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CHARACTERIZATION OF VASCULAR WALL PROGENITOR CELLS AND THEIR ROLE IN THERAPEUTIC ANGIOGENESIS AND MÖNCKEBERG'S SCLEROSIS

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List of Abbreviations

AA and AA-MSCs: aortic arch and aortic arch derived mesenchymal stem cells
ASCs: adult stem cells
BF: blood flow
BM-MNCs: bone marrow mononuclear cells
BM-MSC: bone marrow derived mesenchymal stem cells
BMP: bone morphogenetic protein
CNS: central nervous system
CVCs: calcifying vascular cells
cVW-MSCs: calcified vascular wall derived mesenchymal stem cells
DAB: diaminobenzidine
ECM: extracellular matrix
ECs: endothelial cells
EPCs: endothelial progenitor cells
ESCs: embryonal stem cells
FA and FA-MSCs: femoral artery and femoral artery derived mesenchymal stem cells
FACS: fluorescent activated cell sorting
FGF: fibroblast growth factor
G-CSF: granulocyte colony stimulating factor
GVD: graft versus host disease
HCs: hematopoietic cells
HFSCs: hair follicle stem cells
HITA: human internal thoracic aorta
HLA: human leucocyte antigen
HPF: high power field
HSCs: hematopoietic stem cells
ICM: inner cell mass
IF: immunofluorescence
IHC: immunohistochemistry
IM: intramuscular
ISC: intestinal stem cells

IVF: in vitro fertilization
MAC: medial artery calcification
MAPC: multipotent population of adult stem cells
mESC: murine embryonal stem cells
MoAbs: monoclonal antibodies
MSCs: mesenchymal stem cells
ncVW-MSCs: non calcified vascular wall derived mesenchymal stem cells
OLCs: osteoclast-like cells
OR: odds ratio
PAD: peripheral arterial disease
PB-MNCs: peripheral blood mononuclear cells
PCs: pericytes cells
PDGF: platelet derived growth factor
RT: room temperature
RT-PCR: retro transcription and polymerase chain reaction
SCs: stem cells
SMCs: smooth muscle cells
SP: side population
TA and TA-MSCs: thoracic aorta and thoracic aorta derived mesenchymal stem cells
TEM: transmission electron microscopy
TGF-β: tumor growth factor-beta
VEGF: vascular endothelial growth factor
VPCs: vascular progenitor cells
VSMCs: vascular smooth muscle cells
VWCs: vascular wall cells
vWF: von Willebrand factor
VW-MSCs: vascular wall derived mesenchymal stem cells

Chapter 1

The Vascular System

The human circulatory system is responsible for delivering food, oxygen, and other needed substances to all cells in all parts of the body while taking away waste products. The circulatory system is also known as the cardiovascular system, from the Greek word *kardia*, meaning "heart," and the Latin *vasculum*, meaning "small vessel." The basic components of the cardiovascular system are the heart, the blood vessels, and the blood. As blood circulates around the body, it picks up oxygen from the lungs, nutrients from the small intestine, and hormones from the endocrine glands, and delivers these to the cells. Blood then picks up carbon dioxide and cellular wastes from cells and delivers these to the lungs and kidneys, where they are excreted *(Stranding, 2009)*.

1.1 Histomorphology and classification of blood vessels

The blood vessels of the body (arteries, capillaries, and veins) make up a closed system of tubes that carry blood from the heart to tissues all over the body and then back to the heart. Arteries carry blood away from the heart, while veins carry blood toward the heart. Large arteries leave the heart and branch into smaller ones that reach out to various parts of the body. These divide still further into smaller vessels called arterioles that penetrate the body tissues. Within the tissues, the arterioles branch into a network of microscopic capillaries. Substances move in and out of the capillary walls as the blood exchanges materials with the cells. Before leaving the tissues, capillaries unite into venules, which are small veins. The venules merge to form larger and larger veins that eventually return blood to the heart.

The walls of arteries, veins, and capillaries differ in structure. In all three, the vessel wall surrounds a hollow center through which the blood flows. The walls of both arteries and veins are composed of three coats, but they differ in thickness. The inner and middle coats of arteries are thicker than those of veins.

This makes arteries more elastic and capable of expanding when blood surges through them from the beating heart. The walls of veins are more flexible than artery walls. This allows skeletal muscles to contract against them, squeezing the blood along as it returns to the heart. One-way valves in the walls of veins keep blood flowing in one direction. The walls of capillaries are only one cell thick. Of all the blood vessels, only capillaries have walls thin enough to allow the exchange of materials between cells and the blood (**Fig. 1.1**).

Arteries and veins are classified mainly by their anatomic position and divided in 3 main classes: Resistance vessels (arteries and arterioles); Exchange vessels (capillaries, sinusoid and venules) and at the end capacity vessels (veins) *(Stranding, 2009)*.

All arteries show a common pattern of

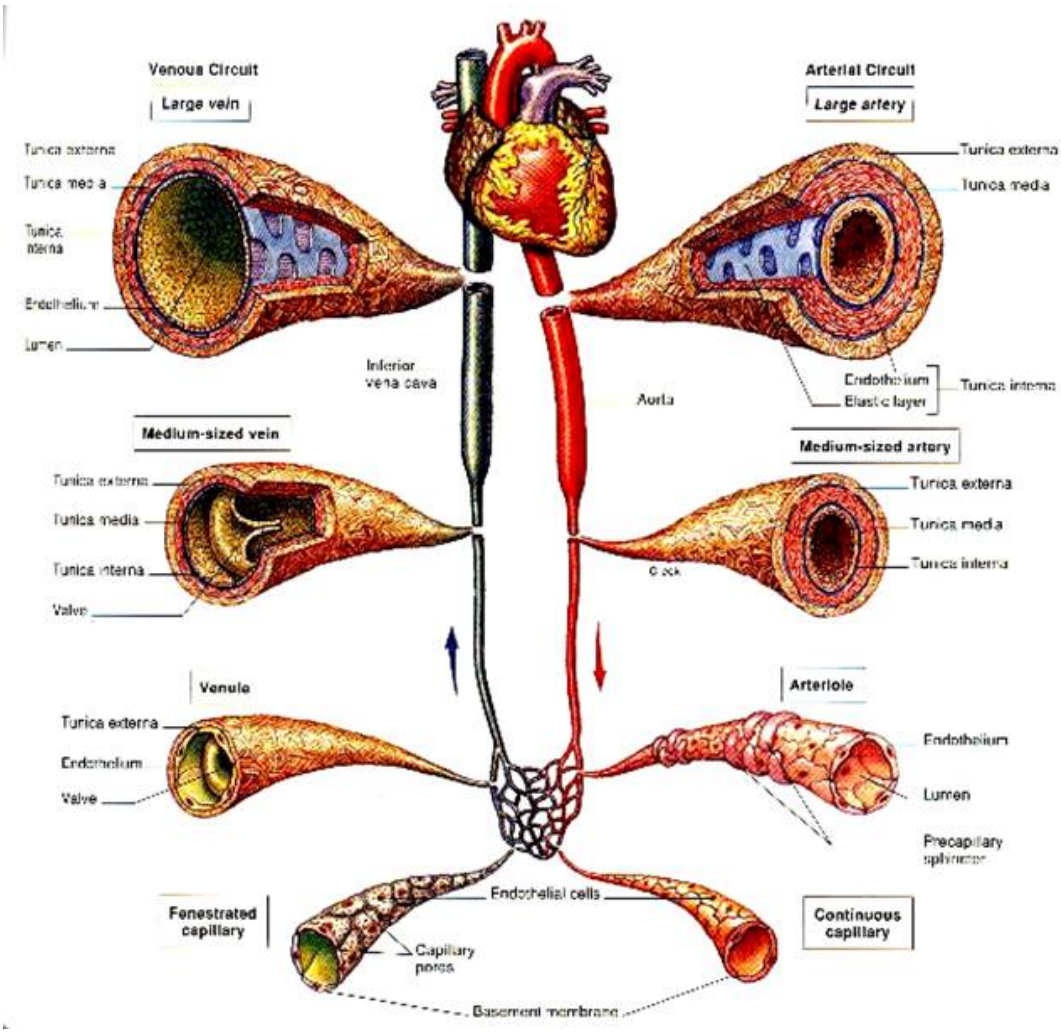

Figure 1.1. Representative scheme of cardiovascular system.

organization and are made up of similar material, thought the proportions vary in different parts of the circulation. The arterial wall is well organized connective tissue structures composed of cells and matrix fibers arranged in three tunicae: the intima, the media and the adventitia (**Fig. 1.2**).

Tunica intima

The innermost subluminal layer consists of endothelium and a variable quantity of underlying cells and matrix elements, constituting, from the lumen to the outer part of the arterial wall: the basement membrane, the sub-endothelial layer and the internal elastic lamina. A continuous monolayer of polygonal flat cells (0,3 to 0,5 μm), the endothelium, lines the luminal surface of all arteries. The endothelium layer extends as a continuous lining right through the circulation, covering all the surfaces which come in contact with the blood-arteries, capillaries, veins, heart valves and endocardial surfaces *(Lévy et al, 1999)*.

Endothelium

The endothelium is continuously submitted to shearing forces related to blood flow and exposed to circulating cells and

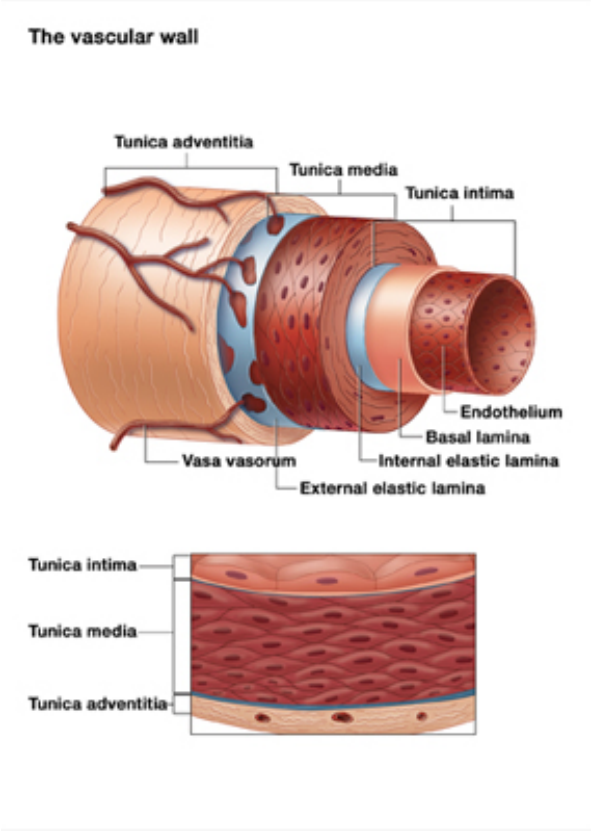

Figure 1.2. The vascular wall architecture.

plasma components In relation to shear stress, endothelial cells tends to be elongated (± 100 μm) in the direction of blood flow, particularly where the latter is rapid, laminar and unidirectional. Where flow is slow, complex, turbulent, or nearly stagnant, the endothelial cells are less distinctly elongated or oriented. In vitro experiments confirmed that cultured endothelial cells are oriented and elongated in the direction of the shear stresses *(Levesque et al, 1985)*. The luminal surface of the endothelial cells, largely smooth and regular *(Clark et al, 1976)*, is covered by a glycoprotein coat constituting the glycocalix responsible of the anti-thrombogenic properties of endothelial surface. In some arteries where the endothelial layer is submitted to high shear stress levels, as in the angle of bifurcations, the rate of renewal of endothelial cells is higher and can reach 10 cells over 100 per day. The endothelial cell ECs have multiple functions such as a permeability barrier *(Pappenheimer, 1953)*, regulation of hemostasis *(Aird, 2001)*, leukocyte recruitment and homing *(Butcher, 1991)*, and vascular wall tone control *(Moncada et al, 1991)*. Furthermore, the wide diversity of antigens found on the endothelial surface may explain the susceptibility of the cell to immunologic injury. Therefore, it appears that the endothelial cell not only serves a multiplicity of primary vascular functions but it is also a highly diversified synthetic cell, the function of which may markedly influence the response of vessels to a number of agent or stimuli *(Lévy et al, 1999)*.

Tunica media

The media is constituted principally of smooth muscle cells (SMCs), elastic fibers, and collagen fibers separated by the internal and external elastic laminae. Arteries are classified as elastic or muscular types according to the relative proportions of these cellular and fibrous components found in the media *(Wolinsky et al, 1967)*. In elastic arteries, matrix fibers, in the form of well defined elastic lamellae and collagen bundles, are abundant and prominent in the media. The conducting vessels of relatively large diameter in close proximity to the heart (such as aorta, brachiocephalic trunk, iliac and the main pulmonary arteries) are examples of elastic arteries. The media of muscular arteries contains fewer connective tissue fibers than that of elastic arteries; smooth muscle cells being the predominant component. The predominant muscular composition of these vessels corresponds to greater capacity to change diameter actively under the influence of neurohumoral stimulation. The relative proportions of elastin and collagen in the aortic media change with distance from the heart and also vary from vessels to vessels. The thoracic aorta contains a greater quantity of elastin than collagen, whereas the abdominal aorta has more collagen than elastin. Yet, the sum of the collagen and elastin along the aorta does not change with distance from the heart. The role of the medial tunica is both to bestow elasticity to the vessel (in big caliber arteries there are abundant elastic fibers and few contractive ones) and contraction (in muscular arteries there are

more muscular than elastic components) *(Lévy et al, 1999)*.

Tunica adventitia

The external elastic lamina is the inner limit of the adventitia; in contrast, the outer limit of the adventitia is often difficult to define: it is usually contiguous with the perivascular connective tissue. The aorta has relatively slight adventitial condensation of fibrous connective tissue whereas the adventitia of the large muscular arteries consists of prominent elastic and collagen fibers in well-organized layers. In these locations, adventitia may be thicker than the media. However, adventitial cells are sparse and mainly fibroblasts; the adventitia also contains few thick elastic fibers. The adventitia contains vasa vasorum and nerves, the former providing nutrition to the adventitia and media; the latter contributing to the regulation of medial smooth muscle function. Vasa vasorum exist only in arteries with diameter larger than 200μm; the outer part of the media is irrigated by the vasa vasorum. Thus, the oxygen and metabolites supply of the smooth muscle cells are provided both from the luminal blood flow (for the inner part of the media) and from the vasa vasorum (for the outer layers of the media). A lymphatic network contained in the adventitia collects the proteins, ions, soluble substances and water coming from the blood and transported through the vessel wall.

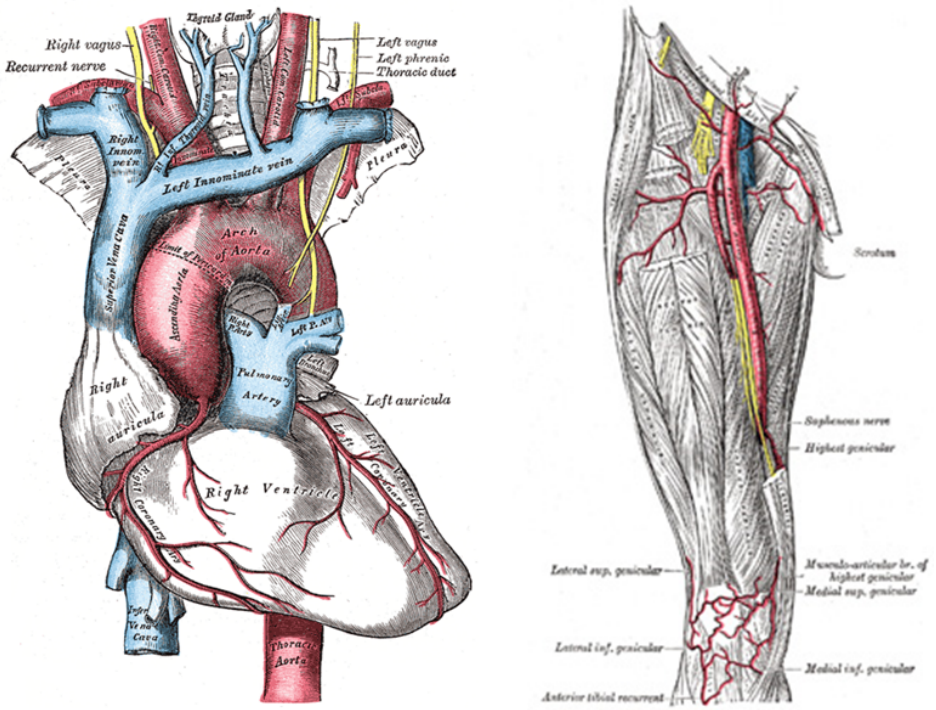

Figure 1.3. Anatomical distribution of AA, TA and FA.

1.2 Anatomy of aortic arch, thoracic aorta and femoral artery

Aorta and Aortic arch

The aorta is an elastic artery, and as such is quite distensible; it consists of a heterogeneous mixture of smooth muscle, nerves, intimal cells, endothelial cells, fibroblast-like cells, and a complex extracellular matrix. The thickness of the aorta encourages an extensive network of tiny blood vessels called vasa vasorum which feed the layers of the aorta. The aortic arch (AA) contains baroreceptors and chemoreceptors which relay information concerning blood pressure and blood pH and carbon dioxide levels to the medulla oblongata of the brain. The arch of the aorta connects the ascending aorta with the descending aorta; it begins at the level of the upper border of the second sternocostal articulation of the right side, and runs at first upward, backward, and to the left in front of the trachea; it is then directed backward on the left side of the trachea and finally passes downward on the left side of the body of the fourth thoracic vertebra, at the lower border of which it becomes continuous with the descending aorta. Its upper border is usually about 2.5 cm. below the superior border to the manubrium sterni. Three elastic arteries originate along the aortic arch: brachiocefalic artery, left common carotid artery, left subclavian artery. It lies within the mediastinum. *(Martini, 2007; Stranding, 2009)* (**Fig. 1.3**).

Thoracic aorta

The thoracic aorta (TA) is contained in the posterior mediastinal cavity. It begins at the lower border of the fourth thoracic vertebra where it is continuous with the aortic arch, and ends in front of the lower border of the twelfth thoracic vertebra, at the aortic hiatus in the diaphragm where it becomes the abdominal aorta. It travels within the mediastinum , on the posterior thoracic wall, slightly to the left of the vertebral column. This vessel supplies blood to branches that service the tissues and organs of the mediastinum, the muscles of the chest and the diaphragm, and the thoracic spinal cord *(Martini, 2007; Stranding, 2009)* (**Fig. 1.3**).

Femoral artery

The femoral artery (FA) is a general term comprising a few large muscular arteries in the thigh. The femoral artery is divided into three parts: the common femoral artery which divides into the deep femoral artery, which provides blood to the thigh, and the superficial femoral artery, which provides blood to the arteries that circulate the knee and foot. The femoral arteries receive blood through the external iliac artery. This connection occurs at the femoral triangle behind the inguinal ligament which is usually near the head of the femur bone. That proximal section of the femoral artery, known specifically as the common femoral artery, leaves the femoral triangle through an apex beneath the sartorius muscle. It then divides into a deep and superficial femoral. The deep vessel is more commonly known as the Profunda FA which provides blood to the

thigh. The other branch is the superficial femoral artery; it connects to the popliteal artery at the opening of adductor magnus or hunter's canal towards the end of the femur *(Martini, 2007; Standing, 2009)* (**Fig. 1.3**).

1.3 Embryological origin of the Vascular Wall Cells (VWCs)

Yolk sac hemangioblast

According to classic embryology studies the first ECs in the gastrulating embryo originate from the lateral and posterior mesoderm. Newly-formed lateral and posterior mesodermal cells migrate toward the yolk sac, where they will differentiate to ECs and hematopoietic cells (HCs) of the blood islands *(Murray, 1932)*. The cells constituting the outer layer of the blood islands assume a spindled morphology and then differentiate into ECs, while the vast majority of the inner cells progressively lose their intercellular attachments as they differentiate into hematopoietic cells *(Shepard et al, 2000)*. The intimate anatomic and chronological association existing between blood and vascular development suggests that ECs and hematopoietic cells share a bipotent mesodermal ancestor called hemangioblast *(Murray, 1932; Sabin, 1920)*. A recent enhancement of the hemangioblast concept, further establishing the close embryonal relationship between the vascular system and the hematopoietic system comes from recent studies supporting the existence of a hemogenic endothelium in the dorsal aorta, a cell committed to the endothelial lineage, but capable, in the mean time, to give rise to hematopoietic cells. Based on the expression of transcription factor Runx1 that regulates the definitive hematopoiesis, mice development studies demonstrate that hemopoietic endothelial cells (Runx1+) represent a subpopulation of the yolk sac endothelium and could also be found in the floor of the dorsal aorta within the aorta-gonad-mesonephros region. The hematopoietic region was so called hemogenic endothelium and generates definitive hematopoietic cells including hematopoietic stem cells *(Ribatti, 2008; Bollerot et al, 2005)*. Tavian et al. in 2001 confirmed this hypothesis in chicken and human embryos as well *(Tavian et al, 2001)*. Unlike what happens in the yolk sac where ECs and HCs appear simultaneously, the aorta is formed at least 1 day before the emergence of the hematopoietic clusters, suggesting the existence of an aorta-specific type of hemangioblast *(Bollerot et al, 2005)*.

