Chapter 2

Phenotypic and Functional Mapping of Mesenchymal Stem Cells Harvested from Different Portions of the Human Arterial Tree

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Additional information is available at the end of the chapter

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Abstract

The human arterial wall contains progenitors and mesenchymal stem cells (MSCs) acting as a postnatal reservoir of stem cells during lifetime. They are nestled in distinct arterial zones close to blood support, that is, the intima and the media-adventitia vasa vasorum plexus, representing vascular stem cell niches. In previous studies, MSCs were successfully isolated from fresh and cadaveric human large- and middle-sized arteries; these cells have a mesenchymal phenotype, self-renewal ability, and tri-lineage plasticity with high endothelial and smooth muscle cell differentiation potential. Here we present an overview of human MSCs derived from the vascular wall (hVW-MSCs) of different anatomical sites focusing on their phenotypic expression, multilineage potency, and stemness properties based on the localization in the arterial tree. We describe the isolation protocols as well as immunophenotyping, functional, and ultrastructure methods used to investigate these cell properties. hVW-MSCs from distinct portions of the vascular tree exhibit distinct phenotypic expression, multilineage potency, and stemness properties. This observation may contribute to explain the regional differences seen in vascular disease; moreover the different attitudes that hVW-MSCs exhibit in vascular differentiation should be taken in consideration whenever cell therapy, regenerative medicine, and tissue engineering strategies are attempted to replace tissues and organs.

Keywords: human arteries, vascular wall, mesenchymal stem cells, endothelial progenitors, smooth muscle progenitors, stem cell niche

1. Introduction

1.1. Mesenchymal stem cells: phenotype, mesodermal differentiation, and immunomodulation properties

The scientific community has been investigating since decades the stemness properties of the mesenchymal stromal/stem cells (MSCs). Due to their multiple properties, MSCs are the favorable candidates for cell- and gene-based therapy, regenerative medicine, and tissue engineering applications. They are a rare and multipotent cell population characterized by self-renewal and multilineage differentiation such as bone, cartilage, and adipose tissue as well as myocytes and bone marrow stromal cells [1, 2]. In 1976, Friedenstein discovered MSCs in the bone marrow [3] as adherent cells able to form colonies starting from single cells and to differentiate in osteoblasts. Several studies demonstrated that it is possible to successfully isolate a similar populations in multiple adult tissues other than the bone marrow including the amniotic fluid [4], cartilage [5], peripheral blood [6, 7], adipose tissue [8, 9], dental pulp [10, 11], fetal membranes [12], umbilical cord [13, 14], and human large- and medium-sized blood vessels such as the aorta and femoral artery [15, 16], pulmonary artery [17], internal mammary artery [18], and saphenous vein [19, 20]. According to the minimal criteria proposed by the International Society for Cellular Therapy [21], human MSCs are in vitro defined by the following properties: spindle-shape fibroblast-like morphology, the capacity to grow in adhesion on plastic surfaces, and to expand under appropriate experimental conditions. Phenotypically, MSCs express an array of surface markers usually detected by flow cytometry and exhibit differentiation capacity toward the tri-potential mesodermal adipogenic, osteogenic, and chondrogenic lineages. Due to the absence of specific markers useful to discriminate MSCs from other cytotypes, many attempts have been made to develop a mesenchymal profile in order to improve the purification and identification of MSCs. MSCs express numerous mesenchymal antigens such as CD73, CD90, CD105, CD44, and CD106 and are negative for the most common hematopoietic lineage markers like CD34, CD45, CD14, CD19, and HLA-DR.

MSCs isolated from different tissues show minimal changes in phenotype and growth; moreover they have been reported to be heterogeneous for their multilineage differentiation potential [22]; tissue-specific MSCs are also more prone to differentiate into one specific type of lineage. A similar behavior was seen in clones derived from MSCs in relation to the state of early commitment [23].

In addition to multilineage mesodermal differentiation, several studies reported the high immunosuppressive property of MSCs both in vitro and in vivo [24]. Although initially described in BM-derived cells [25], the immunomodulatory functions were also described in different human sources [26–28]. The MSC therapeutic effect is exerted not only by their low immunogenicity, migratory capacity, and direct reparative differentiation into cells of the residing tissue but also by the secretion of several bioactive molecules capable to inhibit the inflammatory milieu [29, 30].

1.2. Arterial wall structure

Three concentric layers compose the arterial wall: the intima, the media, and the adventitia. A single and continuous layer of endothelial cells leaned on basal membrane, and a thin

subendothelial matrix characterizes the tunica intima, the most internal layer, in contact with the flowing blood. The tunica media is sandwiched between the intima and adventitia, from which it is separated by the internal and external elastic lamina, respectively. It represents the major component of the vessel wall and contains smooth muscle cells embedded in a matrix rich in elastic fibers, collagens, and proteoglycans. The adventitia is placed externally to the external elastic lamina; it is a loose connective tissue containing fibroblasts, adipocytes, small vascular structures (vasa vasorum), and nerve fibers; the adventitia is critical for numerous functions, that is, dampening the systolic force, nurturing the outer portion of the media, modulating the contractile response, and regulating vascular wall homeostasis.

Based on the architecture, diameter, and function, the arteries are divided into elastic and muscular arteries. Elastic arteries are characterized by large diameters, richness in elastic tissue, and low contractile ability. Pulmonary trunks, aortic arch, and their principal branches, that is, pulmonary, common carotid, subclavian, and common iliac arteries, belong to this category. The medium-sized arteries, called muscular arteries, are characterized by a low blood flow; they have a thin intima, a well-developed internal elastic lamina, and a media that is composed by concentric layers of smooth muscle cells. The peripheral arteries and those of the internal organs such as femoral arteries, external carotid artery, bronchial arteries, and mesenteric arteries are medium-sized arteries.

