculature and were characterized by the expression of endothelial (i.e., CD31 and CD144) and muscular (i.e., CD56) markers. The pericytes were successfully isolated and characterized as CD146+/CD56−/CD45−/CD144− by Corselli et al. (14) and were mainly localized in the macrovasculature, in particular, in the tunica adventitia of both the arteries and the veins (9, 10, 27). All these perivascular cells were able to differentiate to adipose-, osteo-, and chondrocyte phenotypes, and both the MECs and the pericytes showed myogenic differentiation potential (10, 16, 32, 65) as well MSCs, but this has not yet been demonstrated for ACs.

Other perivascular cells, instead, displayed an high angiogenic potential in terms of differentiation to endothelial cells (29, 30, 45), as also shown for some uncommitted MSC populations that could transdifferentiate to the endothelial lineage (12, 43).

A unique definition of vessel-derived precursor cells is quite complicated; however, recently, cells derived from the vasculature that are able to differentiate into all cell types which constitute a functional blood vessel and that display features similar to MSCs have been defined as vascular stem cells (VSCs) (38).

Precursor cells derived from the vasculature display, such as MSCs (53), have antifibrotic, anti-inflammatory, and immunosuppressive properties (11) and could be employed for regenerative medical purposes (10, 16). Several published studies have indicated pericytes as the ideal candidate for the treatment of peripheral ischemia (19), heart infarction (11), or bone regeneration (32); however, the potential of vessel-derived precursor cells in regenerative medicine needs to be further investigated. Another very interesting and emerging aspect is that some precursor cells derived from different compartments of the vessel wall could take part in the pathogenesis of several cardiovascular...
The scientific literature of the last 20 yr has shown an exponential number of articles in which the pig has been used as an animal model. It has been observed that the porcine species displays an elevated degree of biological (anatomic, physiologic, and genomic) similarity to human; in particular, in recent years, complete genome sequencing (3) has allowed researchers to produce several transgenic animal models for biomedical purposes (62).

Overall, due to its anatomy and physiology, the pig is a relevant animal model for studying cardiovascular physiopathology (21, 22, 61, 63, 64) and for preclinical studies on MSC therapy (23, 24, 35, 52).

Although all these features make the pig a good experimental animal model, only recently porcine and human MSC similarities have been described previously (7, 42).

Pasquinelli et al. (45) have shown that fresh aorta segments, harvested from heart-beating multorgan donors, are highly suitable for obtaining MSCs confirming that the artery wall contains different populations of precursor cells (26, 29, 30, 31, 58). Furthermore, several studies have reported that a common ancestor for endothelial and mesodermal precursors, the so-called “mesoangioblast,” could be isolated from the embryonic dorsal aorta (15, 41, 47) and also from postnatal tissue (48, 59). Moreover, in 2010, Juchem et al. (33) described a method for the isolation of pericyte-like cells from the intima layer. These cells have been described as α-SMA, calponin, alcalin phosphatase, pericyte-specific ganglioside (3G5 antibody), and NG2 (venous)-positive cells when cultured and detached from the vascular endothelium.

Taking into account the relevance of MSCs in the cardiovascular physiopathology and regenerative medicine, and the usefulness of the pig animal model in this field, in the present article, we have reported an attempt to establish a simple and highly suitable method for obtaining large numbers of precursor cells, with MSC-like features, derived from the porcine aortic wall.

MATERIALS AND METHODS

All reagents were purchased from GIBCO (Life Technology, Carlsbad, CA) unless otherwise specified.

Isolation and culture of cells from aortic wall. The isolation of cells from the porcine aorta was carried out by modifying the method previously developed and described by us (5) for the isolation of porcine aortic endothelial cells (pAECS). Briefly, thoracic aortic traits (11 ± 2 cm; n = 3) were surgically removed and collected from 3-mo-old pigs, euthanized for other experimental purposes, to generate three primary cell line 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.