The growth of ECs determines confluence of blood islands to form small vessels *(Murray, 1932)* a sort of primitive vascular labyrinth of small capillaries, a process known as vasculogenesis. Interestingly, already at this stage capillaries have acquired an arterial and venous cell fate, indicating that vascular-cell specification is genetically programmed and not only determined by haemodynamic force. During the angiogenesis phase, the vascular plexus progressively expands by means of vessel sprouting and remodels into a highly organized and stereotyped vascular network of larger vessels

ramifying into smaller ones. Nascent ECs channels become covered by pericytes (PCs) and SMCs, which provide strength and allow regulation of vessel perfusion, a process termed arteriogenesis *(Carmeliet, 2005)* (**Fig. 1.4**).

Smooth muscle progenitors

The embryological origin of vascular SMCs is more complex than thought before. According to classic studies the vascular SMCs arise from the portion of the yolk sac mesoderm which is closely associated with differentiating endothelium (splancnic mesoderm). However, several studies have focused on the heterogeneity of normal adult SMCs a characteristic which is further enhanced in the course of vascular diseases *(Hao et al, 2003)*; these functional and structural differences were believed to be a consequence of the intrinsic cell plasticity through which a single SMC can switch its contractile phenotype into a synthetic one in response to chemical and mechanical variations in the local microenvironment; however recent studies of lineage mapping and specific mutagenesis in vertebrate models suggest that the cause of these different response abilities relies on the different SMC embryological origin *(Majesky, 2003)*. Indeed, different vessels, or even different segments of the same vessel, are composed of distinct SMC populations that arise from specific sources of smooth muscle progenitors *(Majesky,*

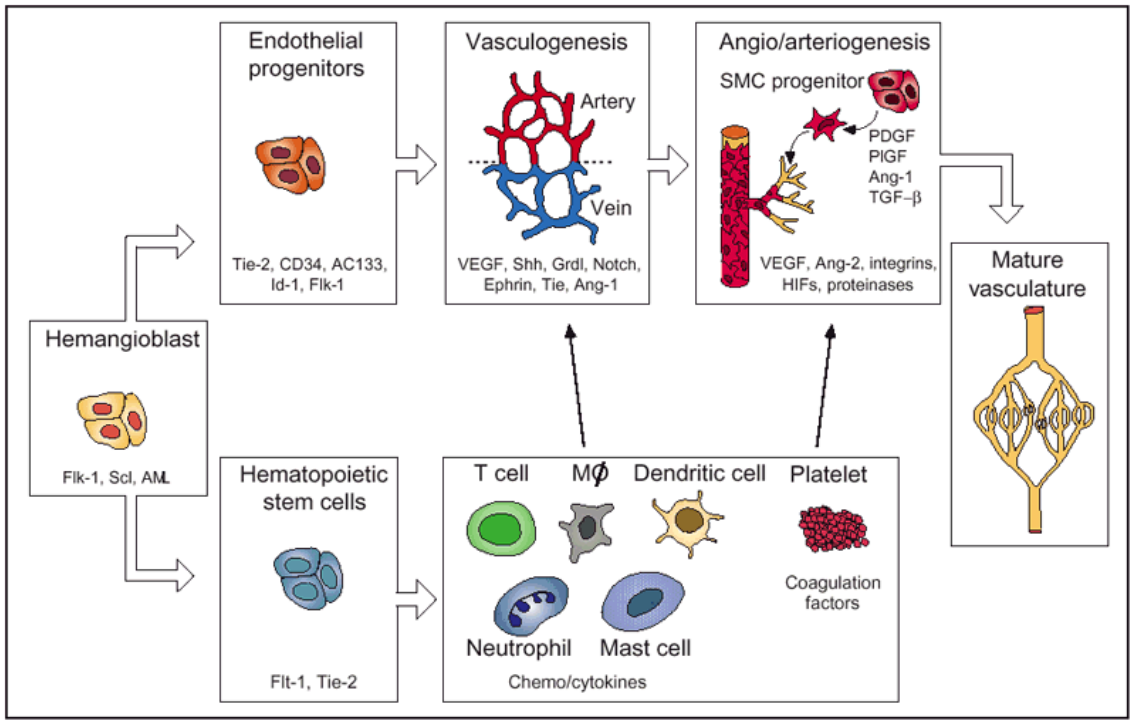

Figure 1.4. Formation of blood vessels.

2007). In 1975, Le Lievre and Le Douarin first reported a detailed fate maps that revealed a neural crest origin for vascular SMCs. From the original location, progenitors migrate and accumulate in the walls of pharyngeal arch arteries, becoming closely associated with ECs of the branchial arch arteries, and differentiate into vascular SMCs *(LeLievre et al, 1975)*. A subset of these cells continues to migrate into the cardiac outflow tract where they mediate septation of the aorta and pulmonary trunk *(Kirby et al, 1983)*. A genetic approach that used a Wnt1-Cre transgenic line crossed with a floxed stop Rosa26 reporter line (R26R) confirmed the important role of neural crest cells in mammalian vascular development by following the activation of a LacZ reporter gene. This study showed that murine neural crest contributes SMCs to the ascending and arch portions of the aorta, the ductus arteriosus, the innominate and right subclavian

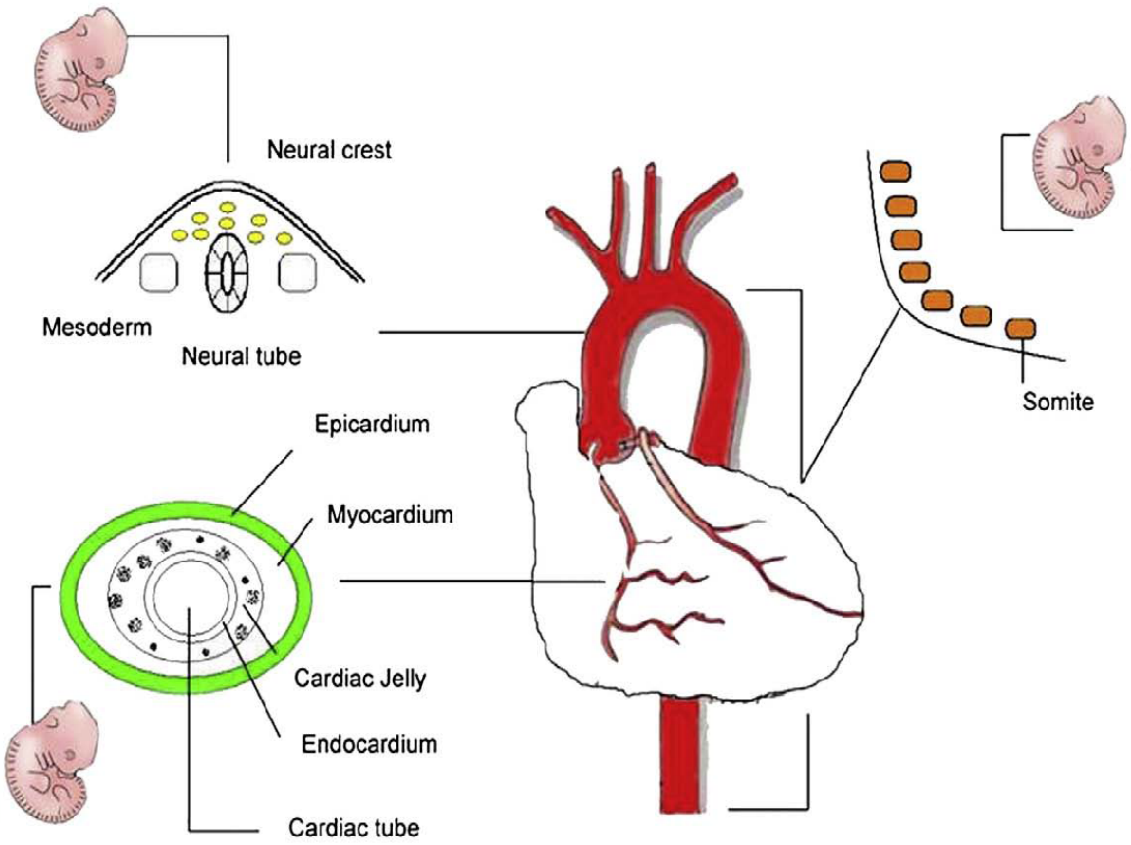

Figure 1.5. Different embryological origin of SMCs (from Pacilli et al, 2009).

arteries, as well as the right and left common carotid arteries while does not contributes SMCs to the descending thoracic aorta, abdominal aorta, coronary arteries, pulmonary arteries, left subclavian artery and distal portions of the internal carotid arteries *(Jiang et al, 2000)*. The development of coronary vessels, in fact, is separate and distinct from that of the systemic vasculature and coronary SMCs derive from progenitors located in the proepicardium *(Wada et al, 2003; Gittemberger-de Groot et al, 1998)*. The SMCs of the descending aorta, instead, derive from somites. The embryonal aorta develops in close proximity to the somites and xenograft and genetic studies show a segmental origin of it. It seems that each somite gives SMC progenitors within a locally restricted spatial domain *(Christ et al, 2004; Hungerford et al, 1996)* (**Fig. 1.5**).

Aortic mesoangioblast

An unexpected finding is that cells derived from the embryonic aorta are able to differentiate "in vitro" into ECs and in most mesodermal tissues. In 2002, Minasi et al. identified a new type of resident stem cell inside the dorsal aorta wall in quail embryos capable of self renewal and to incorporate into mesodermal tissues after grafting of quail or mouse embryonic aorta into host chick embryos. For this reason and for the expression of myogenic and endothelial markers they called this cell mesoangioblast. Based on these data, they proposed that during tissue histogenesis, when vessels penetrate into developing tissues, vessel-associated progenitors, which possibly originate from a common ancestor (mesoangioblast, rather than hemoangioblast) would leave the growing vessels and adopt the fate of the tissue where the vessel has entered responding to environmental stimuli *(Minasi et al, 2002)*. Actually, the existence of mesoangioblasts in adult tissues has not yet been reported, but many research groups are looking at a similar class of blood vessel associated stem cells in humans *(Cossu G et al, 2003)*.

Chapter 2

Stem Cells

2.1 Definition and classification of Stem Cells

Stem cells (SCs) differ from other cells type in the body since it is an *undifferentiated* cell that has the ability to divide *(self replicate)* for indefinite periods—often throughout the life of the organism. Under the right conditions, or given the right signals, stem cells can give rise *(differentiate)* to the many different cell types that make up the organism. That is, stem cells have the potential to develop into mature cells that have characteristic shapes and specialized functions, such as heart cells, skin cells, or nerve cells. Many of the terms used to define stem cells depend on the behavior of the cells in the intact organism *(in vivo)*, under specific laboratory conditions *(in vitro)*, or after transplantation *in vivo*, often to a tissue that is different from the one from which the stem cells were derived. For example, the fertilized egg is said to be totipotent because it has the potential to generate all the cells and tissues that make up an embryo and that support its development in utero. Most scientists use the term pluripotent to describe stem cells that can give rise to cells derived from all three embryonic germ layers, mesoderm, endoderm, and ectoderm. Unipotent stem cell, a term that is usually applied to a cell in adult organisms, means that the cells in question are capable of differentiating along only one lineage. Also, it may be that the adult stem cells in many differentiated, undamaged tissues are typically unipotent and give rise to just one cell type under normal conditions (**Fig. 2.1**). This process would allow for a steady state of self-renewal for the tissue. However, if the tissue becomes damaged and the replacement of multiple cell types is required, pluripotent stem cells may become activated to repair the damage *(Chandross et al, 2001)*.

2.2 Embryonic Stem Cells

Embryonic stem cells (ESCs) are derived from the Inner Cell Mass (ICM) of blastocyst stage of embryos. A normal day-5 blastocysts consisting of 200-250 cells

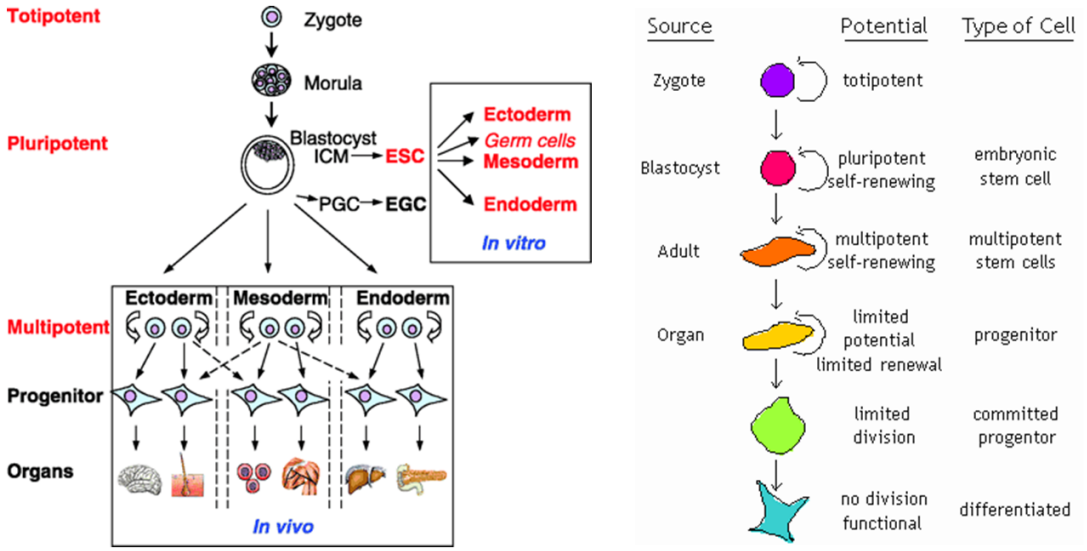

Figure 2.1. Classification and plasticity of SCs.

are used to derive ESC cultures. At this stage, the ICM is composed of only 30 to 34 cells *(Bongso A, 1996)* and are capable to long-term self-renewal, while retaining a normal karyotype *(Reubinoff et al, 2000; Shamblott et al, 2001)* and have the potential to generate any cell type of the body derived from all three embryonic germ layers *(Itskovitz-Eldor, et al, 2000; Schuldiner et al, 2000)*. The derivation of mESCs was first reported in 1981 *(Evans et al, 1981; Martin, 1981)*, but the derivation of human ES cell lines was first reported in 1998 by Thomson and colleagues which produced 5 different ES cell lines from 14 blastocyst embryos obtained by in vitro fertilization (IVF) *(Thomson et al, 1998)*. In the 1990s, ES cell lines from two non-human primates, the rhesus monkey *(Thomson et al, 1995)* and the common marmoset *(Thomson et al, 1996)*, were derived, and these offered closer models for the derivation of human ES cells. Experience with non-human primate ES cell lines and improvements in culture medium for human IVF -produced embryos led rapidly to the derivation of human ES cell lines in 1998 *(Thomson et al, 1998)*. Human ES cells can proliferate for two years through 300 population doublings *(Odorico et al, 2001)* or even 450 population doublings.

ESCs were defined by the presence of several transcription factors and cell surface protein further by cellular morphology. The transcription factors Oct-4 (Octamer-4), Nanog and Sox2 (Sex determining region Y-box2) *(Niwa et al, 2000; Chambers et al, 2003; Avilion et al, 2003)* form the core regulatory network that ensures the suppression of gene that lead to differentiation and the maintenance of pluripotency *(Boyer et al., 2005)*. The cell surface antigens commonly used to identify mESCs are Stage-Specific Embryonic Antigen-1 (SSEA-1), the glycolipids SSEA3, SSEA4 *(Henderson et al., 2002)* and the keratan sulfate

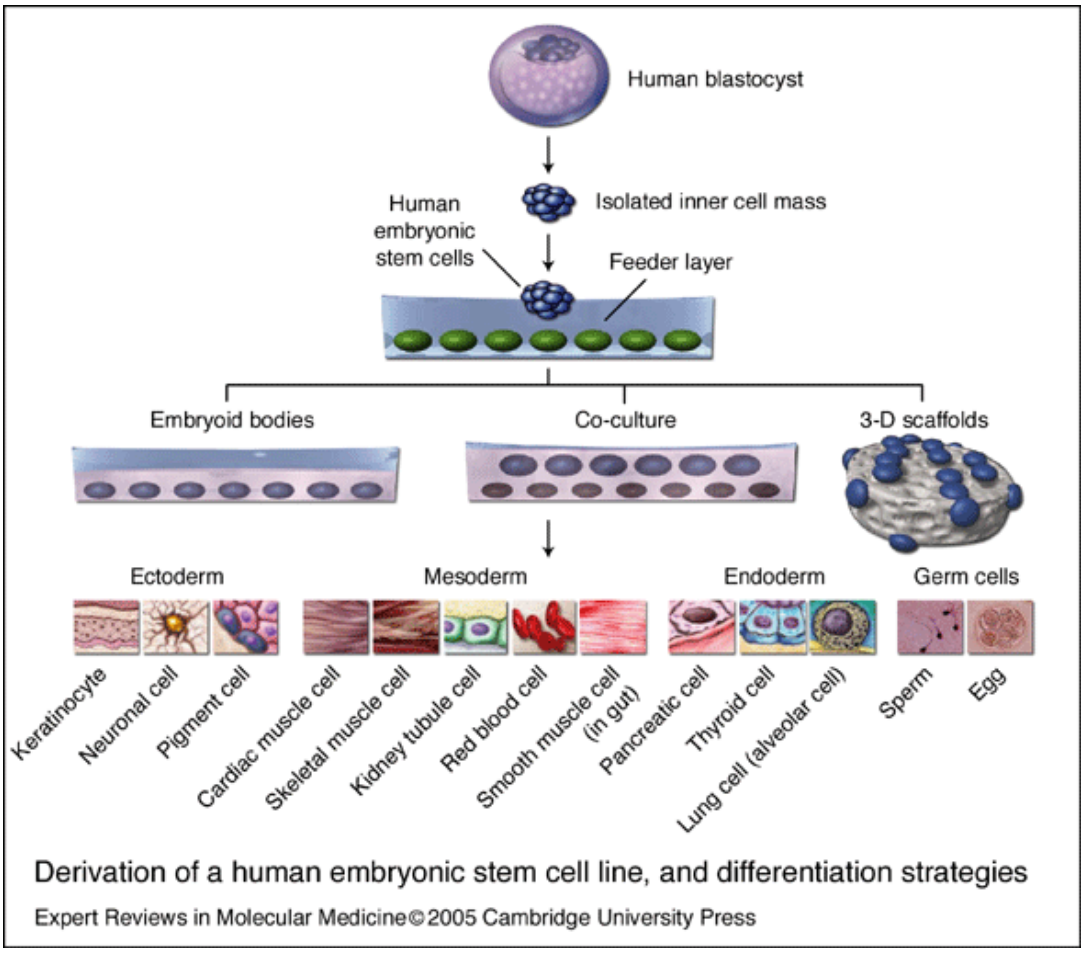

Figure 2.2. Derivation of a human embryonic SC line and differentiation strategies (from Hyslop et al, 2005).

antigens Tra-1-60 and Tra-1-81 *(Henderson et al, 2002; Carpenter et al, 2003)*. Furthermore, ESCs retain their undifferentiated status until they are able to form embryoid bodies; from here they spontaneously start to differentiate. All the characteristics mentioned above makes the ES cell lines adapted for therapeutic use and regenerative medicine. Currently, a major goal for embryonic stem cell research is to control the differentiation of human ES cell lines into specific kinds of cells to be used as the basis for therapeutic transplantation, testing drugs, or screening potential toxins. The most-often discussed is their potential use in transplant therapy to treat a wide range of degenerative diseases. However, treatments for any of these diseases require that human ESCs be directed to differentiate into specific cell types prior to transplant. Thus, at this stage, any therapies based on the use of human ES cells are still hypothetical and highly experimental *(Jones et al, 2000; Pedersen, 1999)*. The potential disadvantages

of the use of human ES cells for transplant therapy include the propensity of undifferentiated ES cells to induce the formation of tumors (teratomas), which are typically benign. Human ESCs could be used to study early events in human development and it may be possible to identify the genetic, molecular, and cellular events that lead to these problems and identify methods for preventing them *(Jones et al, 2000;Rathjien et al, 1998)* (**Fig. 2.2**).