1.3. Mesenchymal stem cells resident in the human artery wall

Recent findings indicate that the adventitia of large- and medium-sized adult human arteries contains resident MSCs with multilineage differentiation capacity acting as a postnatal reservoir of stem cells.

In the human pulmonary artery, human vascular adventitial fibroblasts (hVAFs) were isolated from adventitia showing a strong ability to differentiate in mesenchymal cells. Immunophenotypically, these multipotent cells express vimentin, type-1 collagen, CD29, CD44, and CD105 markers and are negative for the most common monocyte markers. Under appropriate differentiation medium, the hVAFs were committed to adipocytes and osteocytes as well as myogenic cells positive to calponin and alpha smooth muscle cells (αSMA) in response to transforming growth factor-beta 1 (TGF-β1) [17].

Our group has demonstrated for the first time the presence of MSCs in large- and medium-sized vessels, including the thoracic aorta, aortic arch, and femoral artery from healthy and heart-beating donors [15, 16]. The vascular wall resident mesenchymal stem cells (VW-MSCs) were isolated from the adventitia with mechanical and enzymatic digestion and selected using plastic adherence-based cultures. These cultured-isolated cells expressed stemness markers (Notch-1 and Oct-4) and mesenchymal antigens (CD44, CD90, CD105, CD73, CD29, CD166, and STRO-1). As the bone marrow-derived MSCs, these multipotent cells displayed mesengenic potential to differentiate into the cartilage, adipose tissue, and, albeit to a lesser extent, also bone; consistent with their vascular localization, VW-MSCs were able to originate endothelial and smooth muscle cells [16].

In an interesting morphogenetic study performed on adult fresh human internal thoracic artery fragments, the authors [31] identified a CD44+ multipotent stem cell population (VW-MPSCs)

residing in the arterial adventitia; these cells exhibited typical MSC properties, including cell surface antigens (CD44+, CD90+, CD73+, CD34−, and CD45−) without expression of CD146 and platelet-derived growth factor receptor beta (PDGFR-β) pericyte markers and multilineage plasticity into adipocytes, chondrocytes, and osteocytes, when cultured under appropriate differentiation media. Moreover, VW-MPSCs were able to generate smooth muscle cells particularly after TGF-β1 stimulation and pericytes. In vivo experiments performed on SCID mice, coculture of VW-MPSCs, and human umbilical vein endothelial cells (HUVECs) in a (three-dimensional) 3D Matrigel model resulted in the formation of a spontaneous vascular network where pericytes or smooth muscle cells derived from implanted VW-MPSCs cells were incorporated into new capillary-like structures.

The search for inexhaustible sources without ethical restrictions allowed to identify and isolate a population of VW-MSC residents in the human epiaortic wall collected from cadaveric donors; these progenitors were able to support prolonged ischemic injury and to survive in the explanted vascular tissues after 4 days of donor death and 5 years of cryopreservation in liquid nitrogen without losing their stemness characteristics. These multipotent human cadaveric mesenchymal stem cells (hC-MSCs) showed rapid expansion, clonogenic capability, immunomodulatory function, and ability to originate vascular and mesodermal tissues [28]. The possibility of obtaining stem cells from cadavers also represents a demonstration of the ability of these cells to survive adverse conditions, including long-time cryopreservation.

As a further demonstration of this capability, VW-MSCs obtained from abdominal aneurysms and exposed to extremely adverse culture conditions, for example, media acidification, hypoxia, starving, drying, and hypothermia, remained alive while keeping their morphology and stemness features [32].

1.4. Other stem cells resident in the human vascular wall

Other studies have reported the existence of stem cell and stem cell-like populations residing in the vascular wall.

Pericytes or mural cells represent a distinct cell embracing endothelial cells which share the basal membrane [33, 34]; although considered a contractile cell, when seen with electron microscopy, they contain only small quantities of assembled contractile filaments, raising uncertainties as to their actual vascular role. Using functional studies, pericytes have been found crucial for the control of endothelial cell growth and differentiation, capillary tone, caliber, and permeability; they are essential for supporting the capillary stability establishing mutual contacts with endothelial cells and producing proteins of the basal lamina [35].

Pericytes have a heterogeneous morphology, phenotype, and embryological origin (mesodermal and neuroectodermal). These peri-endothelial cells were found in intimal and adventitial niches sharing a common phenotype and multilineage plasticity with MSCs [36, 37]. In situ, they are identified through the expression of CD146, neural glial antigen (NG2), and PDGFR-β; they also express MSC markers (CD44, CD73, CD90, CD105, CD29, and alkaline phosphatase); pericytes do not express hematopoietic and endothelial cell antigens (CD31, CD34, CD45, CD14, and von Willebrand factor (vWF)).

In vitro pericytes acquire several MSC-like properties including a spindle-shape morphology; high proliferation; clonogenicity; ability to differentiate in several mesodermal lineages including the bone, cartilage, adipose tissue, smooth muscle cells, and skeletal muscle [38, 39]; immunomodulation functions [40]; as well as paracrine activity, promotion of the angiogenesis, and tissue regeneration [41, 42]. This finding supports the recent evidences that pericytes may represent the MSC in situ counterpart.

Recently, the presence of a novel stromal cell type called telocyte was documented in several organs and tissues [43]. The main features used to distinguish them from other stromal and interstitial cells are the presence of thin and long telopodes [44] and the co-expression of CD34 and CD117/c-kit, vimentin, PDGFR-α, or PDGFR-β markers [45].