Explanted vessels were washed twice with prewarmed 0.9% NaCl sterile solution to which 1 mg/ml ampicillin was added (Sigma-Aldrich, St. Louis, MO) and 1× amphotericen B were transferred to a sterile 200-ml tube (BD Falcon; BD Bioscience, Franklin Lakes, NJ). The aortas were immediately moved to the laboratory, and all the following procedures were performed under a laminar flow hood; however, before the vessels were transferred under the laminar flow hood, an additional wash was performed using Dulbecco’s phosphate-buffered solution (DPBS; Cambrex Bio-Science, Wakersville, MA) to which 10× antibiotic-antimycotic (no. 152340) was added.

All excess tissue was removed from the vessels taking care to not damage the vessel side branches that were ligated with sterile silk surgical sutures. The aortas were then cannulated with sterile modified syringe cones and silicone tubes to definitively set up a closed system.

The lumen of the vessels was gently washed twice using DPBS to which 10× antibiotic-antimycotic was added to remove the residual blood and were filled with 0.2% collagenase IA (no. C-9891; Sigma-Aldrich) solution. To remove the endothelial layer, after 40 min of incubation at 38.5°C, collagenase solution containing cellular suspension was recovered in a sterile 50-ml tube and the vessel was vigorously washed twice with 15 ml DPBS to which 1× antibiotic-antimycotic was added.

The aortas were refilled with 0.2% collagenase IA solution and were incubated for an additional 4 h at 38.5°C. Collagenase solution containing cellular suspension was then recovered in a 50-ml tube, and the vessel was vigorously washed twice with DPBS to which 1× antibiotic-antimycotic was added. Collagenase enzymatic activity was then stopped by addition of 10% FBS.

Furthermore, the cylinders of the vessels (~10 mm in length) were cut from the aortas for histological and immunohistochemical analysis at time 0, after 40 min, and after 4 h of collagenase digestion.

The cellular suspension, recovered after 4 h of incubation, was centrifuged at 500 g for 10 min. The supernatant was discarded and the pellet was resuspended in high glucose (hg) DMEM to which 10% FBS and 1× antibiotic-antimycotic (hgDMEM-10X) were added for a supplemental wash at 500 g for 10 min. The final pellet was resuspended in 12 ml hgDMEM-10X and seeded in a 75-cm² tissue culture flask (BD Primaria, BD Bioscience).

The cells were cultured overnight (15–16 h) in hgDMEM-10X in a 5% CO₂ incubator at 38.5°C and the culture medium was then replaced with hgDMEM + 1× antibiotic-antimycotic (hgDMEM-1X).

After 3 days of culture with hgDMEM-1X medium, the cells were serum starved overnight (24 h) with hgDMEM + 1× antibiotic-antimycotic.

After serum starvation, the culture medium was replaced with hgDMEM:M199 (1:1) to which 10% FBS + 1× antibiotic-antimycotic (DM medium) were added. Before each change of medium, the cells were extensively washed with DPBS.

When the cells reached 75–80% confluency, they were detached from the flask using a 0.05% trypsin solution, counted, and seeded at a concentration of 10⁵ cell/cm² in 25-cm² tissue culture flasks (BD Primaria; BD Bioscience) in DM medium. Serial passages (P) were carried out until P6.

The cell numbers were evaluated using a hemocytometer and doubling time (DT) was calculated for each passage as DT = ln(CD)/h, where h was the culture time (in h) between two passages and CD was the cell doubling calculated as CD = (ln10/log2N0) / log2, where N is the number of cells at 80–90% confluency and N₀ is the number of cells seeded.

At P3, an aliquot of cells (1 × 10⁶ total cells), prefixed in 70% ice-cold Ethanol, was analyzed for dimension [forward scatter (FSC)] and complexity [side scatter (SSC)] through a Beckman Coulter EPICS XL MCL flow cytometer. The cell cycle was assessed using propidium iodide-stained cells at 50% confluency. The DNA contents 2N (G₀-G₁ phase), 2N (S phase), and 4N (G₂-M phase) were evaluated by ethanol treatment.

Histological and immunohistochemical analysis. Three samples of the aorta wall (at time 0, 40 min, and 4 h) for each animal were immediately fixed in Carson’s formalin. After 6 h, they were embedded in paraffin blocks from which four-micron thick sections were obtained and stained with hematoxylin-eosin and Orcein Van Gieson stain according to McManus and Mowry (40).