2.3 Adult Stem Cells

To resolve ethical concerns regarding the use of ESCs in clinical practices, the scientific community focused the attention on adult stem cells (ASCs). Today, there is new evidence that stem cells are present in far more tissues and organs than once thought and that these cells are capable of developing into more kinds of cells than previously imagined. Adult stem cells, like all stem cells, share at least two characteristics. First, they can make identical copies of themselves for long periods of time; this ability to proliferate is referred to as long-term self-renewal. Second, they can give rise to mature cell types that have characteristic morphologies (shapes) and specialized functions. Typically, stem cells generate an intermediate cell type or types before they achieve their fully differentiated state. The intermediate cell is called a precursor or progenitor cell. Progenitor or precursor cells in fetal or adult tissues are partly differentiated cells that divide and give rise to differentiated cells. Such cells are usually regarded as "committed" to differentiating along a particular cellular development pathway *(Robey, 2000)*. Adult stem cells are rare. Their primary functions are to maintain the steady state functioning of a cell, called homeostasis, and, with limitations, to replace cells that die because of injury or disease *(Hunt et al, 1987; Leblond, 1964)*. Furthermore, adult stem cells are dispersed in tissues throughout the mature animal and behave very differently, depending on their local environment. For example, HSCs are constantly being generated in the bone marrow where they differentiate into mature types of blood cells *(Domen et al, 1999)*. In contrast, stem cells in the small intestine are stationary and are physically separated from the mature cell types they generate. These epithelial crypt cells divide fairly often, but remain part of the stationary group of cells they generate *(Slack, 2000)*. Unlike embryonic stem cells, which are defined by their origin, no one knows the origin of adult stem cells in any mature tissue. Some have proposed that stem cells are somehow set aside during fetal development and restrained from differentiating. As documented below, the list of adult tissues reported to contain stem cells is growing and includes bone marrow, peripheral blood, brain, spinal cord, dental pulp, blood vessels, skeletal muscle, epithelia of the skin and digestive system, cornea, retina, liver, and pancreas. In order to be classified as an adult stem cell, the cell should be capable of self-renewal for the lifetime of the organism. This criterion, although fundamental to the nature of a stem cell, is difficult to prove in vivo. Ideally, adult stem

cells should also be clonogenic and able to give rise to fully differentiated cells that have mature phenotypes, are fully integrated into the tissue and are capable of specialized functions that are appropriate for the tissue. The term plasticity means that a stem cell from one adult tissue can generate the differentiated cell types of another tissue. At this time, there is no formally accepted name for this phenomenon in the scientific literature. It is variously referred to as "plasticity" *(Brazelton et al, 2000; Krause et al, 2001)*, "unorthodox differentiation" *(Bianco et al, 1999)* or "transdifferentiation" *(Anderson et al, 2001; Lagasse et al 2000)*. The differentiated cell types that result from plasticity are usually reported to have the morphological characteristics of the differentiated cells and to display their characteristic surface markers. Many plasticity experiments involve injury to a particular tissue, which is intended to model a particular human disease or injury *(Bjornson et al, 1999; Lagasse et al, 2000; Orlic et al, 2001)*. However, there is limited evidence to date that such adult stem cells can generate mature, fully functional cells or that the cells have restored lost function in vivo *(Lagasse et al 2000)*. Most of the studies that show the plasticity of adult stem cells involve cells that are derived from the bone marrow *(Brazelton et al, 2000; Lagasse et al 2000; Ferrari et al, 1998; Peterson et al, 1999)* or brain *(Bjornson et al, 1999; Clarke et al, 2000)* (**Fig. 2.3**).

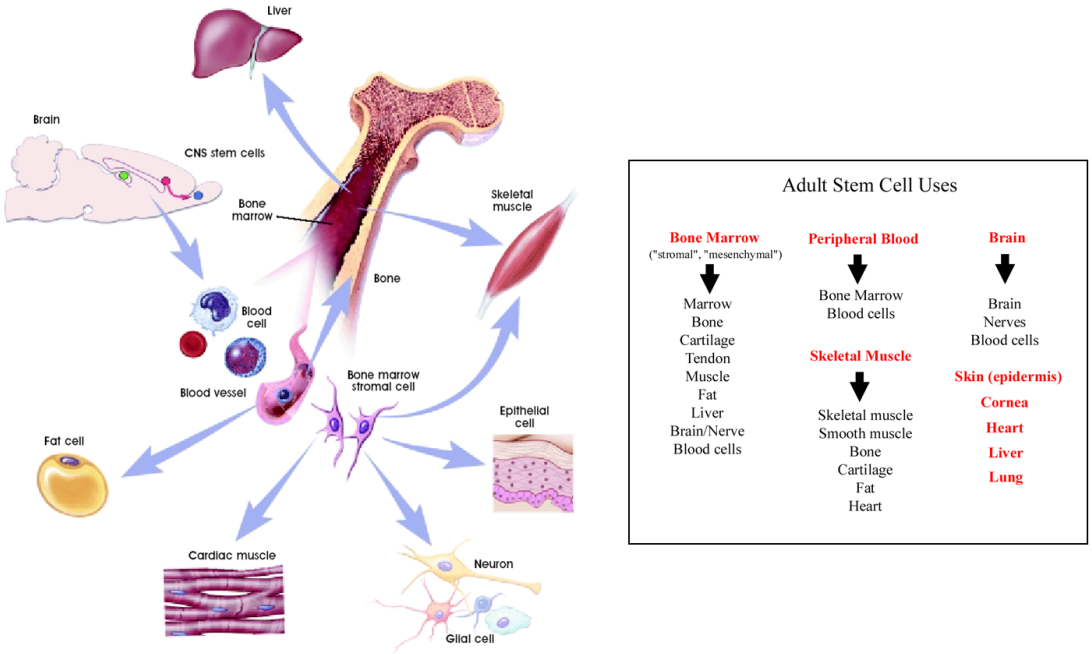

Figure 2.3. Multipotency and use of ASCs (from http://stemcells.nih.gov).

2.4 Mesenchymal Stem Cells

The adult bone marrow contains not only the hematopoietic stem cells, but also Mesenchymal Stem Cells (MSCs) that represent an archetype of multipotent adult stem cell capable of giving arise to a number differentiated mesodermal cells of various type, including chondrocytes, osteocytes, adipocytes, myocytes and bone marrow stromal cells *(Deans et al, 2000; Pittenger et al, 1999)*; that can be promising for their application in regenerative medicine. Interest in MSCs began over 130 years ago when Cohnheim, a German pathologist, suggested that the bone marrow gave rise to fibroblast-like cells during the repair process *(Ross et al, 1970; Petrakis et al, 1961)*. Later in 1976, Friedenstein described first the fibroblast precursor from bone marrows capable of osteogenesis. Since their original description, these bone marrow multipotent progenitors were known with different names *(Castro-Malaspina et al, 1980; Piersma et al, 1985; Kuznetsov et al, 1997; Prockop et al, 1997; Caplan et al, 1994; Conget et al, 1999)*. An attempt to clarify the nomenclature for MSCs has recently been proposed by the International Society for Cellular Therapy and discussed at several international meetings such as Adult Mesenchymal Stem Cells in Regenerative medicine *(MSC 2007, http://www.msc2007.net)*. In bone marrow, MSCs are important components of the HSCs niche. In fact, all niches components like stroma, stromal cells (endothelial cells, adipocytes, macrophages, reticular cells, fibroblasts and osteoprogenitors) and cellular microenvironment, where MSCs are presumed to exist in a small percentage (estimated at about 0.001-0.01%), ensure the survival and growth of HSCs *(Koller et al, 1997; Strobel et al, 1986; Tavassoli et al, 1982)*. Although MSCs were originally isolated from bone marrows *(Friedenstein et al, 1966; Pittenger et al, 1999)*, similar populations reside in a different numbers of adult and fetal *(In 't Anker et al, 2003)* tissues, including the spleen, amniotic fluid, cartilage, muscle,

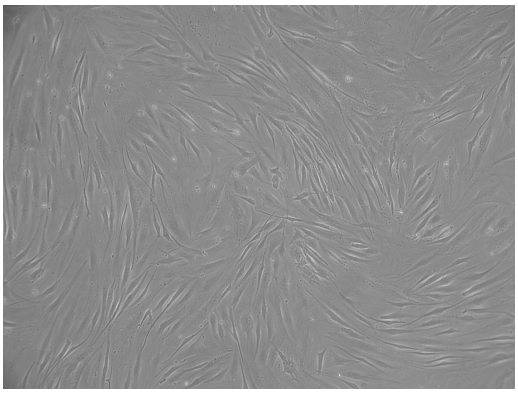

Figure 2.4. Morphological properties of in vitro MSCs (image taken from VW-MSCs culture).

tendons, peripheral blood *(Zvaifler et al, 2000; Kuznetsov et al, 2001)* and tissue adipose *(Zuk et al, 2001; Alhadlaq et al, 2004)* and more recently from deciduous tooth, fetal membrane *(Zhang et al, 2004)* and umbilical cord *(Bieback et al, 2004; Kogler et al, 2004)*. In general, MSCs have been defined by their plastic adherent growth and subsequent expansion under specific culture conditions, by a panel of non-specific surface antigens reported below and by their in vitro e in vivo differentiation potential *(Javazon et al, 2004)* (**Fig. 2.4**).

Although there are not specific markers MSCs, many attempts have been made to develop a cell-surface antigen profile to improve the purification and identification of MSCs. Minimal criteria, to define human MSCs proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy *(Dominici et al., 2006)*, are the positivity for the following antigens:

- CD105: the antibody SH2 identifies an epitope of endoglin (CD105), the Transforming growth factor beta (TGFβ) receptor III presents on endothelial cells, erythroblasts, monocytes, and connective tissue stromal cells which facilitates enrichment of stromal progenitors from bone marrow *(Short et al, 2003)*;
- CD73: a glycoprotein, identified by monoclonal antibody SH3 and SH4, involved in B-cell activation *(Short et al, 2003)*. It is expressed by lymphocytes and endothelial cells;
- CD90 or Thy1: a 25–37 kDa heavily N-glycosylated, glycophosphatidylinositol (GPI) anchored conserved cell surface protein, originally discovered as a thymocyte antigen. Thy-1 can be used as a marker for a variety of stem cells and for the axonal processes of mature neurons. Structural study of Thy-1 lead to the foundation of the immunoglobulin superfamily.

In contrast, the MSCs are negative for other markers of the hematopoietic lineage including CD34 (a transmembrane protein that defined ˜ 1% of normal bone marrow mononuclear cells including hematopoietic precursors/stem cells and normal endothelial cells and its is considered a primitive HSCs marker); CD31(a glycoprotein also designed platelet endothelial cell adhesion molecule-1 (PECAM-1) that is normally expressed on endothelial cells, circulating and tissutal hematopoietic cells including platelets, monocytes/macrophages, granulocytes and B-cells); CD45 (a leukocyte common antigen exclusively expressed on the surface of almost all haematolymphoid cells and their progenitors) and CD14 or CD11b (an immune cell marker).

The identification of a definitive marker that allows the isolation of MSCs from fresh tissue could be very important. Stro-1 is the best-know MSC marker because the cell population negative for Stro-1 is not capable to form colonies. Stro-1 positive cells can become HSC-supporting fibroblasts, smooth muscle cells, adipocytes,

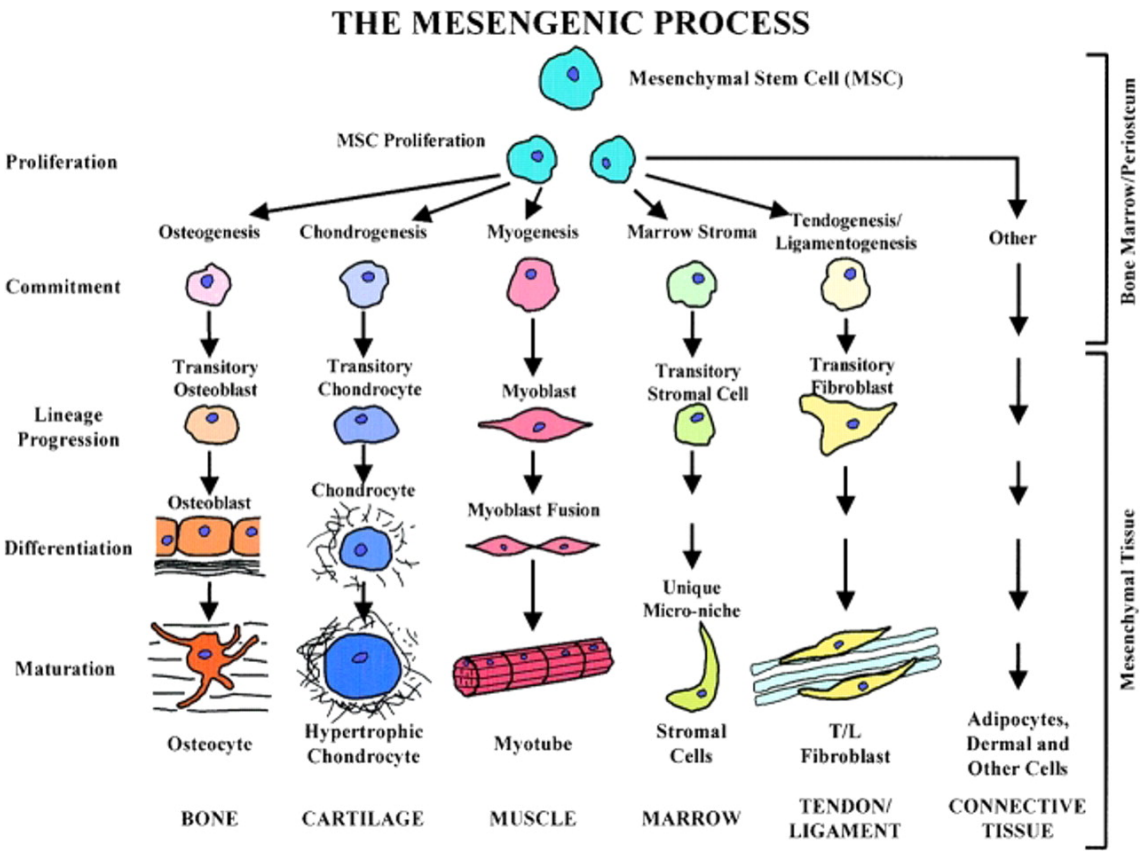

Figure 2.5. The mesengenic process of MSCs (modified from Caplan, 2009).

osteoblasts and chondrocytes *(Dennis et al, 2002)* which reinforce the functional role of MSCs. However, Stro-1 is not specific for these cells and its expression in MSCs is gradually lost during culture expansion *(Gronthos et al, 2003)*, so the Stro-1 labeling to isolate and/or identificate MSCs it is possible only during early passages. In some cases, other cell surface markers have been empirically used to isolate the human bone marrow mononuclear cells by fluorescence-activated cell sorting (FACS) or magnetic bead cell sorting based both on the expression of several markers including CD49b, CD146, CD130, CD200, CD44 and CD166.

Since that were first discovered, several studies demonstrated the multilineage differentiation potential of MSCs populations showing their capacity to develop into terminally differentiated mesenchymal phenotypes including bone (*Bruder et al, 1997)*, cartilage *(Kadiyala et al, 1997)*, tendon *(Young et al, 1998)*, muscle *(Ferrari et al, 1998)*, adipose tissue *(Dennis et al, 1999)* and hematopoietic-supporting stroma *(Prockop et al, 1997)* and showing also a high degree of plasticity *(D'Ippolito et al, 2004, Zhao et al, 2002)* (**Fig. 2.5**). The lineage-committed cell progresses

through several stages of maturation process to a terminal differentiation, which is characterized by the cessation of proliferative capacity and the synthesis of tissue-specific markers, including components of the extracellular matrix (ECM) *(Baksh et al, 2004)*.

In the 1999, Pittenger et al reported that only one-third of the initial adherent BM-MSCs clones are pluripotent (osteo/chondro/adipo) *(Pittenger et al, 1999)*. Furthermore, non-immortalized cell clones have been used to investigate the nature and properties of committed progenitors present in culture of BM-MSCs. This study demonstrated that 30% of all clones exhibited a trilineage (osteo/chondro/adipo) differentiation potential, while the remainder displayed a bi-lineage (osteo/chondro) or uni-lineage (osteo) *(Muraglia et al, 2000)*. The heterogeneity, both in vivo and in vitro studies, could be explained by the notion that MSCs in the bone marrow (and likely in all tissues) are a pool of cells that include MSCs and different subpopulations at different state of differentiation. During differentiation, the proliferative potential of these different MSCs decreases and, depending on the initial state of differentiation, both proliferative and multilineage potential become limited *(Baksh et al, 2004)*.

As regarding the immunological profile, MSCs express intermediate levels of human leukocyte antigen (HLA) class I molecules major histocompatibility complex (MHC) and low levels of class II HLA and Fas ligand; they do not express the costimulatory molecules B7-1, B7-2, CD40, or CD40L. The immunosuppressive nature of MSCs is of clinical relevance in allogeneic transplantation since it could reduce the incidence and severity of graft-vs-host disease (GVD) *(Le Blanc et al, 2003 a, b)*. Clinically, the easy of isolation, expansion potential, migratory capacity and immunosuppressive capability of MSCs has made them a popular cell type for investigating regenerative medicine, gene therapy and tissue engineering. Several studies based on animal transplantation, shown that ex-vivo expanded MSCs were able to differentiate into cells of the residing tissue, to repair damaged tissue and to restore partially its normal function, generating promising results for the treatments of several illness, including bone *(Mauney et al, 2005)*, cardiovascular and brain disease *(Zimmet et al, 2005)*. Recent studies demonstrated that the therapeutic contribution of MSCs transplantation could be caused not only by direct differentiation but also by paracrine activities which supply with large amounts of cytokines and growth factors. These bioactive factors suppress the local immune system, inhibit apoptosis and enhance angiogenesis *(Caplan et al, 2006)*. MSCs are promising also for tissue engineering. Bioengineered structures with a defined shape made with biomaterials like collagen type I, fibronectin, alginate, polylactic acid and alginate can be combinated with MSCs, culturing in bioreactors, it could be possible to obtain tissue and organs *(Stock et al, 2001)* as reported in pre-clinic animal model studies for the treatment of a large bone defects *(Kon et al, 2000)*.

2.5 Stem Cell Niches

It is well know that self renewal and pluripotency are specific skills of adult stem cells, but the idea that extremely specialized external stimuli and microenvironment can affect the regulation of the specific stem characteristic brought several team to go in for it. The niche concept was introduced in 1978 by Schofield studies *(Schofield, 1978)*; his team proved that microenvironment cells has a role in maintaining in a quiescent condition the hematopoietic stem cell, and suggested the presence of a "stem cell niche" in which stromal cells releases growth factors that bind extracellular matrix structures; in this way target cells recognized them by specific receptor *(Gordon, 2008)*. This mechanism permit to localize high concentration of specific growth factors in specific microenvironment areas. Several increasing studies had shown the present of a wide range of humoral factors, cytokines, chemokines and adhesive factors supporting adult stem cells. All those results suggest that stem niche not have only a structural role, but seems to have the intrinsic potential to lead the destiny of the cells in it.

2.5.1 Adult Stem Cells of the Nervous System

More than 30 years ago, Altman and Das showed that two regions of the postnatal rat brain, the hippocampus and the olfactory bulb, contain dividing cells that become neurons *(Altman et al, 1965 and 1969)*. Despite these reports, the prevailing view at the time was that nerve cells in the adult brain do not divide. In fact, the notion that stem cells in the adult brain can generate its three major cell types—astrocytes and oligodendrocytes, as well as neurons—was not accepted until far more recently. Within the past fifteen years, a series of studies has shown that stem cells occur in the adult mammalian brain and that these cells can generate its three major cell lineages *(Gage et al, 1995; Johe et al, 1996; Temple et al, 1999)*. There is now widespread consensus that the adult mammalian brain does contain stem cells. Because there are no markers currently available to identify the cells in vivo, the only method for testing whether a given population of Central Nervous System (CNS) cells contains stem cells is to isolate the cells and manipulate them in vitro, a process that may change their intrinsic properties *(Morrison et al, 1999)*. Despite these barriers, three groups of CNS stem cells have been reported to date. All occur in the adult rodent brain and preliminary evidence indicates they also occur in the adult human brain. One group occupies the brain tissue next to the ventricles, regions known as the ventricular zone and the subventricular zone. The ventricles are spaces in the brain filled with cerebrospinal fluid. During fetal development, the tissue adjacent to the ventricles is a prominent region of actively dividing cells. By adulthood, however, this tissue is much smaller, although it still appears to contain stem cells *(Morshead et al, 2001)*. A second group of adult CNS stem cells, described in mice

but not in humans, occurs in a streak of tissue that connects the lateral ventricle and the olfactory bulb, which receives odor signals from the nose. In rodents, olfactory bulb neurons are constantly being replenished via this pathway *(Luskin, 1993)*. A third possible location for stem cells in adult mouse and human brain occurs in the hippocampus, a part of the brain thought to play a role in the formation of certain kinds of memory *(Eriksson et al, 1998; Gage et al, 1995)* (**Fig. 2.6**).