Based on electron microscopy techniques, telocytes appear to be located in the stem cell niche [46, 47] where they probably serve as nursery for stem and progenitor cells influencing their survival and destiny. Here, telocytes form an intricate 3D network by contacting the resident stem cells, vessels, nerve endings, and neighboring stromal and immune reactive cells; this suggests that they have a potential role in tissue repair and regeneration [43, 48] as well as in tissue homeostasis, development, and immunosurveillance [43].

1.5. Vasculogenic niches

Progenitor cells are nestled in a three-dimensional (3D) hypoxic microenvironment localized in a specific anatomic district keeping them in their native undifferentiated and quiescent state, regulating their self-renewal, differentiation, and destiny. In the better-characterized niches, that is, the bone marrow stem cell niche, progenitors are found close to blood-bedewed areas.

In adult human vascular wall, a "vasculogenic zone" localized in between the media and adventitia was identified. In this hypothetical "vascular niche," endothelial progenitor cells (EPCs) and MSCs have been described along with hematopoietic progenitor cells (HPCs) as well as precursors of smooth muscle cells, fibroblasts, and pericytes [18]. According to this view, the vasculogenic zone contains a complete hierarchy of resident stem cells.

Despite EPCs have been intensely studied for years, there are conflicting results on their true identity; they were initially discovered in the peripheral blood [49] as circulating angioblasts involved in new blood vessel generation in response to various stimuli; it is still unclear whether these cells can also reside permanently in the vessel wall where they are expected to contribute to vascular homeostasis.

Studies performed on human aortic endothelial cells [50], coronary [51], and internal thoracic arteries [15, 18] have demonstrated that postnatal EPCs are localized in between the endothelium and in the innermost portion of adventitia; these observations corroborate the existence of EPCs resident in the human vascular wall. Peripheral blood EPCs express CD45, CD31, CD34, CD133, KDR (vascular endothelial growth factor receptor-2 (VEGFR-2)), Tie-2, the ligand for lectin ulex europaeus agglutinin-1 (UEA-1), and the low density lipoprotein (LDL) receptor [52]; different methods of EPC isolation have been proposed including the colony formation as spindle adherent cell [53]. Moreover, EPCs are hierarchy organized showing

different clonogenicity, variable proliferative potential, ability to differentiate into functional, differentiated, and mature endothelium and to form capillary-like structures under appropriate induction [50, 54].

Within the human media, the presence of postnatal smooth muscle cell progenitors have been hypothesized but not sufficiently demonstrated yet. Most of the knowledge about resident smooth muscle cell progenitors comes from animal models even if some studies hypothesize their presence and role in the human vascular wall. After enzymatic digestion of the human carotid arteries, a multipotent vascular stem cell (MVSC) with in vitro self-renewal, clonogenicity, and plasticity to differentiate into mesodermal and neural lineage was discovered in the tunica media. Additionally, these vascular progenitor cells showed a propensity to give rise to smooth muscle cells after stimulation with basic fibroblast growth factor (bFGF), PDGF-BB, and TGF-β1. Furthermore, in a vascular disease model such as endothelial denudation, the MVSC contributed to the formation of neointima producing new synthetic smooth muscle cells, and deposition of extracellular matrix [55].

The human adventitia of large vessels also contains a branched plexus of vasa vasorum or "vessels of vessels" in close proximity with the vasculogenic niche. These capillary vessels ensure the oxygenation and the nourishment of the deeper layers of vascular wall as well as the removal of waste products. Recently hot spot areas of intensely positive nestin and WT1 vasa vasorum were described by our group [56]; nestin, an intermediate filament of neural stem cells that is under WT1 control, marks endothelial cells that are functional to the vascular niche, possibly regulating mononuclear cell traffic as demonstrated in an ApoE knockout murine model of atherosclerosis [57].

2. Immunophenotype and plasticity of hVW-MSCs derived from human arterial segments

In this chapter, we present an overview of human VW-MSC derived from the vascular wall of different anatomical sites focusing on their phenotypic expression, multilineage potency, and stemness properties based on the localization in the arterial tree. For this purpose, several human variously sized arteries as subclavian, brachiocephalic, common carotid, aortic arch, thoracic, renal, and femoral collected from multiorgan or multitissue donors were employed to recover hVW-MSCs.

2.1. Isolation procedure

After decontamination for 72 hours in an antibiotic mixture, each arterial segment was washed in physiological solution, cut lengthways and into small pieces, and enzymatically digested with 0.3 mg/ml liberase type II (Roche, Milan, Italy) in serum-free Dulbecco's Modified Eagle's Medium (DMEM) (Lonza, Basel, Switzerland) overnight at 37°C using a rotor apparatus. The following day, the remaining digested tissue was filtered using cell strainers (100-70-40 μm) (Becton Dickinson, Franklin Lakes, NJ) pelleted, counted, seeded at $1 \times 10^5/cm^2$ on T75 flasks plates with DMEM plus 20% fetal bovine serum (FBS), and incubated at 37°C in a humidified

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atmosphere with 5% CO_2. After the removal of nonadherent cells using phosphate-buffered saline (PBS), the cells were cultured near confluence changing culture medium every 2 days. For expansion, the cells were detached with trypsin-EDTA (Sigma, Milan, Italy), replated in a new culture flasks in DMEM supplemented with 10% FBS, and expanded in vitro for immunophenotype and differentiation analysis.