For immunohistochemical analysis, 4-μm thick sections underwent avidin-biotin immunoperoxidase labeling to factor VIII-related antigen (vWF), an endothelial cell marker. The sections were dewaxed in...
Table 1. Flow cytometry antibody list used for the immunophenotyping of cells

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Product Number</th>
<th>Species</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45-APC</td>
<td>K252-1E4</td>
<td>Mouse</td>
<td>AbD Serotec</td>
<td>10 μl/10^6 cells</td>
</tr>
<tr>
<td>CD90-APC</td>
<td>Ab139364</td>
<td>Mouse</td>
<td>Abcam</td>
<td>10 μl/10^6 cells</td>
</tr>
<tr>
<td>CD105-FTTC</td>
<td>Ab53318</td>
<td>Mouse</td>
<td>Abcam</td>
<td>20 μl/10^6 cells</td>
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<tr>
<td>CD56-PE</td>
<td>304606</td>
<td>Mouse</td>
<td>Biolegend</td>
<td>10 μl/10^6 cells</td>
</tr>
<tr>
<td>CD44-PerPC</td>
<td>103036</td>
<td>Rat</td>
<td>Biolegend</td>
<td>10 μl/10^6 cells</td>
</tr>
<tr>
<td>CD34 unconjugated</td>
<td>Ab81289</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>1:60</td>
</tr>
<tr>
<td>Anti Rabbit-PE</td>
<td>Ab97070</td>
<td>Goat</td>
<td>Abcam</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Diaphane (Histo Line; Pantiglante, Milan, Italy) and rehydrated by passage through graded alcohols. The endogenous peroxidase was blocked by incubation in hydrogen peroxide 0.3% in distilled water for 30 min at room temperature (RT). The sections were then rinsed in Tris buffer, immersed in citrate buffer (2.1 g citric acid monohydrate/1 liter of distilled water) pH 6.0, and incubated twice for 5 min in a microwave oven at 750 W. They were then left to cool at room temperature for 20 min. To block non-specific binding, the sections were then incubated in a solution containing 5% normal goat serum and 1% bovine serum albumin in phosphate-buffered saline (PBS) for 1 h at RT in a humid chamber. The primary antibody [polyclonal rabbit anti-human vWF (Factor VIII-related antigen; Dako, Milan, Italy)] was used, diluted 1:1,000 in 0.01 M PBS, pH 7.3. The sections were incubated with the antibody overnight at 4°C in a humid chamber; a secondary reagent (biotinylated goat anti-rabbit antibody, diluted 1:200, 30 min at RT; Dako) was followed by ABC Kit (30 min, RT, Vectorstain; Vector, Burlingame, CA). The reaction was developed with 3,3-diaminobenzidine, and Papanicolaou hematoxylin was used as a counterstain. As a negative control, the primary antibody was replaced with an unrelated polyclonal rabbit antibody.

Immunophenotyping. Immunophenotypic analysis of the aorta-derived cells was carried out by incubating 2 × 10⁵ cells (at P3) with each fluorophore-labeled antibody (antibody list and dilutions can be found in Table 1) in 100 μl of DPBS for 40 min at 4°C in the dark. After incubation, the cells were washed twice with DPBS and analyzed with a flow cytometer (FACSAria; BD Biosciences) by collecting 10⁵ events. For CD34 staining, after the first incubation with the primary antibody, the cells were washed and incubated with PE-conjugated secondary antibody for 40 min at 4°C in the dark (dilutions and suppliers listed in Table 1). All data were analyzed using FACSDiva Software (BD Biosciences).

Gene expression analysis. The expression of CD105, CD90, and CD73 was assessed using conventional PCR. β-Actin was amplified as the housekeeping gene.

The RNA was extracted from 2 × 10⁵ cells derived from the porcine aorta media layer at P3 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. Moreover, a 1% agarose gel was used to assess RNA integrity.