2.5.2 Hematopoietic Niche

The notion that the bone marrow contains stem cells is not new. One population of bone marrow cells, the hematopoietic stem cells (HSCs), is responsible for forming all of the types of blood cells in the body. HSCs were recognized as a stem cells more than 40 years ago *(Becker et al, 1963; Till et al, 1961)*. Bone marrow stromal cells—a mixed cell population that generates bone, cartilage, fat, fibrous connective tissue, and the reticular network that supports blood cell formation—were described shortly after the discovery of HSCs *(Friedenstein et al, 1966; Owen et al, 1988)*. The mesenchymal stem cells of the bone marrow also give rise to these tissues, and may constitute the same population of cells as the bone marrow stromal cells *(Pittenger et al, 2001)*. Recently, a population of progenitor cells that differentiates into endothelial cells, a type of cell that lines the blood vessels, was isolated from circulating blood *(Asahara et al, 1997)* and identified as originating in bone marrow *(Shi et al, 1998)*. Whether these endothelial progenitor cells, which resemble the angioblasts that give rise to blood vessels during embryonic development, represent a bona fide population of adult bone marrow stem cells remains uncertain. Thus, the bone marrow appears to contain three stem cell populations—hematopoietic stem cells, stromal cells and endothelial progenitor cells. Two more apparent stem cell types have been reported in circulating blood, but have not been shown to originate from the bone marrow. One population, called pericytes, may be closely related to bone marrow stromal cells, although their origin remains elusive *(Bianco et al, 2001)*. The second population of blood born stem cells, which occur in four species ofanimals tested—guinea pigs, mice, rabbits, and humans—resemble stromal cells in that they can generate bone and fat *(Kuznetsov et al, 2001)*. This replenishment process occurs largely in the bone marrow, where HSCs reside, divide, and differentiate into all the blood cell types. Both HSCs and differentiated blood cells cycle from the bone marrow to the blood and back again, under the influence of a barrage of secreted factors that regulate cell proliferation, differentiation, and migration. HSCs can reconstitute the hematopoietic system of mice that have been subjected to lethal doses of radiation to destroy their own hematopoietic systems *(Becker et al, 1963; Till et al, 1961)*. Over the years, many combinations of surface markers have been used to identify, isolate, and purify HSCs derived from bone marrow and blood. Undifferentiated HSCs and hematopoietic progenitor cells express c-kit, CD34, and

H-2K. These cells usually lack the lineage marker Lin, or express it at very low levels (Lin–/low). And for transplant purposes, cells that are CD34+ Thy1+ Lin– are most likely to contain stem cells and result in engraftment. Two kinds of HSCs have been defined. Long-term HSCs proliferate for the lifetime of an animal. In young adult mice, an estimated 8 to 10% of long-term HSCs enter the cell cycle and divide each day. Short-term HSCs proliferate for a limited time, possibly a few months. Long-term HSCs have high levels of telomerase activity *(Weissman, 2000)*. In adult humans, HSCs occur in the bone marrow, blood, liver, and spleen, but are extremely rare in any of these tissues. Bone marrow stromal cells have long been recognized for playing an important role in the differentiation of mature blood cells from HSCs. But stromal cells also have other important functions *(Friedenstein, 1966)*. In addition to providing the physical environment in which HSCs differentiate, BM stromal cells generate cartilage, bone, and fat (see above). Unlike HSCs, which do not divide in vitro (or proliferate only to a limited extent), BM stromal cells can proliferate for up to 35 population doublings in vitro *(Bruder et al, 1997)*. They grow rapidly under the influence of such mitogens as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor-1 (IGF-1) *(Bianco et al, 2001)*. Like HSCs, BM stromal cells arise from embryonic mesoderm during development, although no specific precursor or stem cell for stromal cells has been isolated and identified. One theory about their origin is that a common kind of progenitor cell—perhaps a primordial endothelial cell that lines embryonic blood vessels—gives rise to both HSCs and to mesodermal precursors. The latter may then differentiate into myogenic precursors (the satellite cells that are thought to function as stem cells in skeletal muscle), and the BM stromal cells *(Bianco et al, 1999)* (**Fig. 2.6**).

2.5.3 Epidermal Stem Cells Niche

Skin epidermis, with its appendix hair follicle structure, is a regenerating organ with a well-organized architecture. Each hair follicle is composed of a permanent portion, which includes sebaceous glands and the underlying bulge area, and a dynamic renewing portion which give arise two stem cells population within the hair follicle and interfollicular regions. The first population, the epidermal stem cell is located in the basal layer of the skin clustered in epidermal proliferation units *(Potten, 1981)*, normally gives rise to stratified skin layers. The second, hair follicle stem cells (HFSCs), resides in a region of the outer root sheath called the "bulge", and it is responsible for the regeneration of hair and sebaceous glands, restore the epidermis after wounding *(Taylor et al, 2000; Rendl et al, 2005)* and that can be activated during the hair cycle in response to injury.

The bulge area act as a niche where HFSCs *(Niemann et al, 2002)* are located, maintained *(Cotsarelis et al, 1990; Sun et al. 1991)* and also responsible for

the long-term replenishment of the interfollicular epidermis. Bulge stem cells are generally quiescent, multipotent and, after their activation, giving rise to daughter cells; the daughter cells retained in the bulge remain as stem cells while other daughter cells migrate down to become hair-matrix progenitors responsible for hair regeneration *(Cotsarelis et al, 1990; Niemann et al, 2002; Oshima et al, 2001; Taylor et al, 2000)*.

Another population of stem cells in skin occurs in the basal layer of the epidermis. These stem cells proliferate in the basal region, and then differentiate as they move toward the outer surface of the skin. The keratinocytes in the outermost layer lack nuclei and act as a protective barrier. A dividing skin stem cell can divide asymmetrically to produce two kinds of daughter cells. One is another self-renewing stem cell. The second kind of daughter cell is an intermediate precursor cell which is then committed to replicate a few times before differentiating into keratinocytes *(Zhu et al, 1999)* (**Fig. 2.6**).

2.5.4 Intestinal Stem Cells Niche

The intestinal epithelium can be divided into two regions, a region containing pericryptal fibroblasts and mesenchyme functional differentiated cells (villa) and a proliferative region (crypt Lieberkühn) which represents the stem cell niche. Intestinal regeneration begins with intestinal stem cells (ISCs), which give rise to four different types of epithelial lineages: columnar enterocytes, mucin-producing goblet cells, Paneth cells, and enteroendocrine cells *(Bjerknes et al, 1999; Winton et al, 2000)*.

ISCs are generally proposed to be located at the fourth or fifth position from the crypt bottom, above the Paneth cells *(Booth et al, 2000; He et al, 2004; Sancho et al, 2004)*. The crypt is a contiguous pocket of epithelial cells at the base of the villus. ISCs and Transit Amplifying (TA) cells within the crypt regenerate the entire villus every 3 to 5 days *(Potten et al, 1990)*. Genetic marker shows that crypts derive from an individual or few ISCs and that each villus is the product of cells the coming from several adjacent crypts *(Gordon et al, 1992)*. There are four to six ISCs per crypt that are located in ring diameters of about four cells from the crypt bottom. Progeny of activated ISCs migrate upwards to become TA cells. When they reach the top of the crypt, TA cells stop proliferating and assume their appropriate positions within the villus structure. During postnatal intestinal regeneration, mesenchymal cells subjacent to epithelial cells play a role in epithelial cell proliferation, differentiation, and apoptosis; BMP4, expressed in the ISC-adjacent mesenchymal cells, is one of the putative niche signals *(He et al, 2004)*; endothelial cells provide ISCs with survival signals such as fibroblast growth factor (FGF) *(Paris et al, 2001)*; myofibroblasts surrounding epithelial cells, supported ISCs "niche" and influence other epithelial cells (Mills et al, 2001). Molecular analysis showed that signal Wnt plays a positive role

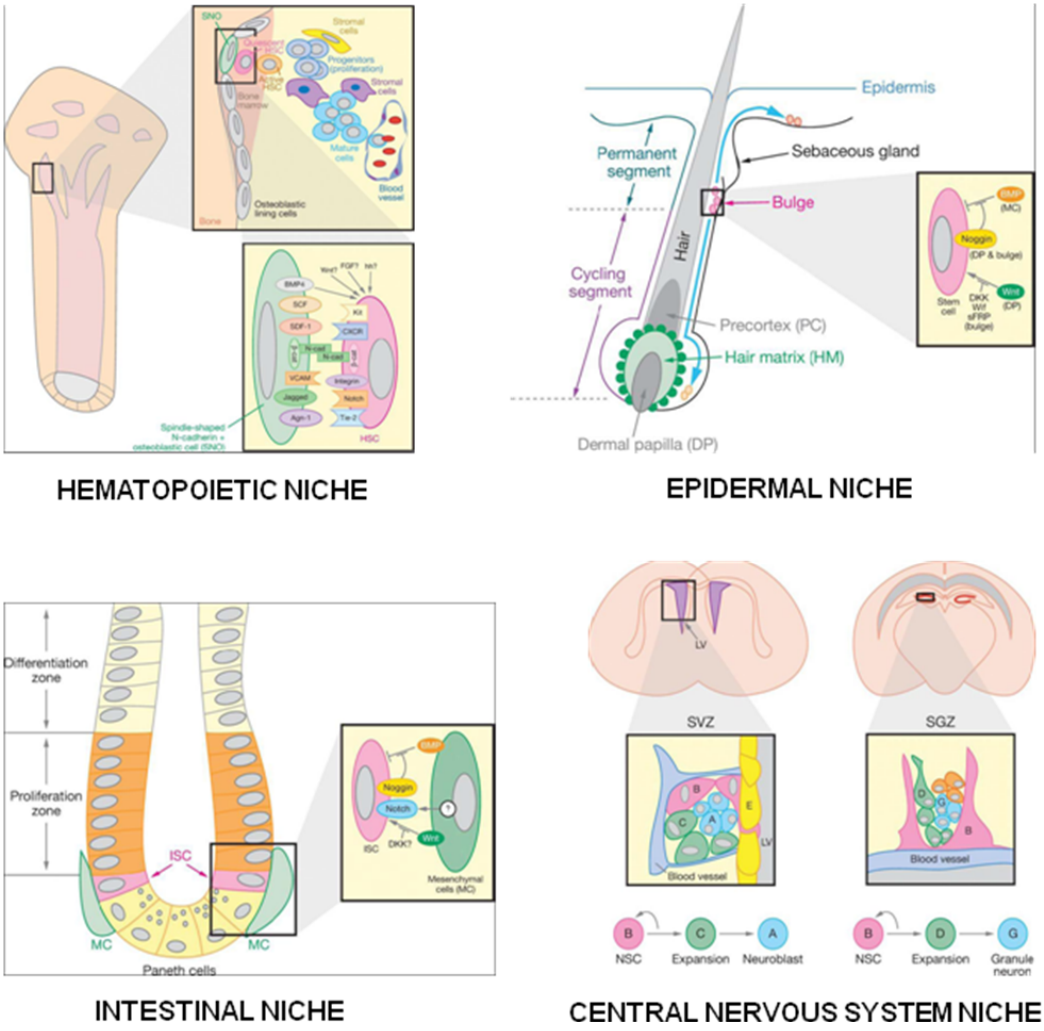

Figure 2.6. In vivo distribution of Stem cells niches (from Li et al, 2005).

in the promotion of ISC activation/self-renewal; in contrast, BMP signaling restricts ISC activation and crypt cell fate *(Haramis et al, 2004; He et al, 2004)* (**Fig. 2.6**).

2.5.5 Skeletal muscle Stem Cells

Skeletal muscle is derived from embryonic mesoderm. To date, at least three populations of skeletal muscle stem cells have been identified: satellite cells, cells in the wall of the dorsal aorta, and so-called "side population" cells (SP). Satellite cells in skeletal muscle were identified 50 years ago in frogs by electron microscopy *(Mauro, 1961)*, and thereafter in mammals *(Schultz, 1976)*. Satellite cells occur on the surface of the basal lamina of a mature muscle cell, or myofiber. In adult mammals, satellite cells mediate muscle growth *(Schultz, 1996)*.

Although satellite cells are normally non-dividing, they can be triggered to proliferate as a result of injury, or weight-bearing exercise.mediated by a rich group of myogenic regulatory elements. A recent report indicates that muscle stem cells may also occur in the dorsal aorta of mouse embryos, and constitute a cell type that gives rise both to muscle satellite cells and endothelial cells. Whether the dorsal aorta cells meet the criteria of a self-renewing muscle stem cell is a matter of debate *(De Angelis et al, 1999)*. Another report indicates that a different kind of stem cell, called an SP cell, can also regenerate skeletal muscle may be present in muscle and bone marrow. SP stands for a side population of cells that can be separated by fluorescence-activated cell sorting analysis *(Gussoni et al, 1999)*.

2.5.6 Stem Cells in the Pancreas and Liver

The status of stem cells in the adult pancreas and liver is unclear. During embryonic development, both tissues arise from endoderm. A recent study indicates that a single precursor cell derived from embryonic endoderm may generate both the ventral pancreas and the liver *(Deutsch et al, 2001)*. In adult mammals, however, both the pancreas and the liver contain multiple kinds of differentiated cells that may be repopulated or regenerated by multiple types of stem cells. In the pancreas, endocrine (hormone-producing) cells occur in the islets of Langerhans. They include the beta cells (which produce insulin), the alpha cells (which secrete glucagon), and cells that release the peptide hormones somatostatin and pancreatic polypeptide. Stem cells in the adult pancreas are postulated to occur in the pancreatic ducts or in the islets themselves. Several recent reports indicate that stem cells that express nestin—which is usually regarded as a marker of neural stem cells—can generate all of the cell types in the islets *(Zulewsky et al, 2001)*. The identity of stem cells that can repopulate the liver of adult mammals is also in question. Recent studies in rodents indicate that HSCs (derived from mesoderm) may be able to home to liver after it is damaged, and demonstrate plasticity in becoming hepatocytes (usually derived from endoderm) *(Lagasse et al, 2000; Theise et al, 2000)*. But the question remains as to whether cells from the bone marrow normally generate hepatocytes in vivo. It is not known whether this kind of plasticity occurs without severe damage to the liver or whether HSCs from the bone marrow generate oval cells of the liver *(Crosby et al, 2001)*. Although hepatic oval cells exist in the liver, it is not clear whether they actually generate new hepatocytes *(Thorgeirsson, 1993)*. Oval cells may arise from the portal tracts in liver and may give rise to either hepatocytes*(Lazaro et al, 1998)* and to the epithelium of the bile ducts *(Germain et al, 1988)*. Indeed, hepatocytes themselves, may be responsible for the well-known regenerative capacity of liver.

Chapter 3

Vascular Wall Niche

The development of new vessels from progenitor cells, i.e., angiogenesis, is not limited to embryogenesis *(Carmeliet, 2003)*. Adult vessels are physiologically exposed to mechanical forces and shear stresses that can damage the vascular wall; as a result vascular wall Cells (VWCs) growth and repair are necessary during life. Furthermore chronic vascular injuries are associated with arterial wall diseases, e.g., atherosclerosis, post-graft arteriosclerosis, aneurysmal dilatation, in-stent restenosis,; under these pathological circumstances not only VWCs but also their progenitors are involved in the early and late arterial wall disease development. As a consequence, the identification and characterization of progenitors involved in physiological homeostasis and pathological vascular remodelling is an issue of great interest involving the modern fields of repair or regenerative medicine and tissue engineering. In 2006 was suggested the existence of a "vasculogenic niche" in the human vascular wall of large and mid-sized blood vessels. The existence of this "vasculogenic zone" has been defined as a vascular mural zone, identified in adult human vascular wall and located at the border between the media and the adventitial layers containing a complete hierarchy of resident stem cells, which may serve as a source for progenitor cells for postnatal vasculogenesis *(Zengin et al, 2006)* (**Fig. 3.1**).

3.1 Vascular wall resident Endothelial Progenitors

Most of the knowledge about resident vascular progenitors comes from animal models, even though more recent studies are shedding light on their presence and role in the human vascular wall.

Pivotal studies by Alessandri and colleagues investigated the angioforming capacity of human embryonal aorta through ring assay. Under this experimental condition, capillary-like structures expressing markers of endothelial differentiation sprouted from the outer layer of aortic rings raising the possibility that immature endothelial precursor cells could be present in the vessel wall.

This hypothesis was confirmed by in vitro differentiation of CD34+/CD31- vessel wall isolated cells into mature endothelial cells *(Alessandri et al, 2001)*. Similar results were recently obtained on fetal tissue. In fact, vascular progenitor cells residing in the human fetal aorta were able to give rise to endothelial and mural cells in response to Vascular Endothelial Growth Factor (VEGF) and Platelet-Derived Growth Factor-BB (PDGF-BB) respectively and to improve neovascularisation in a murine model of peripheral ischemia*(Invernici et al, 2007)*.

In 2005 Ingram et al. found that human mature ECs contain a subpopulation of EPCs allegedly organized in a completely hierarchical manner with different clonogenic and proliferative potential *(Ingram et al, 2005)*. This finding is very intriguing because it refers to an adult cell population which is supposed to have a low turnover in normal vessels; adult ECs are indeed reported to undergo mitosis in less than 1% of the overall cell compartment daily *(Schwartz et al, 1977)*.

A crucial evidence for the presence of EPCs in the adult vascular wall is provided by the study of Zengin et al. who identified in the human internal thoracic artery (HITA) a vasculogenic area located between the media and adventitia layers; according to the authors this area may serve as a source for progenitor cells suited for postnatal vasculogenesis, i.e., the growth of new vessels from pre-existing ones. Consistent with this hypothesis, ring assays demonstrated that CD34+ cells of the HITA-wall form capillary sprouts ex vivo both in the external surface and in the internal lumen *(Zengin*

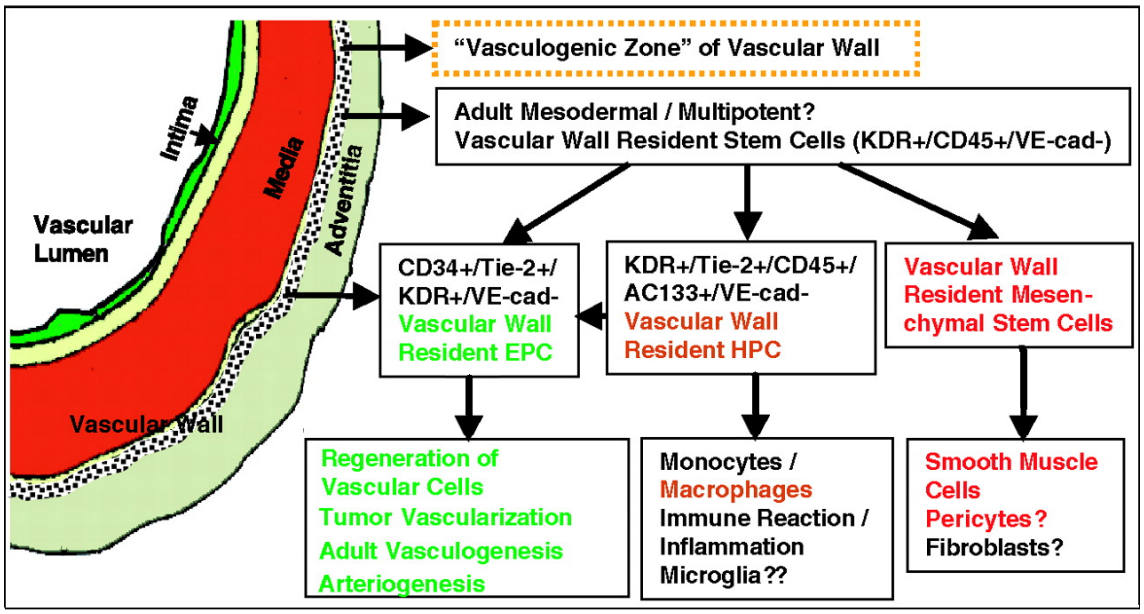

Figure 3.1. The vascular wall niche (from Zengin et al, 2006).

et al, 2006).

3.2 Vascular wall resident SMC progenitors

The integrity and synchronized activity of the smooth muscle layer is also essential for vascular functionality as it insures the correct haematic flow to the peripheral tissues. The composition of adult media reflects the different embryological origins of SMCs. Indeed they differ in morphology, cytoskeletal-contractile protein expression and production of peptide growth factors *(Chamley-Campbell et al, 1979).* Growth and remodelling of blood vessels after birth is due to migration and proliferation of resident progenitor cells according to embryogenesis. Actually little is known about the exact identity and location of SMC progenitors in human vessels even though their existence in the vascular wall of adult animals has been demonstrated.