2.2. Phenotype characterization

To determinate their mesenchymal and stemness identity, early passages (P2 or P3) of each hVW-MSCs cell population was analyzed using flow cytometry accompanied by immunofluorescence detection. For surface antigen, the cells were washed in PBS and stained using the following extensive conjugated monoclonal antibody (moAb) panel: anti-CD90-phycoerythrin-cyanine 5 (PC5), anti-CD105-phycoerythrin (PE), anti-CD73-PE, anti-CD44-fluorescein isothiocyanate (FITC), anti-CD146-PE, anti-CD34-FITC, anti-CD31-PE, anti-CD14-FITC, anti-CD45-allophycocyanin (APC) (all from Beckman Coulter), anti-vWF (DakoCytomation), anti-NG2 (R&D System), anti-PDGFR-β (R&D System), anti-STRO-1 (R&D System), anti-Notch-1 (Santa Cruz Biotechnology), and anti-Oct-4 (Santa Cruz Biotechnology). The anti-mouse IgG-APC (Beckman Coulter) and anti-rabbit IgG-FITC (DakoCytomation) were used as a secondary antibody for the detection of unconjugated primary moAbs. For nuclear or cytoplasmic antigens, the cells were permeabilized with IntraPep Kit (Beckman Coulter). Negative controls were stained with secondary antibodies only. Samples were analyzed using a Navios FC equipped with two lasers for data acquisition (Beckman Coulter) and Kaluza FC Analysis software (Beckman Coulter) for data analysis.

In addition, further antigens were analyzed using a single immunofluorescence staining. In parallel experiments to flow cytometry, 6×10^5 hVW-MSCs were seeded on glass overnight that allowed to adhere, fix, and permeabilize in 2% paraformaldehyde plus 1% Tryton X-100 in PBS for 4 minutes at room temperature (rt). After washing in PBS, the sample was blocked with 1% bovine serum albumin (BSA) in PBS for 30 minutes at rt in humid chamber to reduce nonspecific staining and incubated with antihuman αSMA (1:9000, Sigma); Nestin (1:400, Millipore); fibroblast surface protein (FSP) (1:100, Abcam); and ki-67 (1:100, Novocastra) antibodies. After prolonged washing, the cells were stained with Alexa Fluor-488 (1:250, Life Technology; Carlsbad, CA, USA) secondary antibody in the dark and counterstained with ProLong antifade reagent with DAPI (4,6-diamidino-2-phenylindole, Molecular Probes). All incubations were performed for 1 hour at 37°C in humid chamber; antibodies were diluted in 1% PBS/BSA. Negative controls were stained with secondary antibodies only. Specimens were observed and the pictures captured with Leica DMI6000 B inverted fluorescence microscope (Leica Microsystems; Wetzlar, Germany). No signal was detected in the negative controls.

2.3. In vitro multilineage differentiation

The mesengenic potential of hVW-MSCs was proved inducing the differentiation into osteogenic, adipogenic, chondrogenic as well as angiogenic lineage considering their vascular origin.

For adipogenic differentiation, 6×10^4 hVW-MSCs/well were plated in a 24-well culture plate using the Mesenchymal Stem Cell Adipogenesis Kit (Chemicon International, Temecula, CA, USA) in accordance to the manufacturer's instructions. Induction medium was replaced every 2–3 days alternating with maintenance medium (DMEM 10% FBS and 10 μg/mL insulin). After three complete cycles of induction/maintenance medium (about 3 weeks), the presence of cytoplasmic lipid droplets was assessed by Oil Red O staining and confirmed by transmission electron microscopy (TEM) analysis. Control cells were cultured in DMEM basal medium plus 10% FBS.

For osteogenic differentiation, 6×10^4 hVW-MSCs per well were seeded in a 24-well culture plate using the osteogenic induction medium Mesenchymal Stem Cell Osteogenesis Kit (Chemicon International) plus 10% FBS and cultured for 3 weeks changing the medium every 2–3 days according to manufacturer's recommendations. Control cells were cultured in basal medium (DMEM with 10% FBS). The identification of calcium salt extracellular deposition was evaluated using Alizarin Red staining and confirmed by TEM analysis.

For chondrogenic differentiation, a 3D model was employed; the hVW-MCS cells were pelleted at the concentration of 2.5×10^5 in 15 ml polypropylene conical tubes containing 500 μl of differentiation basal medium chondrogenic (Poietics, Lonza) supplemented with hMSC Chondrogenic Single Quotes (Poietics, Lonza) and 10 ng/ml transforming growth factor-beta 3 (SIGMA, Lonza). For control cells, the same medium without TGF-β3 was used. The medium was refreshed every 2 days for 3 weeks of culture. Each pellet was formalin-fixed, paraffin embedded, and stained with Alcian blue to identify the deposition of extracellular matrix rich in sulfated proteoglycans and confirmed by TEM analysis.

For angiogenic differentiation, 6×10^5 hVW-MSCs were cultured in T25 flasks for 7 days in DMEM plus 2% FBS with 50 ng/ml vascular endothelial growth factor (VEGF; Sigma) as well as in DMEM plus 10% FBS for 25×10^4 control cells. To demonstrate whether VEGF could prompt MSCs to differentiate into the endothelium, a tube formation assay (Matrigel assay) was used for evaluating the ability to form capillary-like structures. At the end of induction, 50 μl of Matrigel (BD Bioscience) solution was dispensed into a 96-well plate and incubated for 30 minutes at 37°C to allow the solidification of the Matrigel solution. Meanwhile, the cells were detached from plastic supports and counted in order to have a final cellular suspension containing 5×10^3 in 50 μl of DMEM. The cellular suspension was seeded onto Matrigel and incubated at 37°C 5% CO_2 taking images after 2, 4, 6, and 24 hours with a camera connected to CKX41 Olympus inverted microscope. HUVECs were used as a positive control.

For each differentiation assay, the control and induced hVW-MSCs were fixed with 2.5% buffered glutaraldehyde directly in culture plate for 20 minutes at rt, scarpered, collected in an microtubes, pelleted, fixed again for 24 hours at 4°C, and processed for TEM analysis.