Table 2. Gene expression analysis porcine primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Forward (5’→3’)</th>
<th>Reverse (5’→3’)</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD105</td>
<td>NM_214031.1</td>
<td>ATACAAGGAGCTGCCATGATCC</td>
<td>TGAAGGTTAGAAGCTGATC</td>
<td>151 bp</td>
</tr>
<tr>
<td>CD90</td>
<td>NM_001146129.1</td>
<td>GACTGCGCCGATTGAAGATAC</td>
<td>GGTAGTGGAGCATGATGACG</td>
<td>180 bp</td>
</tr>
<tr>
<td>CD73</td>
<td>XM_003553250.1</td>
<td>AATGCATGCGGTGATGACTG</td>
<td>ATCCGAGGAGCAGATGGA</td>
<td>131 bp</td>
</tr>
<tr>
<td>β-Actin</td>
<td>AJ312193</td>
<td>GTCCTGCCGGCTCCTGGG</td>
<td>GGTGTCGAGTGGATC</td>
<td>141 bp</td>
</tr>
</tbody>
</table>

CD105: endoglin (ENG); CD90: Thy-1 cell surface antigen (THY1); CD73: 5'-nucleotidase (5'-NT), ecto-5'-nucleotidase.

Table 3. Immunocytochemistry primary antibody list

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Product Number</th>
<th>Species</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>NG2</td>
<td>AB-11160</td>
<td>Rabbit</td>
<td>Immunological Sciences</td>
<td>1:200</td>
</tr>
<tr>
<td>Smooth muscle actin (α-SMA)</td>
<td>1A4</td>
<td>Mouse</td>
<td>Cell Marque</td>
<td>1:500</td>
</tr>
<tr>
<td>Vimentin</td>
<td>sc-32322</td>
<td>Mouse</td>
<td>Santa Cruz</td>
<td>1:100</td>
</tr>
<tr>
<td>Nestin</td>
<td>ASB5922</td>
<td>Rabbit</td>
<td>Chemicon</td>
<td>1:150</td>
</tr>
<tr>
<td>Laminin</td>
<td>L3939</td>
<td>Rabbit</td>
<td>Sigma-Aldrich</td>
<td>1:80</td>
</tr>
<tr>
<td>PDGFR-α</td>
<td>sc338</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>1:100</td>
</tr>
<tr>
<td>PDGFR-β</td>
<td>#3169</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>1:100</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>sc-1506</td>
<td>Goat</td>
<td>Santa Cruz</td>
<td>1:150</td>
</tr>
<tr>
<td>c-Kit</td>
<td>#3308</td>
<td>Mouse</td>
<td>Abcam</td>
<td>1:100</td>
</tr>
<tr>
<td>Oct-4</td>
<td>ab19857</td>
<td>Rabbit</td>
<td>Rabbit Chemicon/Millipore</td>
<td>1:150</td>
</tr>
</tbody>
</table>

One microgram of RNA was retrotranscribed using an iScript cDNA synthesis kit (Bio-Rad Laboratories), following the manufacturer’s instructions, in a 20-μl final volume to obtain cDNA. Primers for CD105, CD90, CD73, β-actin, and expected products are listed in Table 2 and were designed using Beacon Designer 2.07 Software (Premier Biosoft International, Palo Alto, CA).

Amplification of the interest genes was carried out in a 25-μl final volume reaction containing 1 μl of cDNA, 1 mM MgCl₂, 0.8 mM dNTPs, 400 nM of each primer, 1.25 U of GoTaq Flexi DNA polymerase (Promega, Madison, WI), and Green Flexi Reaction Buffer (Promega). The amplification protocol used was: 94°C for 3 min, 40 cycles of 94°C for 15 s, 60°C for 30 s, 72°C for 10 s, followed by a further extension step of 5 min at 72°C.

All PCR products were separated on a 2% agarose gel and visualized by ethidium bromide staining under an ultraviolet light (GelDoc, Bio-Rad).