Sainz et al., in 2006, demonstrated the presence of endothelial and smooth muscle progenitor cells in healthy arteries of adult mice. Researchers selected the progenitors as a Hoechst 33342 negative fraction in flow cytometry on total cells extracted from murine aorta and then differentiate them with VEGF and PDGF-BB or TGF-β1 *(Sainz et al, 2006).*

In another study, immunohistochemical staining revealed that the adventitia in murine aortic roots contains a large numbers of cells expressing stem cell markers as Sca-1. These cells can differentiate into SMCs in a PDGF-BB dependent manner. It seems that vascular wall contains smooth muscle progenitors that can replace dead or malfunctional cells *(Hu et al, 2004).*

According to these results, the SMC progenitor should belong to a multipotent stem cell population since both animal studies obtained a SMC lineage able to give origin to endothelial and smooth muscle cells. Furthermore, from embryological studies emerge the possibility that endothelial and smooth muscle cells may also share a common origin from a bipotent precursor cell *(Yamashita et al, 2000; Ferreira et al, 2007).* Flk1+ murine ESCs *(Yamashita et al, 2000)* and CD34+ human ESCs *(Ferreira et al, 2007)* isolated from embryonic bodies originate endothelial and smooth muscle-like cells in vitro *(Yamashita et al, 2000; Ferreira et al, 2007)* and form functional microvessels in vivo *(Ferreira et al, 2007).* Although single cell isolation procedures and parallel divergence of their progeny were not performed in both studies quoted above, this possibility can not be ruled out.

Another possibility is that some smooth muscle progenitors in adult vessels may be commissioned from pericytes. Howson et al. isolated mesenchymal cells from rat aortas, expressing immature pericyte markers at basal level, that form spheroid colonies "in vitro" when cultured in serum free medium supplemented with basic-Fibroblast Growth Factor (b-FGF), whereas they acquire expression of SMC marker proteins and differentiate in pericytes when exposed to serum, PDGF-BB or when cultured with angiogenic outgrowths of rat aorta and ECs *(Howson et al, 2005).*

Recent studies about postnatal neovascularization suggest the possibility to obtain smooth muscle progenitors also in circulating blood *(Simper et al, 2002)*, bone marrow *(Sata et al, 2002)*, skeletal muscle *(Maika et al, 2003)* and fat tissue *(Rodriquez et al, 2006)*.

Altogether these studies indicate that development of vascular SMCs from progenitors does not occur exclusively during embryogenesis; vascular resident progenitor cells can replace immediately damaged cells owing to normal physiology, and vascular progenitor cells from peripheral tissues are mobilized efficiently in case of severe arterial trauma *(Yamashita et al, 2000)*.

3.3 Vascular wall resident mesenchymal stromal cells

It can not be excluded that additional multipotent progenitors are involved in ECs and SMCs generation; actually an angiogenic and leiomyogenic differentiation has been obtained from human vascular wall resident mesenchymal stem cells *(Pasquinelli et al, 2007 a; Hoshino et al, 2008; Covas et al, 2005)*; also a cell endowed with a multipotency equivalent to that of the mesoangioblast described in the quail embryonal dorsal aorta could be involved *(Minasi et al, 2002; Cossu et al, 2003)*. The vasculogenic area which is present in the vascular wall between the media and adventitia layers presents many niche-like characteristics and therefore could be the place where smooth muscle progenitors are located; this is also in agreement with the hypothesis that this same area could contain stem cells, capable to differentiate into mature ECs, hematopoietic and local immune cells, such as macrophages *(Zengin et al, 2006)*, and MSCs whose presence in the adult vessel wall of animal and human tissues *(Pittenger et al, 1999; Zuk et al, 2001; Jones et al, 2004; Schultz et al, 1994; Erices et al, 2000; Zhang et al, 2004; Alviano et al, 2007)* is still matter of debate.

Tintut at al. first demonstrated that MSCs are present also in the adult animal vessels; in fact, they isolated multipotent cells from the tunica media of the bovine aorta. These cells, also known as calcifying vascular cells (CVCs), display not only osteoblastic potential, but also chondrogenic, leiomyogenic and stromogenic potential under specific experimental conditions *(Tintut et al, 2003)*.

More recently our group described the presence of Vascular Wall resident mesenchymal stem cells (VW-MSCs) in normal human adult thoracic aortas. Cryopreserved thoracic aortas harvested from heart-beating multiorgan donors were initially investigated for evaluating the VWC resistance to cryoinjury: the vast majority of VWCs, i.e. ECs and SMCs, were irreversibly injured *(Pasquinelli et al, 2006)*; interestingly the only cells in the cryopreserved arterial wall which still remained viable at 4 days of organ culture were capillary structures located in between the media and adventitia layers; therefore microvascular stress-resistant cells are endowed in this arterial zone; the immunohistochemical analysis of the CD34+/CD31- vasculogenic layer previously described by Zengin in

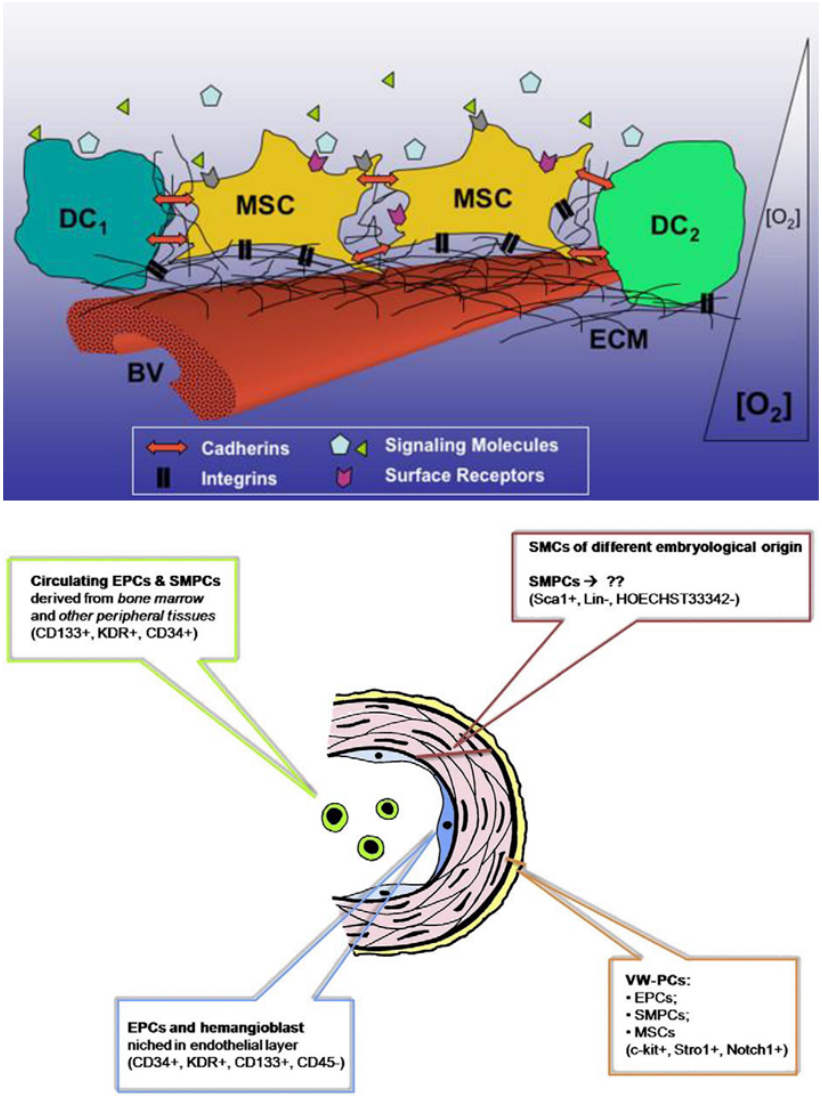

Figure 3.2. Characteristics and cell composition of vascular wall niche (from Kolf et al, 2007 and Pacilli et al, 2009).

HITA confirmed the presence of an analogous well-developed plexus of CD34+ cells also in the aorta and the femoral artery as well; single immunostainings on serial sections supported the presence of CD34+ cells possibly corresponding to the resident CD34+/CD31- endothelial progenitors reported in HITA. Beside its presumptive vasculogenic post-natal ability this area was richly endowed with highly expressing HLA-I *(Pasquinelli et al, 2007 b)* small calibre vessels and therefore could act as a strategic area for the recruitment of blood circulating cells; accordingly we observed the presence of bone marrow-derived CD45+ mononuclear cells; additional experiments also revealed the presence of CD68+ and

S-100+ perivascular cells representing resident macrophages and dendritic accessory cells. Moreover, this area has some characteristics, e.g., a discrete morphology, a strategic location at the interface between media and adventitia layers, an heterogeneous and complex cell composition, an unexpectedly elevated cell proliferation under normal condition, a selective localization of cells expressing the stem cell surface molecule c-kit which makes it unique in the arterial wall and consistent with a niche-like function *(Pacilli et al, 2009)* (**Fig. 3.2**). The niche concept may explain why a cell population sharing many properties with bone marrow-derived MSCs can be isolated and expanded in vitro from the aortic arch, thoracic aorta and femoral artey *(Pasquinelli et al, 2007 a; Pasquinelli et al, 2010)*, pulmonary artery *(Hoshino et al, 2008)* and saphena vein *(Covas et al, 2005)*. Furthermore, the multipotency of these VW-MSCs which have been found to have angiogenic *(Pasquinelli et al, 2007 a)*, adipogenic, osteogenic, chondrogenic and myogenic *(Hoshino et al, 2008; Covas et al, 2005; Pasquinelli et al, 2010)* potential could have implications in the development of ectopic tissues like cartilage, marrow and fat tissue in vascular disease and atherosclerosis.

Chapter 4

Therapeutic Potential Of Vascular Progenitor Cells

4.1 Angiogenesis

Vessel formation occurs mainly through two mechanisms: vasculogenesis and angiogenesis. Vasculogenesis represents de novo formation of blood vessels during embryonic development. As previously described, mesoderm derived stem cells (hemangioblast) first form aggregates (blood islands) and then develop into primitive hematopoietic and endothelial cells (angioblasts). The angioblasts, undifferentiated precursor cells, proliferated and differentiated in situ into endothelial cells to form a vascular labyrinth *(Carmeliet, 2000)*. In postnatal life adult angioblasts seem to be present even if their location and characterization must be determine. On the other hand, the formation of new vessels from preexisting vessels is called angiogenesis, which is more frequent in adult life although vasculogenesis may also occur. Angiogenesis is a tightly regulated process required for a number of physiological processes like wounf healing, ovulation and menstruation as well as embryonic development. Excessive angiogenesis is seen in a wide range of diseases including tumors, inflammatory disease and diabetic retinopathy *(Kiumura et al., 2003)*. Angiogenesis is a very complex mechanism including different steps. It starts with vasodilatation, a process involving nitric oxide. The increased permeability leads to a preexisting vessel destabilization and to a loss of the endothelial cell interactions. Different angiogenic growth factors are released. These molecules activate metallo proteinases, which are crucial for extracellular matrix degradation. After this, endothelial cells proliferate and migrate to assemble in solid cords that finally acquire a lumen. Subsequently, pericytes and SMCs migrate in order to reorganize the cellular interactions and complete the new formed vessels architecture. Angiogenic sprouting is controlled by a balance of activators and inhibitors. Important activator factors are VEGF,

FGF, members of TGF-β family, IL-8 and Angiopoietin-I. Inhibitors factors are interferon-α, β, γ, IL-12, angiostatin and endostatin *(Carmeliet, 2000)* (**Fig. 4.1**).

4.2 MSCs and angiogenic differentiation

Nowadays, the more reliable hypothesis is that hemangioblast derived form mesenchymal stem cells; this hypothesis is supported by the fact that MSCs retain angiogenic and hemogenic ability even at adult life. Reyes at al., in 2002 have isolated a multipotent population of adult stem cells, called MAPC (multipotent adult progenitors cells) from the adult bone marrow, which are able to differentiate into endothelial cells and other mesodermal tissues *(Reyes at al., 2002)*. Recent studies demonstrated that also adult mesenchymal stem cells are endowed with angiogenic ability. Oswald et al. showed that human BM-MSC are able to differentiate into cells with phenotypic (vWF, KDR and Flt expression) and functional features of endothelial cells when cultivated under confluence, presence of 2% FCS and VEGF 50ng/ml for 7 days *(Oswald et al., 2004)*. The angiogenic ability is not only restricted to bone marrow derived cells, but also to MSC isolated from different sources such as vassel wall *(Pasquinelli et al, 2007 a)*, term amniotic membrane *(Alviano et al, 2007)*, dental pulp (Marchionni et al, 2009), intestine *(Lanzoni et al, 2009)*, adipose tissue *(Lin et al, 2010)*. Not surprisingly, fetal and adult vascular progenitor cells are the best candidate for neoangiogenesis *(Alessandri et al 2001; Invernici et al, 2007; Pasquinelli et al, 2007 a, Campagnolo et al, 2010)*. We recently reported that adult thoracic aorta derived MSCs exhibit the ability to differentiate into endothelium in vitro. In accordance with Oswald et al. *(Oswald et al., 2004)* we used a three dimensional Matrigel semisolid matrix to assay angiogenesis. After 20 hrs of culture the MSCs were able to form capillary-like structures but this ability was strictly depended on VEGF induction; controls always proved negative. VEGF induction was accompanied by increased cell expression of KDR, as documented by flow cytometry and RT-PCR assays. At immunofluorescence, vWF intensely stained the cytoplasm of cells with a rounded morphology (12 ± 3 %). Likewise, electron microscopy of the 20 hr samples showed features consistent with a basic endothelial cell employment, i.e., collections of micropinocytotic vesicles and caveolae, tight junctions, and immature Weibel-Palade bodies *(Pasquinelli et al, 2007 a)*.

4.3 Therapeutic angiogenesis for Peripheral Arterial Disease

Peripheral arterial disease (PAD) is a common cause of disability and mortality. Up to one third of patients are not susceptible to traditional revascularization and may benefit from stem cell therapies. Furthermore, replacement of dead cells exclusively by proliferation of terminally differentiated neighboring endothelial cells in case of massive loss of endothelium is not sufficient to recover the structural and functional integrity of the endothelial lining. In such conditions the

support and crucial contribution of stem cells and endothelial or smooth muscle progenitors is necessary. Progenitors can reside in the vascular wall itself or can be mobilized from the bone marrow and delivered in the blood; in any case, progenitors concentrate in the area of tissue damage responding to local microenvironmental signals; tissue repair is then achieved by several still debated mechanisms, which include extracellular matrix remodeling, angiogenesis and differentiation of progenitors into mature and functional cells.

Accumulating evidence shows the ability of mobilized EPCs to repair injured vessels of animal models *(Sata et al, 2002; Shimizu et al, 2001; Hillebrands et al, 2001; Werner et al, 2002; Xu et al, 2003; Griese et al, 2003; Werner et al, 2003)*; however, one potential limitation to the use of autologous stem cells in clinical application is their low number and the documented decline in the number and function with aging *(Riha et al, 2005; Sethe et al, 2006)* and disease *(Hristov et al, 2003; Fadini et al, 2006)*. These shortcomings can be overcome by increasing the number of circulating EPCs through pharmacological administration of growth factors, such as granulocyte colony-stimulating factor (G-CSF) or VEGF, which activate progenitor cell releasing factors that are able to promote the mobilization of stem cells from the bone marrow. Alternatively, Mononuclear cells from peripheral blood (PB-MNCs) can be harvested for in vitro expansion and differentiation and then

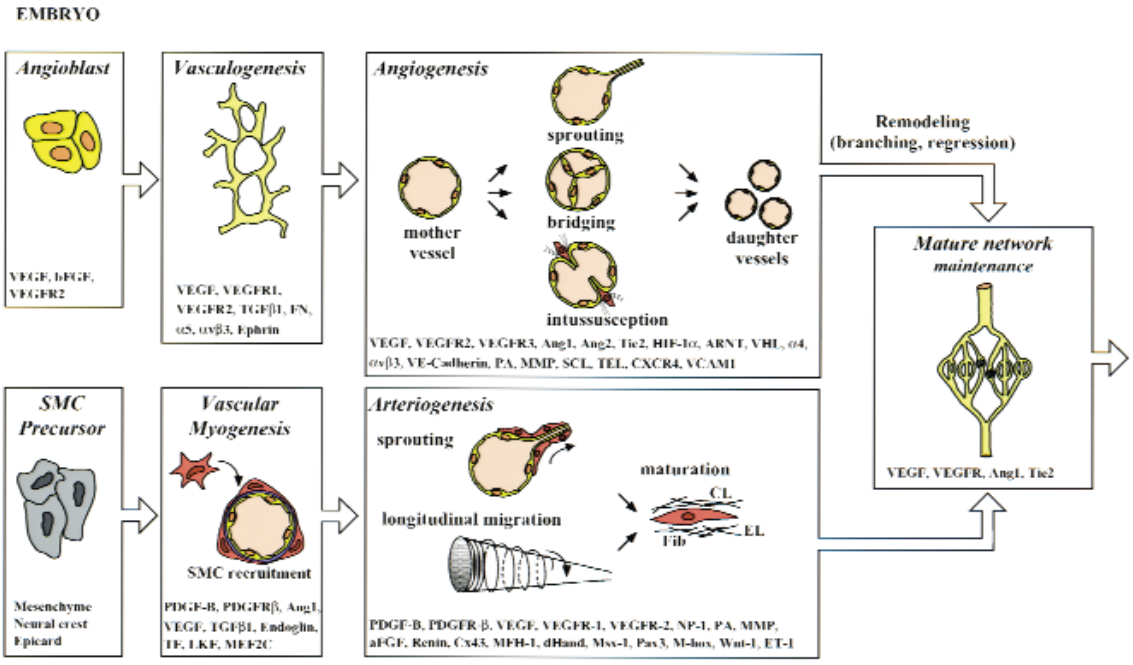

Figure 4.1. Angiogenesis and arteriogenesis (from Carmeliet, 2000).

transfused back into the patient. Thus, stem cell therapy is a realistic option for the treatment of vascular diseases.

Transfusion of ex vivo expanded EPCs *(Wassmann et al, 2006)* or endogenous mobilization of them *(Werner et al, 2002; Kong et al, 2004; Vasa et al, 2001)* enhances reendothelialization in different models of endothelial denudation. Moreover, EPCs may also contribute to angiogenesis in wound healing, tissue ischemia, or myocardial infarction. Delivery of bone marrow derived Lin-/c-kit+ cells onto the border of an infarct promotes angiogenesis in situ *(Orlic et al, 2001)*, and intramuscular (IM) injection of bone marrow-mononuclear cells (BM-MNCs) into the ischemic limbs of rat and mouse induces collateral vessel formation *(Iba et al, 2002; Kalka et al, 2000)*. Some in vivo functional approaches have already been applied in clinical trials, mainly involving patients with myocardial infarction; these trials are extensively reviewed elsewhere *(Boyle et al, 2006; Oettgen et al, 2006)*. In a recent review, Pacilli et al have focused on the less covered field of promoting angiogenesis in peripheral vascular disease through the use of stem cell, protein, and gene therapies; innovative attempts at using stem cell engineered biomaterials are also dealt with *(Pacilli et al, 2010)*.

A more recent metanalysis of the literature examined all 37 clinical trials on autologous cell therapy in patients with PAD; they found that autologous cell therapy was effective in improving surrogate indexes of ischemia, subjective symptoms and hard endpoints (ulcer healing and amputation). On the contrary, G-CSF monotherapy was not associated with significant improvement in the same endpoints. Patients with thromboangitiis obliterans showed some larger benefits than patients with atherosclerotic PAD. Furthermore, the intramuscular route of administration and the use of bone marrow cells seemed somehow more effective than intrarterial administration and the use of mobilized peripheral blood cells. Anyway, all these procedures were well tolerated and generally safe *(Fadini et al, 2010)*.

On the whole, the results obtained so far may help in finding a way to successfully manage threatened ischemic limbs. Most studies seem inadequately categorized in their clinical presentation and, therefore, are of little practical significance for the expert vascular surgeon. Larger, placebo-controlled, randomized multicenter trials need to be planned and conducted to confirm these findings. In order to obtain clinically useful information and to promote meaningful research in this promising field, more strict interaction between "pure" researchers and specialized vascular surgeons should be encouraged. Candidates for therapeutic angiogenesis should be chosen from the really threatened ischemic limb category in order to avoid possible misinterpretation with a benign course of a mild arteriopathy. Such patients should be unfavorable candidates for other kinds of known vascular procedures in order to avoid ethical conflicts in treating them. In this sense, therapeutic angiogenesis treatment may be tested on patients undergoing surgical or endovascular revascularization and compared with patients

treated similarly without the adjunct of this novel therapy, thus comparing possible differences in the healing of ischemic lesions. To this end, vascular centers with high turnover and expertise in treating peripheral vascular disease should be involved in designing and carrying out the clinical arm of research into these innovative strategies *(Pacilli et al, 2010)*.