2.4. Results and Discussion

Human VW-MSCs derived from the vascular wall of different anatomical sites such as subclavian, brachiocephalic, common carotid, aortic arch, thoracic, renal, and femoral arteries showed the distinctive features of mesenchymal stem cells such as the fibroblast-like

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spindle-shaped morphology, growth in adhesion on plastic culture flasks, and high proliferative capacity. Further stemness skills such as the capability to form spheroids when grown in suspension were found in all vascular segments, while the clonogenic activity was reserved to the brachiocephalic artery and thoracic aorta only (**Figure 1**).

Focusing on hVW-MSC phenotype, flow cytometry and immunofluorescence analysis revealed that the 90% of hVW-MSCs derived from each vascular segment express the typical mesenchymal markers (CD44, CD90, CD105, CD73, CD146, and STRO-1) even if the same antigens were reduced to 80% in renal and femoral arteries, and no expression of CD146 and STRO-1 was seen in these same segments.

After cell isolation, a contamination with mature endothelial cells (CD31+ and vWF+) was found when hVW-MSCs were derived from the aorta and its branches; they were losing during the culture passages and completely absent in isolates from distal and peripheral arteries. In each segment, vascular and hematopoietic antigens (CD31, CD14, and CD45) were expressed by a minority of the isolated cells; CD31 was seen in less than 11% of hVW-MSC and CD14 and CD45 in less than 3%; in contrast, the vWF endothelial marker expression gradually increased from 26% in aortic branches to 60% in the thoracic aorta, while it was

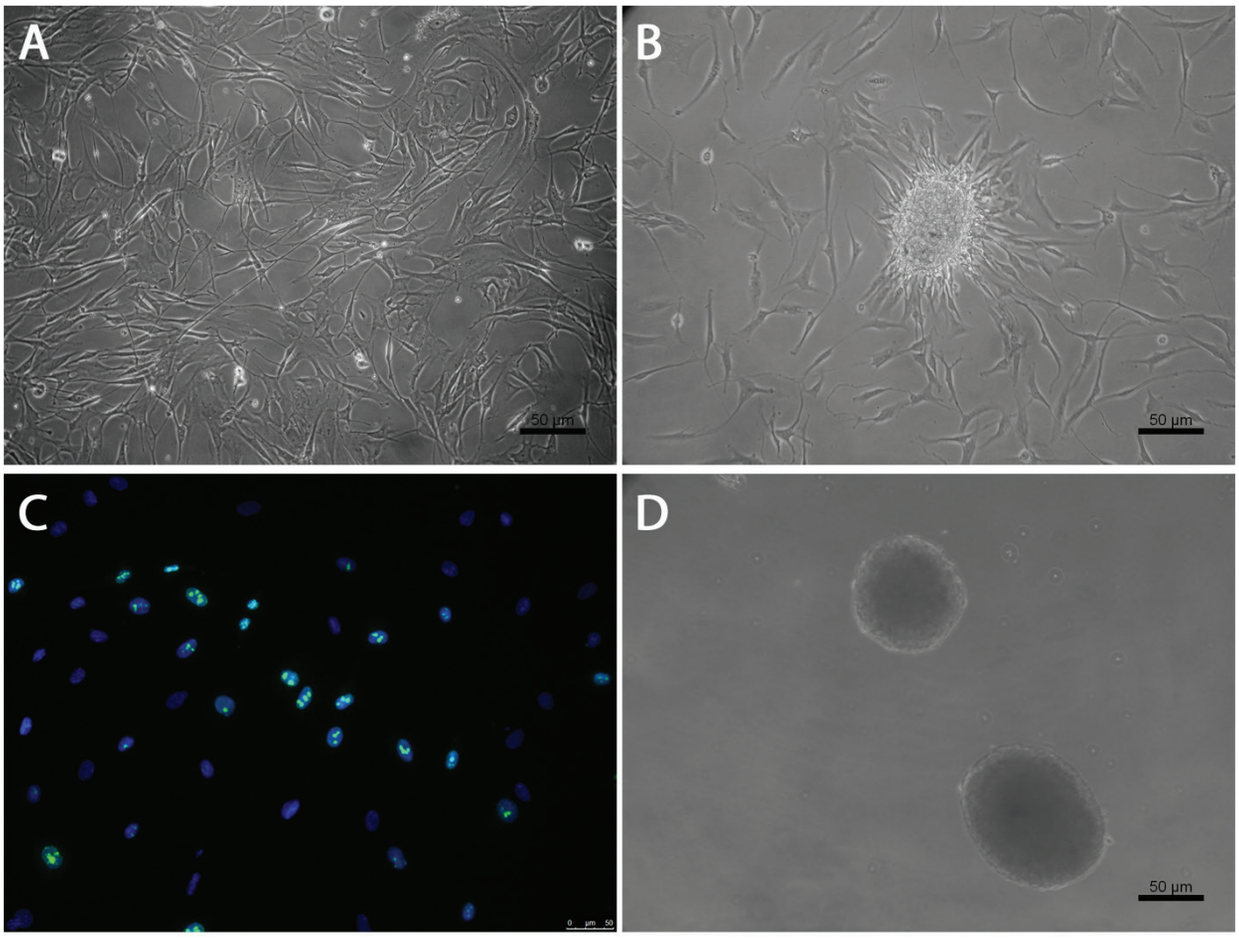

Figure 1. Representative images of hVW-MSC in adhesion to plastic support (A) and their stemness features including clonogenicity (B), high proliferation (C) and ability to form spheroids (D). Scale bars: 50 µm.

completely absent in distal (renal) and peripheral (femoral) arteries. Besides, a subset of CD34+ EPC was seen in the cell harvested from almost all segments; the CD34+ cells peaked in subclavian (26%) and renal (22%) arteries; this observation strengthens the belief that EPCs are resident in the human vascular wall.