Immunocytochemistry. Indirect immunofluorescence procedures were used to study cells at P3 cultured on eight-well CultureSlides (BD Falcon; BD Bioscience) or plastic 24-multiwells (BD Primaria; BD Bioscience). The cells were washed in PBS and fixed in 4% paraformaldehyde in 0.1 M Sørensen phosphate buffer for 20 min at RT. Subsequently, fixed cells were blocked in PBS containing 0.3% Triton-X 100 (Merck, Darmstadt, Germany), 2% donkey normal serum (Sigma-Aldrich) for 1 h at room temperature and then were incubated 48 h at 4°C in a humid atmosphere with the primary antibodies diluted in blocking solution. After being rinsed in PBS (2 × 10 min), the cells were incubated with fluorochrome-labeled secondary antiserum diluted in PBS, 0.3% Triton-X 100 for 30 min at 37°C. The primary and secondary antibodies are listed in Tables 3 and 4, respectively. For nuclear staining, the cells were first washed in PBS and were then incubated 15 min in PBS containing 1 μg/ml Hoechst 33258, 0.3% Triton-X 100. After being rinsed in PBS, the cells were finally mounted in glycerol containing 0.1% 1,4-phenylenediamine (Sigma-Aldrich). Negative controls were carried out by primary antibody omission.
Differentiation potential of aortic-derived cells. At P3, the cells were seeded in a 24-well plate (BD Primaria; BD Bioscience), and their differentiation potential was assessed for adipogenic, osteogenic, and chondrogenic lineages.

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 21 days, the cells were stained with OilRedO, Alizarin Red, and Alcian Blue (all purchased by Sigma-Aldrich) as indicated by the manufacturer’s protocol.

In vitro coculture of aorta tunica media-derived cells and human umbilical vein endothelial cells (HUVECs) on extracellular matrix. The coculture experiment was carried out using μ-Slide Angiogenesis (ibidi, Martinsried, Germany) coated with undiluted Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix (cat. no. A1413201; GIBCO). Extracellular matrix coating was carried out for 3 h in a humidified incubator at 38.5°C, 5% CO₂.

Cocultures were carried out using HUVECs (GIBCO, Life Technologies), and cells derived from the porcine aorta media layer for 18 h.

Before coculture, the HUVECs were stained with a green fluorescent dye (PKH67 fluorescence cell linker kit; Sigma) and porcine aorta media layer-derived cells with a red fluorescent dye (PKH26 fluorescence cell linker kit; Sigma) following the manufacturer’s instructions.

After the staining procedure, the cells were mixed in a 1:5 ratio (porcine aorta media layer derived cells: HUVECs) and 10⁵ total cells were seeded for each well of the μ-Slide Angiogenesis in a total volume of 50 μl of Endothelial Cell Growth Media MV2 (Promocell, Heidelberg, Germany):Pericyte Growth Media (Promocell) (1:1, vol:vol).

Table 4. Immunocytochemistry secondary antibody list

<table>
<thead>
<tr>
<th>Secondary Antibody</th>
<th>Species</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-mouse RRX</td>
<td>Donkey</td>
<td>Jackson ImmunoResearch</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-goat RRX</td>
<td>Donkey</td>
<td>Jackson ImmunoResearch</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-rabbit RRX</td>
<td>Donkey</td>
<td>Jackson ImmunoResearch</td>
<td>1:100</td>
</tr>
<tr>
<td>Anti-rabbit Alexa Fluor 488</td>
<td>Donkey</td>
<td>Molecular Probes</td>
<td>1:600</td>
</tr>
</tbody>
</table>