In the future we cannot exclude that the injection of native vascular progenitor cells could provide a new way to treat ischemic diseases. Some groups have successfully tested in vivo the myogenic and vasculogenic properties of Vascular Progenitor Cells (VPCs) by using a murine model of peripheral ischemia and observed that VPCs transplantation into ischemic muscles significantly ameliorates the clinical outcome of ischemic mice *(Invernici et al. 2007, Campagnolo et al. 2010)*

Chapter 5

Role of Vascular Progenitor Cells in Vascular Calcification

5.1 Biology of vascular calcification

The arterial wall is one of the most frequent location of pathological insults in humans; the balance between injury and repair is altered by acute and chronic stresses, the atherosclerosis being the major disease of the arterial wall; vasculitis, aneurysmal dilatation, dysplasia and even neoplasms are additional examples of diseases that can affect irreversibly the arterial wall. As in bone, endothelial, mesenchymal, and hematopoietic cell lineages control vascular mineral accumulation, with cellular activities entrained to morphogenetic, metabolic, inflammatory and mechanical demands placed on each vascular segment *(Abedin et al, 2004)*. Vascular calcification was previously considered to be a purely degenerative, passive process, without biological regulation. However, the current view is that vascular calcification is a biologically regulated process that, like osteogenesis, involves both activators and inhibitors. The concept that vascular calcification is related to osteogenesis is not unexpected, given the interaction of vascular and bone cells in normal embryonic skeletogenesis. In embryonal development, endochondral ossification follows invasion of neoangiogenic vessels into calcified cartilage matrix. Once the vasculature is established, preosteoblasts, originating from the angiogenic pericytes surrounding the vasculature or from blood-borne mesenchymal cells, differentiate and initiate mineralization *(Gerber et al, 2000)* (**Fig. 5.1**).

Vessel wall osteoprogenitor cells known as calcifying vascular cells (CVCs) can form bone matrix proteins and calcified nodules, analogous to osteoblastic differentiation in bone. These cells have been isolated from the tunica media of bovine and human arteries, and both in-vitro tissue culture models and mouse models of vascular calcification have been established*(Jacoby et al, 2000)*. Ectopic

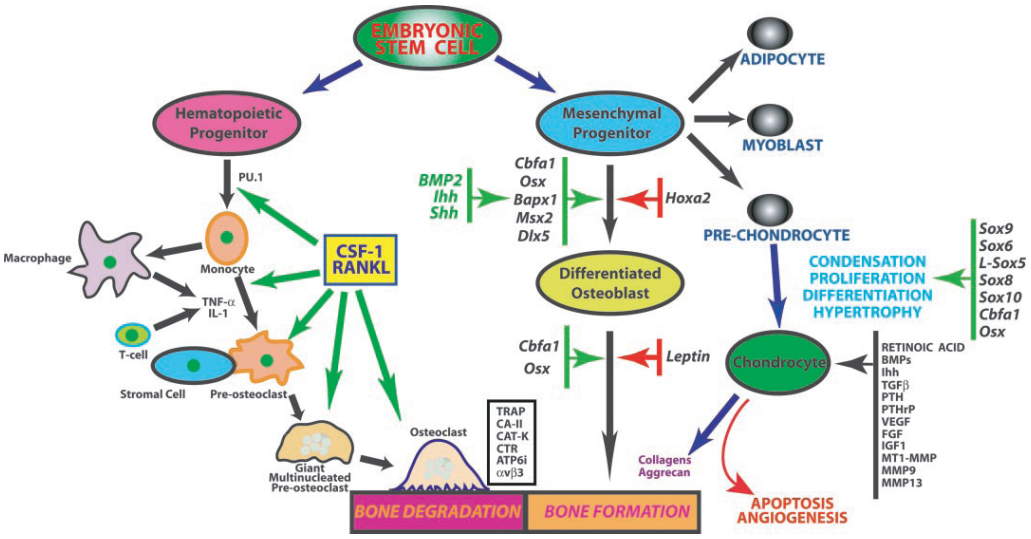

Figure 5.1. Outline of the major genetic, molecular, cellular, and endocrine mediators of bone remodeling (from Doherty et al, 2004).

bone in the artery wall is dramatic evidence of mesenchymal cell plasticity. Eghbali-Fatourechi and colleagues recently identified circulating osteoblastic cells using antibodies to osteocalcin (OCN) or alkaline phosphatase (AP). OCNpos cells consist of two distinct populations: one population exhibits low forward/side scatter, consistent with a small cell phenotype with low granularity, and a second population has higher forward/side scatter (larger and more granular cell). The smaller, low granularity population also co-expresses CD34, whereas the larger, more granular cells are CD34 negative. While the concentration of OCNpos cells increased with age, levels of CD34pos cells tended to decrease, leading to a significant increase in the ratio of OCNpos:CD34pos cells in peripheral blood. Since CD34pos cells include endothelial precursor cells that may be important in the response to vascular injury, this increase in the OCNpos:CD34pos ratio in circulating cells raises the possibility that with aging, the response to vascular injury may potentially result in vascular calcification, rather than repair *(Eghbali-Fatourechi et al, 2007)*. Given the potential overlap between osteoblastic and endothelial cells it is also possible that precursor cells in the vasculature give rise to osteoblastic progenitors.

Several models postulating mechanisms for the formation and/or inhibition of calcification have now been proposed *(Doherty et al, 2003)*. These are: 1) the active model; 2) the passive physicochemical model; and 3) the arterial Osteoclast-like cells (OLC) model. The active model of arterial calcification evolved partly from the observations, stimulated in large part by studies of Bostrom et al. *(Bostrom et al, 1993)*, who reported the existence of pluripotent arterial cells that they named CVCs. The BMP signaling pathway is

critical to proper bone formation. Marshall Urist *(Urist, 1965)* was the first to discover that BMPs can cause transdifferentiation of mesenchymal cells into osteoblasts, which in turn can mediate ectopic bone formation. This finding has been replicated by numerous other investigators in different models *(Wozney et al, 1988; Attisano et al, 2002)*. Cbfa1 is one of the transcription factors that regulates osteoblastic differentiation and bone formation *(Komori et al, 1997; Ducy et al, 1997)*. Among the many gene targets of BMP signaling identified to date, beside cbfa1, other transcription factors, Distal-less5 (Dlx5) and osterix (Osx), are now considered to be the master genes essential for differentiation of mesenchymal progenitors into terminally differentiated osteoblasts. As regarding the third model, morphological evidence for OLCs is supported by numerous evidences *(Doherty et al, 2004)*. Collectively, these studies suggest that there are OLCs in arteries, but the origin of these cells is unknown. It is possible that OLCs could differentiate from "resident" pluripotent arterial cells, but it is equally likely that they originate from hematopoietic precursors, such as stem cells or cells that have differentiated from mononuclear phagocytic lineage precursors *(Doherty et al, 2004)*. As regard the passive mechanism, it is thought that the onset of vascular calcification could be determine by a number of proteins which inhibit precipitation or chelate/sequester ions to lessen their bioavailability. It seems likely that in arterial calcification, proposed models are not mutually exclusive.

5.2 The Mönckeberg sclerosis

Vascular calcifications have been divided into four forms that involve 1) the atherosclerotic intima, 2) the media of medium and large sized arteries, 3) cardiac valves, and 4) a widespread form known as calciphylaxis *(Vattikuti et al, 2004)*. The second form, known as medial artery calcification (MAC) may be found in conjunction with atherosclerotic lesions or it may occur independently *(Shao et al, 2006)*. It occurs in patients with normal serum calcium and phosphorus levels and this distinguishes it from vascular calciphylaxis which is related to high serum calcium and phosphate levels that result in passive mineral deposition onto elastic fibers *(Vattikuti et al, 2004)*. Of these forms, MAC has been most strongly associated with cardiovascular events *(Guzman, 2007)*.

There are important similarities and differences between the two major types of calcification in arteries (following atherosclerosis and MAC) *(Doherty et al, 2004)*. Atherosclerosis is characterized by chronic arterial inflammation instigated and exacerbated by disordered lipid metabolism and other well-characterized risk factors *(Libby, 2002)*. Large postmortem studies have revealed that atherosclerosis begins surprisingly early and is ubiquitous in middle-aged and older adults *(Strong, 1986)*. Considerable confusion has arisen in the literature from failure to properly distinguish two distinct types of arterial calcification: medial arterial calcification, and calcification associated with atherosclerotic plaque. There are several basic lines

of evidence consistent with the conclusion that medial calcification is not the same entity as plaque calcification, including histomorphological, clinical, epidemiological, genetic and animal studies *(Doherty et al, 2004)*. There may be some common elements mediating medial and atherosclerotic calcification; the two are not necessarily mutually exclusive. Mineral deposition in arteries can occur either as a component of atheroma or in the absence of atherosclerotic plaque. In atherosclerotic plaque, calcification typically forms in the intima, most often near the base of the plaque adjacent to the medial layer of the artery. Calcifications near the luminal surface may result in erosions and have been implicated in plaque disruption and thrombosis. In contrast, calcification of the medial layer of arteries occurs independently of atherosclerosis *(Proudfoot et al, 2001)*. This type of calcification, initially referred to as Mönckeberg's sclerosis *(Mönckeberg, 1903)*, has been frequently observed radiographically with an appearance that has been likened to railroad tracks *(Lachman et al, 1977)*. Medial calcification can occur in otherwise normal young patients with no

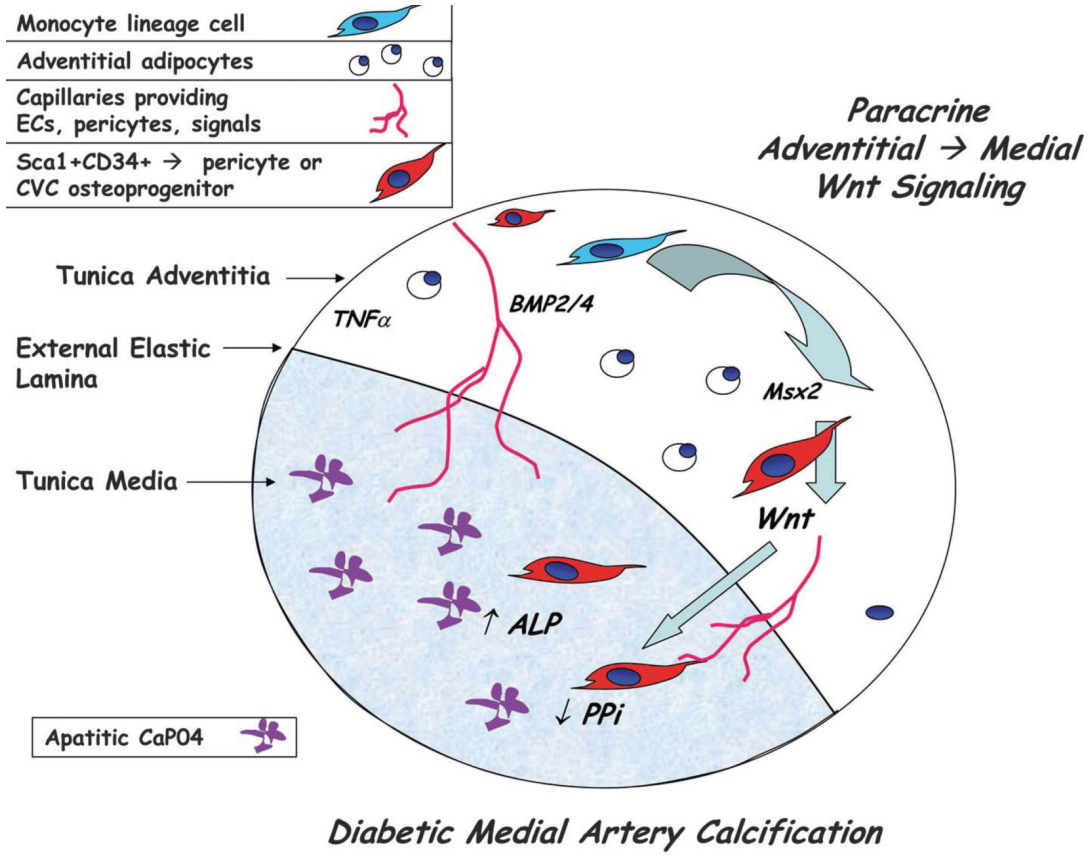

Figure 5.2. Evolving model of diabetic MAC (from Shao et al, 2006).

overt metabolic disease *(Mori et al, 1992; Top et al, 2002)*, increases linearly with age *(Elliott et al, 1994)*, and is common in elderly patients *(Lachman et al, 1977; Tohno et al, 1996)*. Medial calcification is observed with particularly high frequency and severity in disorders characterized by generalized metabolic, electrolyte, or pH derangements *(Mallick et al, 1968)*. The Mönckeberg's sclerosis typically affects arteries that are less prone to develop atherosclerosis, such as the abdominal visceral arteries, arteries to the thyroid and breast, and the arteries supplying the extremities, but it is also frequently encountered in the aorta.

Vascular calcification in its early stages develops by a process of matrix vesicle formation then partially replaced with osteoid synthesized by osteoblast-like cells of mesenchymal origin, such as the vascular smooth muscle cells (VSMC) or pericytes. The subsequent bone mineralization is dependent on neovascularization – angiogenesis from the adventitial vasa vasorum, which acts as a custom delivery system to supply the media with oxygen and nutrients but also systemic hormones, cytokines and inflammatory cells to the remodeling media and the atherosclerotic intimal disease of atherosclerosis including ossification. The location of CVCs in these arteries is both in the medial and intimal layers. It is important to note that in health the vasa vasorum normally penetrates the media but not the intima. In disease the vasa vasorum invade excessively not only the media but also the intima and this may help to explain the detrimental process of intimal ossification.

Emerging evidence indicates that vascular osteogenic signals, initiated by adventitial BMP2/Msx2 actions, are concentrically conveyed to the calcifying tunica media via the vasa vasorum *(Shao et al, 2005; Hu et al, 2004)*. Dysmetabolic signals upregulate BMP2/4 production by pericytes and endothelial cells in the vessel wall; this promotes adventitial Msx2/Wnt signaling. Subsequently, enhanced adventitial Wnt production (increased Wnt3a and Wnt7a, decreased Dkk1) augments medial nuclear β-catenin accumulation, alkaline phosphatase activity, and osteogenic differentiation. The mural CVC, a macrovascular myofibroblast related to the microvascular pericyte, is thought to be the resident osteoprogenitor. Stro-1 *(Collett et al, 2005)* and adventitial Sca1+ mesenchymal progenitors contribute to medial and intimal disease processes, and can undergo osteogenic differentiation in response to BMP2/Wnt signaling. However, the lineage relationship between Sca1+ progenitors and the CVC is currently unknown; speculation based on studies of aortic mesoangioblast development suggests that CVCs arise from Sca1+ cells *(Esner et al, 2006)*. It remains possible that the osteogenic potential of vascular progenitors is programmed within the adventitia but is elaborated only when these progenitors migrate with the vasa into the tunica media *(Hu et al, 2004; Zemecke et al, 2005)*. This model was first hypothesized in diabetic pathology; it is not possible to exclude a similar mechanism in spontaneous calcification (**Fig. 5.2**).

Chapter 6

Aim of the Thesis

The existence of a capillary-rich vasculogenic zone has been identified in adult human arteries; in this area it has been postulated that MSCs may be present amidst the endothelial progenitors and hematopoietic stem cells *(Zengin et al, 2006)*. This hypothesis is supported by several studies claiming to have found the in vivo reservoir of MSCs in post-natal tissues *(da Silva Meirelles et al, 2006 and 2008)*. Growing evidence does indeed indicate a developmental relationship between pericytes and MSCs *(da Silva Meirelles et al, 2008; Doherty et al, 1998; Farrington-Rock et al, 2004; Covas et al, 2008; Crisan et al, 2008)*; moreover, it has been suggested that pericytes may represent the MSC in situ counterpart. The presence of multipotent progenitors is not restricted to microvasculature; vascular wall resident MSCs have already been isolated from human large vessels such as the adult thoracic aorta *(Pasquinelli et al, 2007 a)*, pulmonary artery *(Hoshino et al, 2008)*, saphena *(Covas et al, 2005)* and umbilical veins *(Covas et al, 2003)*.

We have first demonstrated that the thoracic aorta harvested from multiorgan donors yields MSCs endowed with angiogenic ability *(Pasquinelli et al, 2007 a)*; now, we investigate whether embryonic-like cells may persist in the adult life and whether, according to the embryological development of the aorta, MSCs may reside in more proximal and peripheral arterial segments collected from healthy donors as well. Thus we evaluated stem cell properties of VW-MSCs, i.e. the efflux ability of DNA-binding dye Hoechst 33342, the clonogenicity and the ability of growth as spheroids under appropriate culture conditions and their multipotency. Furthermore, we assessed an in vivo pivotal study in order to evaluate the neoangiogenic ability of vascular wall derived MSCs and their behaviour in a murine model of hindlimb ischemia.

Due to multipotency, VW-MSCs may participate not only in physiological renewal, providing rapid targeted rescue for regeneration after injury by giving rise to new ECs and SMCs, but also in pathological conditions. The vascular

wall is one of the most frequent location of pathological insults in humans; the atherosclerosis is the major disease of the arterial wall, but also calcifications are diffuse. The mechanisms determining the development of arterial calcification remain largely unexplored. Several models have been proposed among which the active contribute of pluripotent arterial cells named calcifying vascular cells. We focused our attention on a less known pathology, the Mönckeberg sclerosis, an asymptomatic arterial calcification which is often confused with end stage atherosclerosis. It is no widely accepted that atherosclerotic calcification and Mönckeberg sclerosis have two distinct identities and consequently two different etiology *(Doherty et al, 2004)*. On the basis of our and other studies, we retain that calcification of the medial layer of artery wall arises independently from atherosclerosis and VW-MSCs could play an important role in the onset of the calcification.

Thus, we studied a wide series of human adult femoral arteries collected from healthy multiorgan donors which were examined in Light Microscopy (LM) on Hematoxilin-Eosin (H-E) sections to evaluate the presence of heterotopic calcification and quantify this anomalous phenomenon in healthy population. Moreover, we recovered personal and clinical data from each donors in order to find or exclude an eventual correlation between the Mönckeberg sclerosis and the commonly recognized risk factors of atherosclerosis such as diabetes, hypercholesterolemia, tabagism, and hypertension. Furthermore, we have investigated osteogenic ability of progenitors cells isolated from vessels affected by Mönckeberg sclerosis.

Chapter 7

Materials and Methods

7.1 Arterial samples and vascular niche characterization in immunohistochemistry

Fresh vascular samples were harvested from heart-beating multiorgan donors; the mean donor age was 39 years; 2 were females and 6 males. After procurement and decontamination, the arterial segments were fixed in buffered formalin, dehydrated and paraffin embedded. Four-μm-thick sections were used for immunohistochemical studies to localize VW-MSCs in situ involved into vascular wall remodeling using antibodies (Abs) directed against molecules commonly expressed by SCs. The slides were processed according to the non-biotin-amplified method with kit reagents (NovoLinkTM Polymer Detection System, Novocastra Laboratories Ltd., Newcastle Upon Tyne, United Kingdom). Briefly, sections of vessels were deparaffinated, rehydrated through decreasing concentrations of ethanol (from 100% to 70%) and rinsed in distilled water. Then, the specimens were subjected to an antigen retrieval treatment to enhance antigenicity and allow epitope unmasking with citrate buffer pH 6.0 at 120^{o}C, 1atm for 21 minutes. After cooling and washing, the endogenous peroxidase activity of the tissue was quenched for 10 minutes at room temperature (RT) with 3% hydrogen peroxide solution in absolute methanol in the dark. After washing with Tris Buffered Saline (TBS) 1X, the slides were blocked with NovocastraTM Protein Block for 5 minutes in a wet chamber to reduce the non-specific binding of primary antibody and polymer reagent and rinsed twice with TBS 1X. Successively, the samples were incubated with primary antibodies diluited in 1% Bovine Serum Albumin (BSA) in Phosphate Buffer Saline (PBS) over night (o.n.) at 4degC. Vascular tissue sections were stained using monoclonal antibodies (moAbs) against Oct-4 (1:100, Chemicon Int, Tamecula, CA, USA), Stro-1 (1:100, R&D System, Milano, Italy), Sca-1 (1:10, Cederlane Laboratories, Hornby, Ontario, Canada), Notch1 (1:50, clone c-20, SantaCruz, CA, USA). ASMA (1:9000,

clone 1A4, Dako cytomation), CD34 (1:80 clone QBEnd-10, Dako), CD45 (1:600, clone 2B11+PD7/26, Dako), CD68 (1:200, clone PG-M1, Dako), S-100 (1:5000, Dako), CD117 (1:100, Dako), ki-67 (1:200, clone MM1, Novocastra). After washing, the slides were further incubated for 30 minutes at RT with NovocastraTM Post Primary Block to enhance penetration of the next polymer reagent, rinsed in TBS 1X and after that incubated with NovoLinkTM Polymer for 30 minutes at RT. After washing, the enzyme activity was visualized with 3,3-diaminobenzidine (DAB), as substrate, prepared from NovocastraTM DAB Chromogen and NovoLinkTM DAB Substrate Buffer, rinsed in distilled water to stop the reaction and counterstained with Gill's hematoxylin. Subsequently, the samples were dehydrated, coverslipped and viewed in a light microscopy using the Image-Pro Plus® 6 software (Media Cybernetics, Inc., Bethesda, MD, USA). Images were digitalized through a video camera (JVC 3CCD video camera, KY-F55B, Jokohama, Japan) connected with a Leitz Diaplan light microscope (Wetzlar, Germany). Original images were taken at 10X. Negative controls were performed by omitting the primary antibodies.