Regarding the pericyte phenotype, about 94% of hVW-MSCs derived from the aortic portion and its branches expressed the PDGFR-β surface antigen; this percentage was drastically reduced to about 0.8 and 1.8% in renal and femoral arteries, respectively. As to NG2, a proportional increase, from 33 to 75.2%, was seen in hVW-MSC recovered from aortic branches to the thoracic aorta remaining very low in the other segments (4 and 10%). hVW-MSCs derived from the aorta (aortic arch and thoracic aorta) and its branches (subclavian, brachiocephalic, and common carotid) display a hybrid phenotype, that is, mesenchymal/pericytic, coherent with their presumed origin from pericytes of the adventitial vasa vasorum; on the contrary hVW-MSCs derived from distal and peripheral (renal and femoral) arteries present an almost pure mesenchymal phenotype without significant evidence of pericyte marker expression; this finding suggests that, in these districts, hVW-MSCs may have a different origin; either telocytes or perivascular fibroblasts could be robust candidates.

The analysis of ancestral antigens highlighted that Oct-4 and Notch-1 were constitutively expressed in a high percentage (54.6 and 38.5%, respectively) of hVW-MSC in all arteries and were significantly expressed (until to 88.9% for Oct-4 and 61% for Notch-1) in direction of the thoracic aorta; the same markers were not detected in hVW-MSC derived from renal and femoral arteries. Nestin and αSMA immunostainings were used to explore intermediate and contractile filaments. Few nestin-expressing hVW-MSCs were observed except for brachiocephalic, common carotid, and thoracic arteries where nestin-positive cells increased; a similar trend was seen also for αSMA that was found diffusely expressed in the brachiocephalic artery exclusively. The high density of cells expressing stemness markers, that is, nestin, Oct-4, and Notch-1, in thoracic segments as well as aortic arch may explain why intimal sarcomas, the most undifferentiated tumors of the vascular wall, primarily affect large vascular trunks [58].

To determine the percentage of cycling cells, a single immunofluorescence staining for ki-67 proliferation marker was performed. The semiquantitative analysis revealed that hVW-MSCs were highly proliferating when derived from the thoracic aorta (92.3%); the percentage of ki-67 proliferating cells decreased when hVW-MSCs were recovered from femoral (50.6%), subclavian (40%), renal (37.9%), common carotid (11.9%), and brachiocephalic (6.9%) arteries. All arteries, antigens, and percentage of expression analyzed are listed in **Table 1** and mapped in **Figure 2**.

To prove the multipotency into adipo-osteo-chondrocytes, hVW-MSCs derived from brachiocephalic, thoracic, renal, and femoral vascular segments were stimulated using appropriate experimental conditions; in addition, their angiogenic potential was also investigated considering their vascular origin (**Figure 3**). Results were analyzed using Oil Red O for adipogenic, Alizarin Red for osteogenic, and Alcian blue for chondrogenic differentiation; ultrastructural analysis was used for definitive confirmation. The mesengenic and angiogenic potentials are reported in **Table 2**.

	CD90	CD105	CD73	CD44	CD146	STRO-1	CD34	CD31	CD14	CD45	vWF	PDGFR-B	NG2	FSP	Notch-1	Oct-4	Nestina	ASMA	KI-67
Subclavian artery	93.3	99.4	99.5	99.6	45.3	82.1	26.1	4.4	1.3	0.9	26.3	97.7	42.3	Few	38.5	54.6	Few	Few	40
Brachiocephalic artery	90.8	99.6	99.7	99.8	12.2	73.4	6.8	1.3	0	0.3	33.4	96.4	33	Diffuse	33.9	36.4	Diffuse	Diffuse	6.9
Common carotid artery	99	99.5	99.6	99.3	25.5	96.4	9.34	11.2	3.1	1.5	37.2	95	65.1	Few	49.1	72.5	Diffuse	Few	11.9
Aortic arch	95.6	97.9	97.1	98	29.7	82.9	11.1	3.7	1.3	1.3	47	97.8	63.7	Few	58.7	78.1	Diffuse	Few	18.8
Thoracic aorta	99.2	98.8	99	99.5	14.5	82.9	7.1	5.6	1.3	1.2	60.7	85.6	75.2	Few	61	88.9	Diffuse	Few	92.3
Renal artery	81	NA	NA	91	NA	0.8	22.8	0.6	NA	1	NA	0.8	9.8	NA	0.4	NA	NA	Few	37.9
Femoral artery	82.7	99.95	NA	100	0.7	0.7	0.7	NA	NA	0.6	Negative	1.8	4	Few	1	NA	Negative	Few	50.6

Table 1. Phenotypic characterization of MSCs derived from human vascular wall (hVW-MSCs).