Fig. 1. Swine aorta wall digestion. Swine aorta. At time 0 (A, D, and E), the intima shows a flattened unicellular layer (arrow in D) of endothelial cells in which there is a positive (brown stain) cytoplasmic immunohistochemical reaction to FVIII-related antigen (arrowhead in A); the underlying tunica media contains scant fibroblasts and a prevailing extracellular matrix rich in elastic fibers (red stained by Orcein Van Gieson stain; E). In both sampling times after collagenase digestion the endothelial layer is lost (B and C) and the lumen is lined by a lytic luminal wall area (□ in F and G) showing moderate and thin (40 min of digestion; B and F) or severe and thick (4 h of digestion; C and G) areas of edema (*) in the extracellular space separating the elastic fibers and the fibroblasts with condensed nuclei (arrowheads in F and G). A, B, and C are, respectively, an inset (×63) of D, F, and G. A, B, and C: immunohistochemical staining of the FVIII-related antigen, ×63. D, F, and G: hematoxylin-eosin, ×10. E: Orcein Van Gieson, ×10.
Images of the coculture experiment were acquired using dual-channel confocal microscopy. The sections were scanned with a Nikon Ti-E fluorescence microscope coupled with an A1R confocal system (Nikon). The following lasers were used: multi-Ar (457/488/514) laser with exciting wavelengths for DyLight 488 and 561 diode-pumped solid state laser (DPSS) with exciting wavelengths for RRX. The images were acquired with a ×20 objective and 1,024 × 1,024 pixel resolution, using Nis-Elements AR 3.2 software.

Data analysis. All data reported are means ± SD of the three cell lines derived from pig aortas (n = 3). If multiple experiments were performed on the same cell line, the data were pooled to increase precision.

RESULTS AND DISCUSSION

Aortic wall digestion. In this study, we reported a new method for MSC-like cell isolation based on the enzymatic treatment of the pig aorta; filling the vessel with a collagenase IA solution, we were able to detach cells from the lumen of the vessel and then easily recover them leaving the external layers.
attaching at the protocol, the cells derived from the aortic media layer started to elongate thin arms at their ends (Fig. 2A). When subconfluent, the cellular space separating the elastic fibers and fibroblasts with digestion of the aortic wall showing edema in the extracellular space (Fig. 1E) expressed CD105, CD90, and CD73.

Twelve to thirteen days after seeding, we obtained 3.85 ± 0.42 × 10^6 total cells in a 75-cm² culture flask (70–80% of confluence) from the thoracic aorta (11 ± 2 cm). During this period, the cells slowly stuck to the culture flask and began to divide, as has been described for pericytes (17, 19).

The cells then displayed a mean replication time between P1 and P2 of 36.5 ± 8.0 h that gradually increased to 60.8 ± 2.1 h between P5 and P6 (Fig. 2E) as reported for both pericytes, in particular between 3 and 5 wk of culture (19), and ACs (14). Cells at P6 reached a cumulative cell doubling number of 10.7 ± 0.9.

At P3, the cells corresponded to a unique population in terms of dimension and internal complexity as indicated by flow cytometry analysis (Fig. 2F). Analysis of the cell cycle revealed that 57.9 ± 5.0% of cells were in the G0-G1 phase, 23.7 ± 3.6% of the cells were in the S phase and 15.1 ± 1.3% of the cells were in the G2-M phase (Fig. 2G). Data analysis revealed that the cells possessed a standard cell cycle for diploid cells.

**Immunophenotyping.** To assess the expression of the cell-surface antigens, cells at P3, which were 80% confluent were harvested and incubated with antibodies against mesenchymal and hematopoietic markers and were then analyzed on a flow cytometer (FACS). The FACS analysis (Fig. 3A), revealed that cells, grown on culture flasks, expressed the typical markers of MSCs, such as CD44 (93.4 ± 2.0% of positive cells), CD90 (87.9 ± 5.4% of positive cells), and CD105 (86.5 ± 6.1% of positive cells). Conversely, the antigen profiles were negative (<2%) for the hematopoietic markers CD34 (0.7 ± 0.4% of positive cells) and CD45 (1.5 ± 0.4% of positive cells), as required for MSCs (20). In addition, the cells expressed CD56 (95.9 ± 3.5% of positive cells), which is considered a natural killer and neuronal and muscle marker but has also been described as a marker of subsets of MSCs derived from human bone marrow (4, 8) and menstrual blood (51).

Results refer to a single experiment, representative of three independent cell populations.

**Gene expression analysis.** The MSC markers (6) CD105 and CD90, and CD73 expression were evaluated using porcine bone marrow (4, 8) and menstrual blood (51).