7.2 Progenitor cells isolation, cell culture and flow cytometry characterization

Cell isolation. Fresh arteries were furnished by operating room of Vascular Surgery of the S.Orsola-Malpighi General Hospital of Bologna for the vascular stem cells isolation. Each segment was minced at approximately 5-cm-long pieces, longitudinally cut, providing an exposed surface area measuring about 25-30 cm2 and washed with saline solution under a laminar flow. The harvested homograft segments were mechanically minced with a scraper and enzymatically digested with 0.3mg/ml liberase type II (Roche, Milan, Italy) in DMEM serum free (Lonza, Basel, Switzerland) for 30 minutes at RT. The sample was subsequently incubated with the same concentration of Liberase type II at 37^{o}C in 5% CO2 humified atmosphere for other 30 min and subsequently at 37^{o}C for other 4 hours using a rotor apparatus. After digestion, the homogenate was recovered, filtered through a 100-70-40-μm nylon mesh cell strainer (Becton Dickinson; Franklin Lakes, NJ), pelleted, counted with Neubauer hemocytometer and seeded at 1x106/cm2 on collagen-I coated 12-well plates with DMEM supplemented with 20% of heat inactivated FBS (Lonza) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) and subsequently incubated at 37^{o}C in a humified atmosphere with 5% CO2. Nonadherent cells were removed after 72 hours by washing with PBS. Culture media was changed every 3 days until testing. When cells were near confluence (approximately 1-2 weeks), they were detached with 0.25% trypsin-EDTA (Sigma, Milan, Italy), replated at a density of 5.000-6.000 cells per cm2 of surface area as passage 1(P1), maintained in DMEM supplemented with 10% FBS and

subcultured for expansion and in vitro analysis.

Immunophenotyping. Flow cytometry analysis was performed to assess the cell phenotype of VW-MSCs at passage 3-5 using the following MoAbs: anti-CD29-fluorescein isothiocyanate (FITC), CD31-phycoerythrin (PE), CD34-PE, CD146-PE, CD44-FITC, CD45- allophycocyanin (APC), CD73-FITC, CD90-phycoerythrin-cyanine 5 (PC5), CD105-PE, CD166-PE (all from Beckman Coulter, Fullerton, CA, USA), von Willebrand Factor (vWF, clone F8/86, Dako Cytomation, Glostrup, Denmark), STRO-1 (clone STRO1, R&D Systems), Notch-1 (clone C-20, Santa Cruz Biotecnology), Oct-4 (clone C-10, Santa Cruz Biotecnology). The following secondary MoAbs were used after cell staining with unlabeled primary MoAbs: anti-mouse IgG-APC (Beckman Coulter), anti-rabbit IgG-FITC (Dako Cytomation). To reveal vWF and Oct-4, the cells were fixed and permeabilized with the Intrapep Kit (Beckman-Coulter) and subsequently incubated with anti-mouse IgG-FITC (Dako Cytomation). Negative controls were performed using appropriate conjugated irrelevant antibodies. Samples were analyzed using a Cytomics FC500 flow cytometer equipped with two lasers (Beckman Coulter). Results were analyzed using the CXP Software (Beckman Coulter, Fullerton, CA, USA).

Hoechst SP labeling. To investigate the side population phenotype among VW-MSC populations, cells at 3-5 passages were labeled with Hoechst 33342. Briefly, cells were suspended in room-temperature Hanks' balanced saline solution containing 2% fetal bovine serum and 2 mM HEPES buffer at 5x106 cells per milliliter. Cells were prewarmed to 37^{o}C, and Hoechst 33342 added to a final concentration of 5μg/ml. Cells were incubated for 90 minutes, centrifuged, and resuspended in cold buffer at 5x106 per milliliter. Cells were then kept on ice and analyzed within 6 hours of labeling. Propidium iodide was added to the cells at 2 μg/ml immediately prior to analysis. For some experiments, cells were preincubated with the ABCG2 inhibitor verapamil (100μmol/L) at 37^{o}C for 30 minutes prior to Hoechst 33342 addition.

7.3 Stemness analysis

7.3.1 Bell shaped nuclei

Recent studies have reported that leukemic or normal colonic embryonic-like stem cells have a peculiar bell-shaped nuclear morphology reflecting specific stem cell division *(Kroschinsky et al, 2008; Gostjeva et al, 2006)*; accordingly, we investigated whether this feature might also be present in VW-MSCs. To address this issue, all vascular populations were recovered at passage 4 and plated at 3x103/cm2 on collagen-I coated slide chambers (BD Labware, Franklin Lakes, NJ, USA); cells were fixed in 2% paraformaldehyde for 4 minutes at RT, and mounted with Pro-long anti fade with DAPI mounting solution (Molecular Probes, Milan, Italy). Samples were observed under a Leica fully automated inverted fluorescence microscope DMI6000

B using a DAPI filter (Leica Microsystems, Milan, Italy). At least 200 nuclei/sample were counted on a minimum of 10 HPF. The percentage/sample of cells having bell-shaped nuclei was determined by counting the total number of bell shaped nuclei and calculating the percentage of cells with this characteristic out of the total number of counted nuclei. The count was performed independently by two operators on the same samples and then a mean of the individual results was calculated.

7.3.2 Clonogenic Assay

To establish colony-forming units cells, VW-MSCs at passage 5 were plated in cell culture Petri dishes (Corning, NY, USA) coated with collagen I (Sigma) at approximately 1 cell to 10 cells/cm^2 and left growing till 100-150 cells per colony was achieved. Colony growth was visualized directly on plate after crystal violet staining; culture dishes were washed with PBS to remove growth medium; then colonies were fixed overnight in buffered formalin, stained with 0.1% crystal violet in 20% methanol for 30 minutes at RT, washed with distilled water, dried and observed. The percentage of clonogenic cell subsets was estimated by counting colonies derived from all seeded cells.

7.3.3 In vitro spheroid formation and characterization: immunofluorescence, molecular and electron microscopy analysis

Stem cell ability to form spheres is a well known acquisition *(Ingram et al, 2005)*. To determine whether VW-MSCs had this growth capacity, adherent VW-MSCs at passage 5 were suspended in culture medium and plated at 2x104 cells/well in ultra-low attachment 24-well plates. After a few days, numerous spheres were observed under light microscopy. The spheres were recovered and processed for immunofluorescence microscopy (IF), transmission electron microscopy (TEM) and RT-PCR gene expression analysis.

Immunofluorescence analysis.

Single spheres were embedded in a 2% agar gel, mounted on a cork dish with a small amount of OCT mounting medium and frozen in liquid isopentane cooled in liquid nitrogen at -160^{o}C. Frozen blocks were stored at -80^{o}C until use. Immunofluorescent staining was performed on 6 /mum-thick sections cut with a cryostat at -20^{o}C. Frozen sections were dried at RT and fixed in cold acetone. After a second drying step, the sections were washed and incubated with 1% BSA in PBS to block unspecific reactive sites. Immunostaining was performed by labeling the samples with MoAbs against CD133 (1:80, Miltenyi biotec, Bergisch Gladbach, Germany), CD34 (clone QBEND-10, 1:80, Dako Cytomation), c-kit (1:200, SantaCruz Biotechnology) Stro-1 (1:100, R&D System), Sca-1 (1:10, Cederlane Laboratories), Oct-4 (1:100, Chemicon), CD44 (clone G44-26, 1:50, BD), Notch1 (1:50, clone c-20, SantaCruz) for 45 minutes at 37^{o}C and then incubating with FITC-conjugated polyclonal rabbit anti-mouse immunoglobulins for 45 minutes at 37^{o}C. Finally coverslips were mounted with

Pro-long anti fade with DAPI mounting solution and the samples were observed under a Leica fluorescence microscope DMI6000 B using appropriate filters. Original images were taken at 10X. For negative control, the sections were processed omitting the primary antibody, and no signal was detected.

Transmission electron microscopy.

The subcellular characteristics of cells aggregated into spheroids were investigated by TEM. The recovered samples were pelleted, fixed in 2.5% phosphate-buffered glutaraldehyde for 24 hours at 4^oC, followed by post fixation with 1% buffered osmium tetroxide (O_sO_4) for 1 h at RT. They were then washed with 0.15 mol/L phosphate buffer, dehydrated in a graded series of ethanol and embedded in epoxy resin; thin sections were counterstained with lead citrate and uranyl acetate and then observed with a transmission electron microscope Tecnai 12 (FEI Company, Milano, Italy).

Scanning electron microscopy.

To analyze the three-dimensional architecture of spheres, they were seeded on polylysin-coated glass coverslips for 6 hours and then processed for SEM analysis. The adherent spheroids were washed with 0.15 mol/L phosphate buffer, pH 7.4, and then fixed with 2.5% phosphate buffered glutaraldehyde (TAAB Laboratories, England, UK) pH 7.4 for 24 hours at 4^oC. Afterwards, the spheres were washed and post fixed in 1% phosphate buffered (O_sO_4) , rinsed in distilled water and dehydrated in a graded series of ethanol. To dry the samples, they were first incubated in a solution of 50% absolute ethanol/50% hexamethyldisilazane (HMDS) (Fluka Analytical, Sigma) and then in pure HMDS; samples were dried at RT. To observe the samples, they were mounted on aluminium stubs with silver paste and coated with a 10 nm thick gold film in a sputtering device (Balzers Union FL9496, Furstentum, Liechtenstein); samples were finally observed with a Philips SEM 505 (FEI Company) at 15 kV.

RT-PCR Gene expression analysis.

VW-MSCs isolated from human TA constitutively expressed the embryonic stem cell marker Oct-4, molecules involved in stem cell critical regulatory pathways, i.e., c-kit, BCRP-1, BMI-1, and KDR, as well as, to a lower extent, hematopoietic stem cell transcripts, e.g., CD133. We also investigated which regulation pathways are predominantly expressed in stem cell forming spheres among c-kit, Notch-1, BCRP-1, BMI-1, SOX-2, Oct-4, CD133 and KDR genes. Total RNA was extracted from spheroids using the RNA extracting reagent TRI reagent (Ambion Applied Biosystems, Austin, TX, USA, www.ambion.com) according to the manufacturer's instructions. Reverse transcription reactions were performed in a 40 μl volume with 4 μg of total RNA and the RT-PCRs with 1μl of this solution. PCR primers are listed in **Table 7.1** and were purchased from Invitrogen; RT reagents were furnished by Ambion Applied Biosystems and PCR reagents by Qiagen (Milan, Italy). The PCR products were separated by electrophoresis on a 2% agarose

Table 7.1. Primers for reverse transcription and polymerase chain reaction.

GENE	Primers Sequence	Amplicon length (bp)	Ta^{o}C
Oct-4	REV 5'-CCACATCGGCCTGTGTATAT-3' FW 5'-CTCCTGGAGGGCCAGGAATC-3'	380	60
BMI-1	REV 5'-CATTGCTGCTGGGCATCGTAAG-3' FW 5'-GGAGACCAGCAAGTATTGTCCTTTTG-3'	369	62
BCRP-1	REV 5'-CTGAGCTATAGAGGCCTGGG-3' FW 5'-GTTTATCCGTGGTGTGTCTGG-3'	652	60
CD133	REV 5'-GTACAACACTACCAAGGACAAGGCGT-3' FW 5'-CTGGGGCTGCTGTTTATTATTCTG-3'	337	62
KDR	REV 5' TTTGTCACTGAGACAGCTTGG-3' FW 5' TATAGATGGTGTAACCCGGA-3'	555	62
c-kit	REV 5' CATACAAGGAGCGGTCAACA-3' FW 5' GTCTCCACCATCCATCCATC-3'	275	57
SOX-2	REV 5'-GCGCCGCGGCCGGTATTTAT-3' FW 5'-ACCGGCGGCAACCAGAAGAACAG-3'	208	60,5
Notch-1	REV 5'-TGGCATCAGCTGGCACTCGTCC-3' FW 5'-CCGGCTGGTCAGGGAAATCGTG-3'	496	62
β2 μglobulin	REV5'-ATCTTCAAACCTCCATGATG-3' FW 5'-ACCCCCACTGAAAAAGATGA-3'	114	58
ASMA	REV 5'- GTGATCTCCTTCTGCATTCGGT-3' FW 5'- CTGAGCGTGGCTATTCCTTC-3'	372	60
CALP2	REV 5'-GCAGGTCTGAGGGTCTGAAG-3' FW 5'-CCAGTCAACCAAGGGTCTGT-3'	470	60
CALD	REV 5- GCTGCTTGATGGGTCGATTTGA-3' FW 5'- GTCACCAAGTCCTACCAGAAGA-3'	Low: 744 High: 1508	60

gel with 1x Tris-acetate-EDTA buffer, stained with ethidium bromide and photographed under ultraviolet light.

7.4 Multilineage differentiation potential

7.4.1 Adipogenic commitment: in vitro procedure, Oil red O staining and TEM analysis

Twenty thousand cells/cm^2 were cultured for 3 weeks in adipogenic differentiation medium; DMEM was supplemented with 10% FBS, 0.5 mmol/L isobutyl-methyl xanthine (IBMX), 200

μmol/L indomethacin, 1μmol/L dexamethasone and 20 μg/mL insulin (all reagents were from Sigma). Induction Medium was replaced every 2-3 days and alternated with maintenance medium (DMEM 10% FBS and 20 μg/mL insulin). Negative controls were cultured in standard condition (DMEM plus 10% FBS).

Morphological features of adipogenic differentiation were observed by inverted light microscopy and TEM, whilst the cytoplasmic presence of lipid droplets was assessed by Oil Red O staining. For TEM analysis, cells were gently washed with 0.15 mol/L phosphate-buffer, fixed directly in plates with 2.5% phosphate-buffered glutaraldehyde for 10 min at RT, scraped, collected and centrifuged for 10 min at 1800 rpm. Pellets were left in 2.5% phosphate-buffered glutaraldehyde for 24 hours at 4^{o}C and processed as described above. For Oil Red O staining, cells were fixed in 10% neutral buffered formalin, rinsed in 60% isopropanol and then stained with Oil red O solution for 30 min at RT. Cells were rinsed in 60% isopropanol again, lightly stained with hematoxylin, rinsed with distilled water, and mounted in aqueous mounting medium.

7.4.2 Osteogenic commitment: in vitro procedure, von Kossa staining and TEM analysis

Five thousand cells/cm^2 were cultured in DMEM supplemented with 15% FBS, 10 mmol/L β-glycerophosphate, 0.17 μmol/L ascorbic acid, and 0,1μmol/L dexamethasone (Sigma) for 3-4 weeks; the medium was replaced every 2-3 days. Controls were cultured in basal medium (DMEM with 10% FBS). Osteogenic differentiation was assessed by TEM (as described above) and by Von Kossa stain to identify calcium salt extracellular deposition. Briefly, cells were fixed in 10% neutral buffered formalin, washed in distilled water and incubated in 1% silver nitrate solution for 20 min under ultraviolet light. After several washing steps with distilled water, samples were incubated for 5 min at RT in a solution of 5% sodium thiosulfate, rinsed in distillated water, counterstained with 1% nuclear fast red solution, dehydrated and mounted using a permanent mounting medium (Canadian Balsam, Sigma).

7.4.3 Chondrogenic commitment: in vitro procedure, Type II Collagene IHC and TEM analysis

Two hundred and fifty thousand cells were gently pelleted in 15 ml polypropylene tubes (Corning) and cultured for three weeks in complete chondrogenic differentiation medium (Lonza) containing 10 ng/ml TGFβ3 (Lonza) by feeding twice a week.

Controls were cultured in chondrogenic differentiation medium without TGFβ3. To verify VW-MSCs' chondrogenic differentiation potential, pellets were processed for TEM (as described above) and for histological analysis to evaluate collagene type II synthesis. Briefly, pellets were formalin-fixed and paraffin embedded; 3 μm-thick sections were stained with hematoxilyn and eosin and examined at light microscopy.

To examine the expression of type

II collagen, dewaxed sections were digested with pepsin (0.5 mg/ml Tris-HCl, pH 2.0) for 15 at RT; endogenous peroxidase was blocked with absolute methanol containing 1.5% H_2O_2. Sections were incubated with mouse MoAb anti-human collagen type II (1:200 in 1% BSA-PBS; Chemicon Int) in a wet chamber at 4^{o}C overnight. The antigen-antibody reaction was revealed using the Novolink Polymer detection System (Novolink) according to the manufacturer's protocol. The signal was developed with diaminobenzidine cromogen and sections were counterstained with hematoxylin. Negative controls were performed by omitting the primary antibody.

7.4.4 Leiomyogenic commitment: in vitro procedure, IF and gene expression analysis of smooth muscle markers, TEM analysis

Three thousand five hundred cells/cm^2 were plated on Collagen I Biocoated culture six-well plates (BD) in SmGM-2 (Lonza). After 24 hours, the medium was changed for differentiation medium containing SmGM-2 plus 10 ng/ml TGF-β1 (Sigma) and 5 ng/ml PDGF-BB (Sigma). Cells were cultured for 14 days, feeding the medium every 3 days. Cell controls were cultured in SmGM-2 without additional growth factors.

To evaluate smooth muscle marker expression, IF, RT-PCR and TEM analysis were performed.

VW-MSCs were washed with PBS, fixed and permeabilized with 2% paraformaldehyde 0,1% TRITON in PBS for 4 min at RT. Aspecific antibody binding sites were blocked by incubating with 1% BSA in PBS for 30 min at RT. Cells were incubated over night at 4^{o}C in a humidified chamber with the following anti human mouse monoclonal antibodies (alpha smooth muscle actin, 1:9000, clone 1A4; H-caldesmon, clone h-CD, 1:75; calponin, clone CALP, 1: 40; vimentin, 1: 260, clone V9; all purchased from Dako Cytomation). Antigen-antibody reactions were labeled with rabbit fluorescein-conjugated anti mouse IgG (Dako Cytomation) for 45 min at 37^{o}C. After several washes, samples were mounted and nuclei counterstained with Pro long antifade reagent with DAPI (Molecular Probes). Negative controls were done by omitting the primary antibodies.

The expression of smooth muscle specific genes, such as alpha-smooth muscle actin (ASMA), calponin 2 (CALP2) and caldesmon (CALD), was evaluated by RT-PCR. Total RNA was extracted from induced and non induced cells and reverse transcription was performed as described above. The primers listed in **Table 7.1** were selected for amplification using GoTaq Hot Start Polymerase (Promega Corporation, USA); for detection of β2-μglobulin, ASMA, CALP2, and CALD, the following parameters were used: 94^{o}C for 3 min and then 30 cycles of 40 sec at 94^{o}C for denaturation, 30 sec at 60^{o}C for annealing, 90 sec at 72^{o}C for polymerization, and 5 min at 72^{o}C. The PCR products were separated by electrophoresis on a 1.5% agarose gel with 1X Tris-acetate-EDTA buffer, stained with ethidium bromide and photographed under ultraviolet light.

TEM analysis of VW-MSC leiomyogenic differentiation was performed as described above.

7.5 Experimental model of hind limb ischemia and cell therapy

Ten Male CD1 Foxn1nu/nu mice (Charles River Laboratories, Wilmington, Mass) were housed at constant RT (24 ± 1°C) and humidity (60 ± 3%). At 6-week-old, under the effect of 2,2,2-tribromoethanol anesthesia (880 mmol/kg body weight intraperitoneally; Sigma), the mice underwent unilateral limb ischemia on the left limb by ligation of the femoral artery. Subsequently, 2x104 TA-MSCs (passage 4) resuspended in 30 μl of medium or vehicle (DMEM, 30 μl) were injected into 3 different points of the ischemic adductor muscle (n. 5 mice per group).