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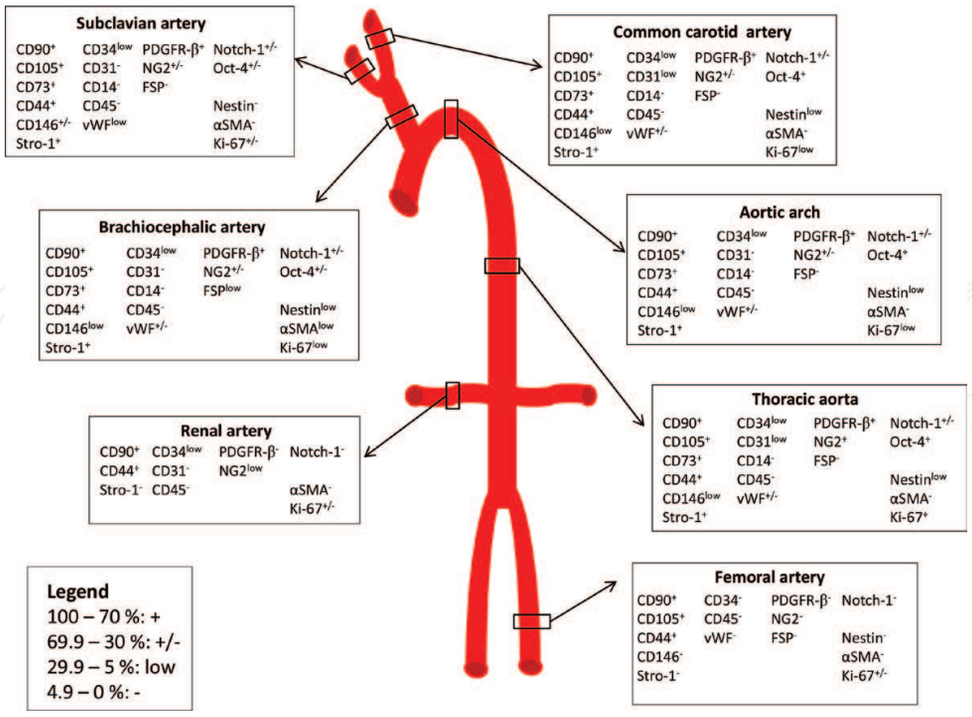

Figure 2. Phenotypic mapping of hVW-MSCs resident in human artery wall.

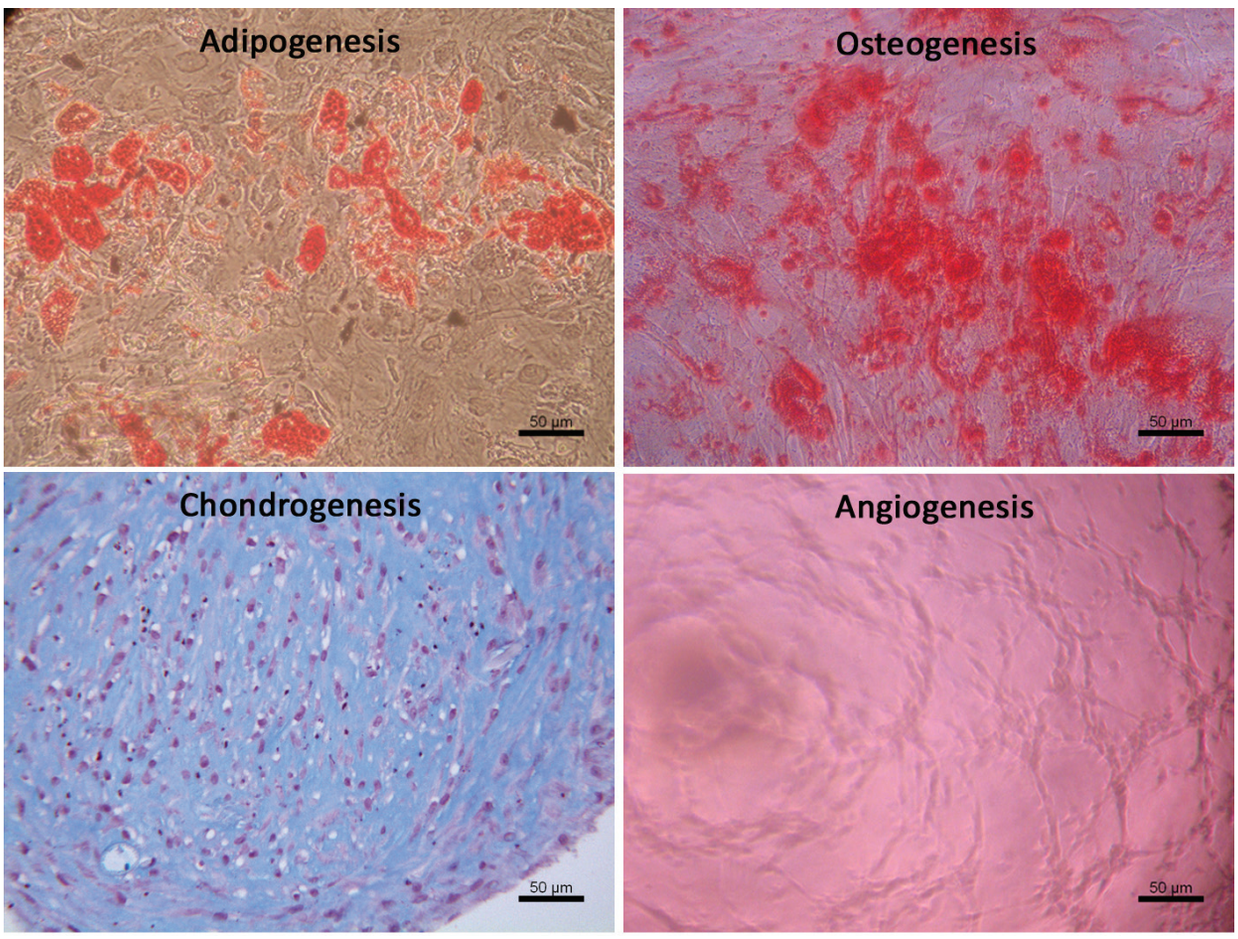

Figure 3. Representative images of hVW-MSCs potential to differentiate into mesodermal and angiogenic lineages. Scale bars: 50 µm.