**Immunocytochemistry.** The cells at P3 were stained with different antibodies (Table 3). To exclude the contamination of endothelial and vascular smooth muscle cells, immunofluorescent labeling for PECAM-1 and smooth muscle myosin heavy chain were performed; no positive cells were detected (Fig. 4, G and H). Instead, the cells were positively stained by antibodies against vimentin, nestin, laminin, PDGF-Rα and -β, NG2, and α-SMA, as shown in Fig. 4, A-C, E, F, and I-L. This antigenic profile is very similar to that described for pericytes in culture (14, 18, 26, 49). In particular, it has been reported that freshly isolated pericytes could lack α-SMA expression (56); however, with in vitro culture, α-SMA is expressed and morphology (Fig. 2B) as reported for MSCs (6). In the earliest passages (P1-P5), the cells could form spheroidal structures (Fig. 2C and D) above the adherent cells, as has already been reported for MSCs and perivascular cells (16, 19, 26).
Fig. 4. Immunocytochemical characterization of cells derived from the pig aorta. In each panel, the cultured cells were stained with different antisera; nuclei were always stained with Hoechst 33258 (blue). A–I: single immunostaining for the indicated antisera. J–L: NG2-IR (L), α-SMA-IR (M), and merged image (N). Scale bar = 20 μm. Vim, vimentin; nest, nestin; lam, laminin; c-kit, CD117; PDGFR, platelet-derived growth factor receptor; PECAM, PECAM-1 (CD31); myo, smooth muscle myosin-heavy chain; oct4, Oct family member 4; NG2, NG2 chondroitin sulfate proteoglycan; SMA, α-smooth muscle actin.
combined with NG2, and PDGFRβ is considered a pericyte marker (18). Furthermore, pericyte-like cells isolated from the intima layer by Juchem et al. (33) displayed positivity for α-SMA and NG2, as did our cells, suggesting the possibility that pericyte-like cells with similar features could reside in different sites of the vessel wall.

Moreover, cells isolated using our method displayed nuclear positivity staining for the stem cell marker octamer-binding transcription factor 4 gene (Oct4); they were negative for the expression of c-kit (Fig. 4D), as previously reported for pig adult MSCs (7, 42).

**Differentiation potential of aortic-derived cells.** Cells derived from the pig aorta media layer were able to differentiate towards adipo-, osteo-, and chondrocyte phenotypes as do MSCs (20), pericytes (18), ACs (14), and mesoangioblasts (50). The cells displayed an elevated osteogenic (Alizarin Red stain), chondrogenic (Alcian Blue stain), and adipogenic (OilRedO stain) differentiation potentials after 21 days of treatment with differentiating culture media (Fig. 5).

**In vitro coculture of cells derived from the porcine aorta tunica media with HUVECs on extracellular matrix.** To show that cells derived from the porcine aorta tunica media displayed pericyte-like properties, we cocultured them with HUVECs on a presolidified growth factor-reduced extracellular matrix, performing a tube formation assay as a functional in vitro assay to differentiate pericytes from other MSCs (6).

Our results showed that cells derived from the porcine aorta cultured for 18 h on extracellular matrix are able to form a cord-like structure (Fig. 6, C and D) as has already been shown for vascular wall multipotent stem cells by Klein et al. (34). If cocultured with HUVECs, our cells were able to form a capillary like-structure (Fig. 6, E–J), as do HUVECs alone (Fig. 6, A and B). In particular, cells derived from the porcine aorta media layer are incorporated into network-like structures, and they are aligned and codistributed, as shown in Fig. 6, E and F, with endothelial tubular-like structures. These data suggest, according to Blocki et al. (6), that cells derived from the porcine aorta media layer using our method had pericyte-like properties.

**Conclusion.** In the present article, we reported a novel simple and highly suitable method for isolating a particular subset of mesenchymal cells with pericyte-like features from the pig aorta media layer. All characterization data confirmed that the method allowed the isolation of a predominant cell phenotype positive for several MSCs and perivascular cell markers.