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	Adipogenesis	Osteogenesis	Chondrogenesis	Angiogenesis
Brachiocephalic artery	High and diffuse	High and diffuse	High and diffuse	High
Thoracic aorta	High and diffuse	Moderate and diffuse	High and diffuse	Moderate
Renal artery	NA	High and diffuse	Moderate and diffuse	High
Femoral artery	High and diffuse	Low and patchy	Moderate and diffuse	Low

Table 2. Mesengenic and angiogenic potential of hVW-MSCs derived from human vascular wall.

In particular, adipogenesis had the same high efficiency in each investigated artery. In hVW-MSC cytoplasm, Oil Red O staining revealed the presence of multiple lipid droplets that intensely stained red; the lipid droplets increased in number and size with the time of stimulation; adipocyte formation was confirmed by TEM analysis. Osteogenesis was very intense and diffuse in brachiocephalic and renal arteries, moderate but equally diffuse in the thoracic aorta and almost absent in the femoral artery. Changes in cell morphology as well as progressive deposition of calcium were seen during the induction period and confirmed at the end of treatment by Alizarin Red staining. TEM revealed osteoid matrix and hydroxyapatite crystals in the extracellular space. A successful chondrogenesis was documented using Alcian blue staining and TEM observation. In the brachiocephalic artery, thoracic aorta, and, with lesser intensity, femoral and renal arteries, hVW-MSCs were prone to produce an alcianophilic proteoglycan-rich extracellular matrix accompanied by the presence of clear, glycogen-rich, cytoplasm vacuoles. TEM displayed proteoglycan particles and bodies in the matrix and adherent to the cell plasma membrane. The distinctive features of each mesodermal commitment were not observed in uninduced hVW-MSCs used as controls. These results highlighted quantitative functional differences among hVW-MSCs collected from distinct vascular segments. The different attitudes to differentiate should be helpful for explaining pathological events occurring in specific arterial districts. For example, osteogenesis and chondrogenesis are efficient in hVW-MSCs derived from the common carotid artery and thoracic aorta; this high efficiency may have an impact on the type of calcification seen in atherosclerosis where the calcified plaque represents the result of an active process that involves hVW-MSC reversibly primed by the inflammatory context; on the contrary the inefficient angiogenesis and low osteo-chondrogenesis seen in hVW-MSCs derived from the femoral artery could explain the prevalent occurrence of non-atheromatous calcified arterial lesions seen in this vascular bed and ultimately explain the burden of trophic and ischemic lesions observed in patients with peripheral arterial obstructive disease.

Angiogenesis was assayed using a 3D semisolid model. In brachiocephalic and renal arteries, hVW-MSCs pretreated with VEGF rapidly aligned themselves emitting thin cytoplasmic projections; they formed an intricated and evident capillary-like network when seeded on Matrigel after 6 hours. A similar attitude was seen in hVW-MSCs from the thoracic aorta, while it was decreased in cells from the femoral artery. In untreated hVW-MSCs used as control, most of the cells remained single and dispersed in the culture medium without any hint of tube formation. These differences in angiogenic potential are essential when repair or regenerative cell therapies are to be established; in this case a source of hVW-MSCs capable of responding to the angiogenic stimulus effectively would be preferable.

3. Conclusion

The human vascular wall contains progenitors and stem cells that reside in distinct niches identified in the intima, media, and adventitia. Different anatomic portions of the vascular tree were used to perform a phenotypic and functional sketch of mesenchymal stem cells harvested from the human arterial wall. Although it is well known that the bone marrow remains the best hMSC source, MSCs can be isolated from almost all the arterial districts; subclavian, brachiocephalic, common carotid, aortic arch, thoracic aorta, renal, and femoral arteries are sources of stem cells residing in their wall as the lack of CD45 expression demonstrates consistently. Based on their topographical derivation, hVW-MSCs show a hybrid phenotype (mesenchymal/pericytic) in the aorta and its branches or pure mesenchymal phenotype in distal and peripheral arteries and contain a subset of CD34+ EPCs resident in the vascular wall of all investigated segments and a high cellular density expressing ancestral markers in thoracic segments as well as aortic arch districts. Furthermore, hVW-MSCs show a different predisposition to differentiate in a specific mesodermic lineage rather than another. This aspect should be considered for future clinical applications based on regenerative cell therapies and be helpful to improve the knowledge on pathological events occurring in specific arterial districts.

Abbreviations

MSCs	Mesenchymal stromal/stem cells
hVAFs	Human vascular adventitial fibroblasts
αSMA	Alpha smooth muscle cells
VW-MSCs	Vascular wall resident mesenchymal stem cells
VW-MPSCs	CD44+ multipotent stem cell population
PDGFR-β	Platelet-derived growth factor receptor beta
TGF-β1	Transforming growth factor-beta 1
hC-MSCs	Human cadaveric mesenchymal stem cells
NG2	Neural glial antigen
vWF	von Willebrand factor
3D	Three dimensional
EPCs	Endothelial progenitor cells
HPCs	Hematopoietic progenitor cells
KDR	Vascular endothelial growth factor receptor-2
UEA-1	Ulex europaeus agglutinin-1
LDL	Low-density lipoprotein
MVSC	Multipotent vascular stem cell
bFGF	Basic fibroblast growth factor

PBS	Phosphate-buffered saline
DMEM	Dulbecco's Modified Eagle's Medium
FBS	Fetal bovine serum
moAbs	Monoclonal antibodies
PC5	Phycoerythrin-cyanine 5
PE	Phycoerythrin
FITC	Fluorescein isothiocyanate
APC	Allophycocyanin
rt	Room temperature
BSA	Bovine serum albumin
FSP	Fibroblast surface protein
DAPI	4,6-Diamidino-2-phenylindole
VEGF	Vascular endothelial growth factor
TEM	Transmission electron microscopy
HUVECs	Human umbilical vein endothelial cells

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