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**Fig. 5.** Differentiation potential of the chondrogenic, osteogenic and adipogenic lineages of cells after 21 days in induction media. If opportunely stimulated, the aorta tunica media-derived cells can differentiate toward chondro (A–D), osteo (E–H), and adipocyte (I–L) phenotypes. For the chondrogenic and osteogenic differentiation, the cells were grown mainly as aggregates and produced cartilage extracellular matrix (Alcian Blue staining for aggrecan; C and D) and calcium-rich deposits (Alizarin Red staining; G and H, respectively). Control staining of undifferentiated cells is shown, for chondro- and osteogenic differentiation in A, B, E, and F, respectively. Cells stimulated for adipogenic differentiation grown as a monolayer producing oil droplets (OilRedO staining; J, K, and L). Undifferentiated cells stained for OilRedO are shown in I, A, C, E, and G: representative pictures of a cultured well of a 24-well plate. B, D, F, H, and I, and J: ×10. K: ×20. L: ×40.

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Several studies have already described the presence of specialized niches of precursor cells in the vasculature wall (10, 13, 44, 47, 57); furthermore, it has been described that the tunica media of the arteries and veins contains pericytes (10, 47), possibly in relation to the presence of the vasa vasorum (2, 47).

Overall, not only were pericytes and ACs isolated and described as precursor cells from the vessel wall as were MECs and, in particular, mesoangioblasts in the embryonic dorsal aorta and then in postnatal tissue, (10, 41, 44, 47, 48, 59).

MSCs from the pig have been used in the in vivo study of cardiovascular diseases confirming that the use of these cells improved the pathological status at different levels (1, 23, 24, 35). Recently, the elevated similarity between human MSCs and porcine MSCs has been demonstrated, even if not all the antibodies against typical mesenchymal markers are available for this species (7). This elevated similarity confirmed the importance of using the pig as a valuable preclinical model (62).

Porcine aortic vascular precursor cells (pAVPCs), isolated and cultured as described in the present study, stained positively for CD44, CD90, and CD105, and negatively for CD34 and CD45. Gene expression analysis revealed the presence of CD105, CD90, and CD73 transcripts. Moreover, these cells showed the ability to differentiate toward adipo-, osteo-, and chondrocyte phenotypes. This typical antigenic/gene expression profile added to a multipotent differentiation potential (20) confirmed the mesenchymal origin of our cells.

CD56 (neural cell adhesion molecule-NCAM) is also expressed in pAVPCs and is widely considered a natural killer, neural and muscle cell marker (4). A subpopulation of CD56-positive MSCs derived from bone marrow (4, 8) and a population of CD56-positive MSCs derived from menstrual blood have recently been described (51).

Furthermore, immunocytochemistry demonstrated the absence of contamination by fully differentiated vascular cells (e.g., endothelial and smooth muscle cells). The ability of our method to select a pure population of MSC-like cells could be ascribed in part to the elimination of contaminant cells from the intimal layer and to the fact that, with this method, we digested only about one-third of the media layer without damaging the adventitial one. Therefore, the selection could also be due to the high-antibiotic and starvation steps used for the culture of the cells. In fact, the intrinsic feature of MSCs is resistance to injury, including its high resistance to xenobiotics (36, 46). Furthermore, it has recently been described that, under severe cellular stress conditions, it is possible to isolate multipotent cells from adipose tissue (25); our approach could be considered a stressful method, taking into account the high antibiotic-

Fig. 6. In vitro coculture with HUVECs on presolidified extracellular matrix. Culture of HUVECs on extracellular matrix results in network-like structure formation as shown in A (phase) and B (green fluorescence). Cells derived from the porcine aorta media layer are able to form cord-like structures if cultured alone on extracellular matrix as shown in C (phase) and D (red fluorescence). E–J: images obtained from the in vitro coculture of cells derived from the porcine aorta tunica media with HUVECs on a presolidified extracellular matrix. Porcine cells are able to form with the cocultured HUVEC network-like structures in which they are incorporated and are able to align with endothelial cell tube-like structures, as shown in I and J. E, G, and I: phase contrast images of porcine cells: red fluorescence). Scale bar: B and D: 150 μm; F: 200 μm; H and J: 50 μm.
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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

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

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