7.5.1 Assessment of blood flow recovery, neoangiogenesis and therapeutic potential of VW-MSCs

Eco color Doppler. Blood flow recovery was followed up by laser Doppler flowmetry measurements. At 7 and 14 days postischemia, the superficial blood flow (BF) of both feet and limb was measured in anesthetized mice by color laser Lisca Doppler (Peri-med AB, Järfälla, Sweden), and the ratio of perfusion between ischemic to nonischemic limb and foot was calculated.

Immunohistochemistry analysis. Two weeks after induction of hindlimb ischemia, mice were euthanized and perfused with 4% formalin. Adductors and gastrocnemii were excised from hischemic and non hischemic limb and paraffin embedded for immunhistochemical analysis. Three μm sections of paraffin-embedded muscles, harvested at day 14 after ischemia were processed for neoangiogenesis and immunohistochemical analysis as described above.

Capillary density

The samples were stained with isolectin B4 to recognize ECs; positive capillaries were counted on the whole surface of tissue sections and the results were reported as numbers of capillaries for square millimeters.

Hypoxia

Mice muscles were stained with HIF-1α (1:4000, Novus Biological) to evaluate the contribute of vascular progenitor cells in ameliorating hypoxic conditions. We calculated the percentage/sample of HIF-1α positive cells comparing to the total number of nuclei (positive and negative) on a minimum of 5 random HPF; the fold change values were obtained by the ratio between correspondent ischemic and controlateral non ischemic muscles. We followed the protocol described in 7.1 paragraph except for the antigen retrieval performed microwave with TRIS-EDTA (50mM-0,2mM) and the incubation with primary antibody carried on for 90 minutes at RT.

Engrafment and differentiation in preexisting vessels

We evaluated the engrafment of transplanted cells and the neovascularization through specific anti-human mitochondria stain (1:100, Chemicon) and

anti-human CD34 stain (1:80, Dako Cytomation). Furthermore we evaluated the in situ level of VEGF (1:50, Abcam), and muscle regeneration using a selective anti mouse PAX7 (1:50,R&D System) in order to quantify the satellite cells activation.

Statistical analysis

We performed statistical analysis on all experiments. Values are presented as mean ± SEM. Analyses were performed with GraphPad Prism 4.0 software (GraphPad Software, San Diego, Calif). Statistical significance was assessed by use of unpaired t test for comparison between 2 groups. For analysis of blood flow recovery in which measurements were repeated on the same subject at different time points, repeated measures ANOVA was used, followed by Bonferroni post hoc test. $P < 0.05$ was interpreted to denote statistical significance.

7.6 Femoral arteries collection, classification and statistical analysis

We evaluated the frequency of vascular calcification on femoral artery tissue sections obtained from vascular segments recovered by healthy multiorgan donors and collected at Regional Cardio Vascular Tissue Bank in Bologna from January 2005 to March 2010. A small portion of each sample was formalin fixed, paraffin embedded, cutted with microtome and processed for hematoxylin and eosin staining. The sections were observed at light microscope and the respective donors were classified as "calcified" or "non calcified" on the basis of the evidence of calcification area in the tunica media, a condition known as Mönckeberg Sclerosis. Successively we divided all donors in different age classes and recovered their clinical information concerning diabetes, tabagism, hypercolesterolemia and hypertension status in order to eventually correlate these clinical conditions with the presence of vascular calcification calculating the correspondent Odd Ratio values.

7.7 Isolation, flow cytometry characterization and osteogenic commitment of calcifying vascular cells

After light microscopy analysis, we selected one calcified femoral segment unsuitable for long term banking and used it for VW-MSCs isolation as previously described (paragraph 7.2). At 3^{rd} passage VW-MSCs were processed for flow cytometry analysis to evaluate the expression of mesenchymal (CD44, CD90, CD166,CD105, CD166, CD29) endothelial (CD31, CD34, KDR) and stemness (Oct-, Stro-1, Notch-1, Sca-1) markers.

Furthermore, VW-MSCs derived from calcified vessel (called cVW-MSCs) were taken at passages 3 and differentiated towards osteogenic lineage according to a consolidate protocol described by Alviano et al *(Alviano et al, 2007)*. In vitro osteogenesis was verified with von Kossa stain and TEM analysis to evaluate the presence of calcium deposit. Furthermore, the results were compared with those obtained from osteogenic commitment of VW-MSCs derived from non

calcified vessels (called ncVW-MSCs).

7.8 Immunohistochemistry assay and statistical analysis

To localize in situ the osteoprogenitor cells, we performed an immunohistochemistry (IHC) analysis on 5μm-thick sections of vessels against one of the master transcriptional factors regulating osteoblat differentiation such as Osterix (Osx) using the polyclonal antibody Osx (1:100, Abcam, Cambridge, UK). The antigen-antibody reaction was revealed using Novolink Polymer detection System (Novolink, New Castle, UK) following the manufacture protocols. Samples were observed under a Leitz Diaplan light microscope (Wetzlar, Germany) and positive cells were counted both in tunica media and in tunica adventitia on a minimum of 10 HPF. The percentage of Osx positive cells was calculated on the total number of cells in each HPF. Non parametric one-way ANOVA (Kruskal-Wallis test) was used to detect differences between tunica media and adventitia scores for Osx positivity and clinical condition such as hypertension or absence of risk factors; analyses were performed using Graph Pad Prism version 4.0.

Chapter 8

Results

Multidistrict human mesenchymal vascular cells: stemness characteristics and pluripotency

8.1 Arterial samples and vascular niche characterization in immunohistochemistry

More recently our group described the presence of VW-MSCs in normal human adult thoracic aortas. After cryopreservation, the vast majority of VWCs, i.e. ECs and SMCs, were irreversibly injured *(Pasquinelli et al, 2006)*. Interestingly the only cells in the cryopreserved arterial wall which still remained viable after 4 days of organ culture were capillary structures located in between the media and adventitia layers; therefore microvascular stress-resistant cells CD34+/CD31- are present in this arterial zone. This area was richly endowed with highly expressing Human Leukocyte Antigen class I antigen small calibre vessels *(Pasquinelli et al, 2007 b)*, and therefore could act as a strategic area for the recruitment of blood circulating cells. In accordance with this we observed the presence of bone marrow-derived CD45+ mononuclear cells. Additional experiments on femoral arteries confirmed these results and also revealed the presence of CD68+ and S-100+ perivascular cells representing resident macrophages and dendritic accessory cells. Moreover, this area has certain characteristics — e.g. discrete morphology, a strategic location at the interface between media and adventitia layers, heterogeneous and complex cell composition, an unexpectedly elevated cell proliferation under normal conditions, selective localization of cells expressing the stem cell surface molecule c-kit — which makes it unique in the arterial wall and consistent with a nichelike function (**Fig. 8.1**).

To further strengthen the concept that in vivo this area exerts a niche-like

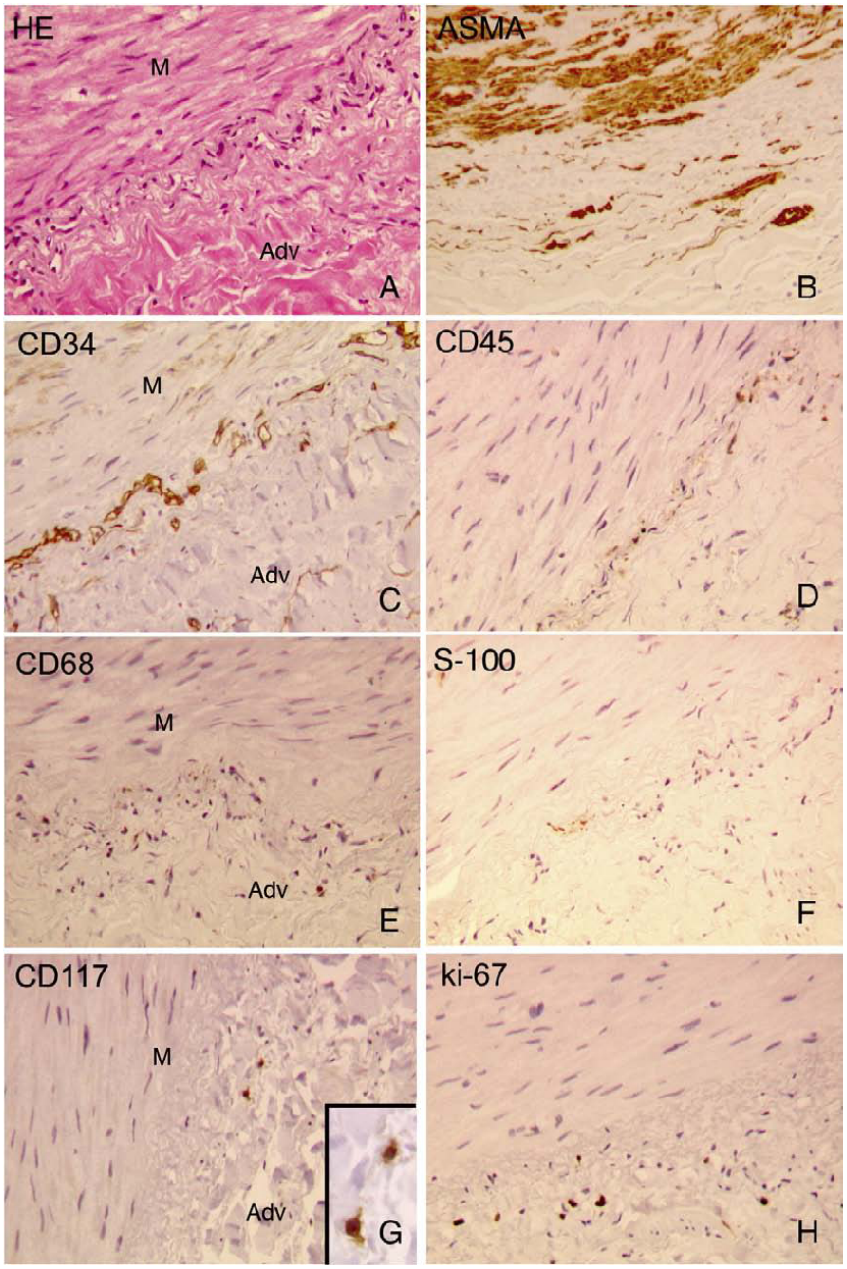

Figure 8.1. Vasculogenic niche of the human femoral artery.

function, we accomplished an immunohistochemical study of stemness markers on arterial wall sections for each district (AA, TA, FA). Regardless of vascular segments, we found that Notch-1, Stro-1 and Sca-1 positive cells were distributed along the previously described vasculogenic-niche; also small, rounded and Oct-4 positive cells appear to localize in this area (**Fig. 8.2**).

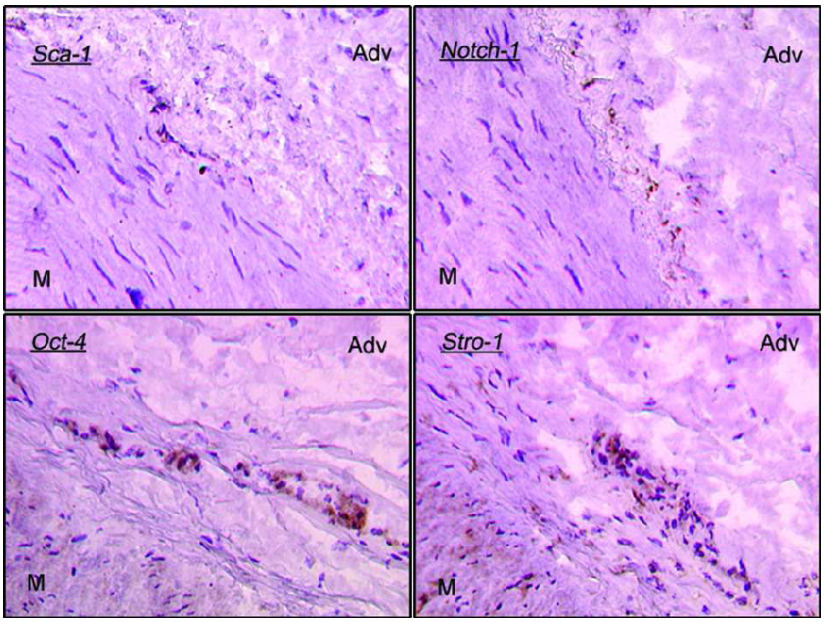

Figure 8.2. Representative picture of perivascular-niche.

8.2 Progenitor cells isolation, cell culture and flow cytometry characterization

From anatomically distinct segments we succeeded in isolating cells with morphological and immunophenotypical properties of MSCs; primary cell cultures were established from two AA, four TA and three FA, thus demonstrating that MSC populations are present in arteries and have different *in vivo* extents and architecture, i.e., an elastic and muscular typology. Depending on the starting amount of arterial tissue used to establish the cultures, conluence was reached within 5 days. The cells were spindle-shaped and had a tendency to grow in a whorled pattern (**Fig. 8.3**). No significant differences, as judged by morphological, immunophenotype and functional analysis, were observed among cultures generated from arterial segments leading to different anatomical districts or architectures; because the amount of tissue available for the study varied considerably, comparative analysis to establish MSC yield differences among arterial sources was not possible.

Regardless of the vascular source, cells derived from the human arterial wall had an immunophenotype profile consistent with that of MSCs; examples of flow cytometry surface molecule single analysis are shown in **Fig. 8.4**. All the populations expressed typical mesenchymal antigens such as CD29, CD44, CD90, CD73, CD105, and CD166; on the contrary hematopoietic lineage (CD45), hematopoietic progenitor (CD34) and endothelial cell (CD31, vWF, CD146) markers were negative. Stemness markers, i.e., Oct-4, Notch-1 and Stro-1, were intensely expressed.

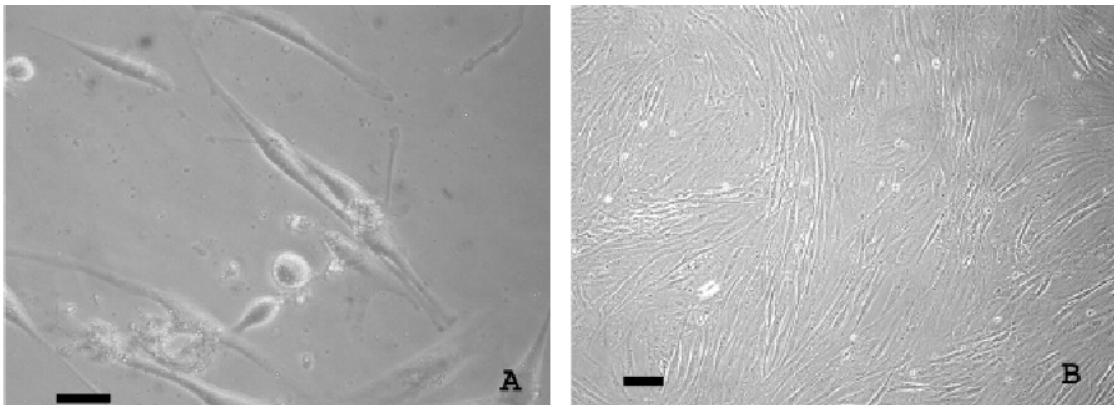

Figure 8.3. W-MSCs morphology at 4th culture day (left) and at 3rd culture passage (right).

8.3 Stemness analysis

A defining property of stem cells is low fluorescence after staining with Hoechst 33342. VW-MSCs were stained with Hoechst 33342 in presence or absence of verapamil and processed for flow cytometry analysis. We found that approximately the 1% of all VW-MSCs posses the SP phenotype; efflux of Hoechst 33342 dye was inhibited by verapamil, confirming the specificity of the staining (**Fig. 8.5**).

Other features of the more undifferentiated stem cells are the presence of bell-shaped nuclei and their clonogenic ability, both features related to symmetric divisions occurring during the cell cycle. Under a DAPI filter, the total number of bell-shaped nuclei were counted and later compared with the total number of seeded cells. Regardless of the vascular source, we found that a percentage ranging from 0.30 ± 0.05% of VW-MSCs present bell-shaped nuclei (**Fig. 8.6A**), a morphological characteristic of embryonic-like stem cells. This result suggests that primary cultures of VW-MSCs are heterogeneous even though they present similar growth and immunophenotypical characteristics; as indicated by DAPI identification of cells with bell-shaped nuclei, a small proportion of hierarchically higher cells exists; this finding was confirmed by other in vitro assays aiming to evaluate more established stemness characteristics of VW-MSCs.

To investigate the clonogenic ability of VW-MSCs, single cells were seeded in a 100 mm Petri dish via limited dilution. At this low density culture condition we noted that cells initially displayed a particular phenomenon known as shrink effect, a typical suffering response to non optimal growing conditions; the cells exhibited membrane wrinkling and flattening; only stress-resistant cells managed to survive in such an unfavorable contest. We found that 0.36% ± 0,03% of total VW-MSCs retained a clonogenic ability in adult life (**Fig. 8.6B** and **8.6C**); again, the clonogenic assay shows that,

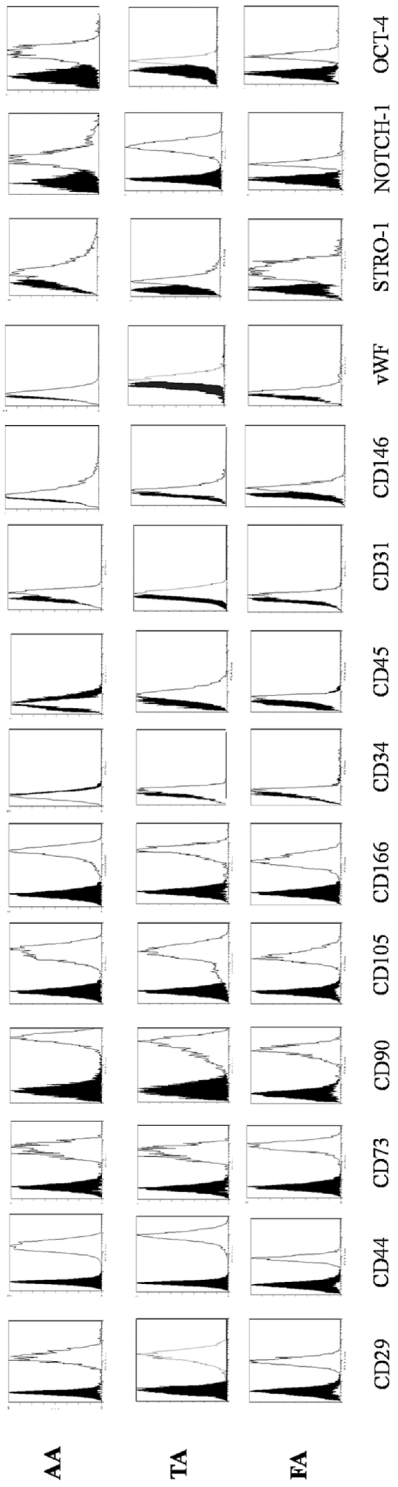

Figure 8.4. Flow cytometry analysis of VW-MSCs immunophenotype.

regardless of the arterial origin or similarities in growth and immunophenotypical characteristics, VW-MSCs are heterogeneous and contain a subset of more undifferentiated cells according to the stage of embryological development. This intrinsic ability is in agreement with current views about the clonal origin of some diseases like tumors.

When VW-MSCs were seeded in ultra-low attachment plates, they formed agglomerates that were able to generate growth-forming spheres (**Fig. 8.6D**). Cells constituting spheres expressed stem cell markers, including c-kit, Stro-1, Notch-1, Sca-1, and mesenchymal markers such as CD44 as documented by IF analysis (**Fig. 8.6E**), whereas they were negative to hematopoietic progenitor, CD133 and CD34 markers. Furthermore, RT-PCR analysis confirmed stem cell gene expression and demonstrated that at passage 5, VW-MSCs lose the expression of BCRP-1 and BMI-1 previously described at passage 3 in thoracic aorta MSCs *(Pasquinelli et al, 2007 a)*, whilst the expression of c-kit and Oct-4 persisted during extended subculture (**Fig. 8.6F** and **8.6G**).

TEM analysis of spheres showed undifferentiated cells with large, irregularly-shaped nuclei, dispersed chromatin, and prominent nucleoli; the cytoplasm contained mitochondria and profiles of rough endoplasmic reticulum which in some cells were distended by the presence of moderately dense material; in the intercellular space we occasionally found collections of extracellular membrane vesicles and primary cilia documenting intense extracellular membrane traffic; primitive intercellular junctions were occasionally seen and gap junctions as

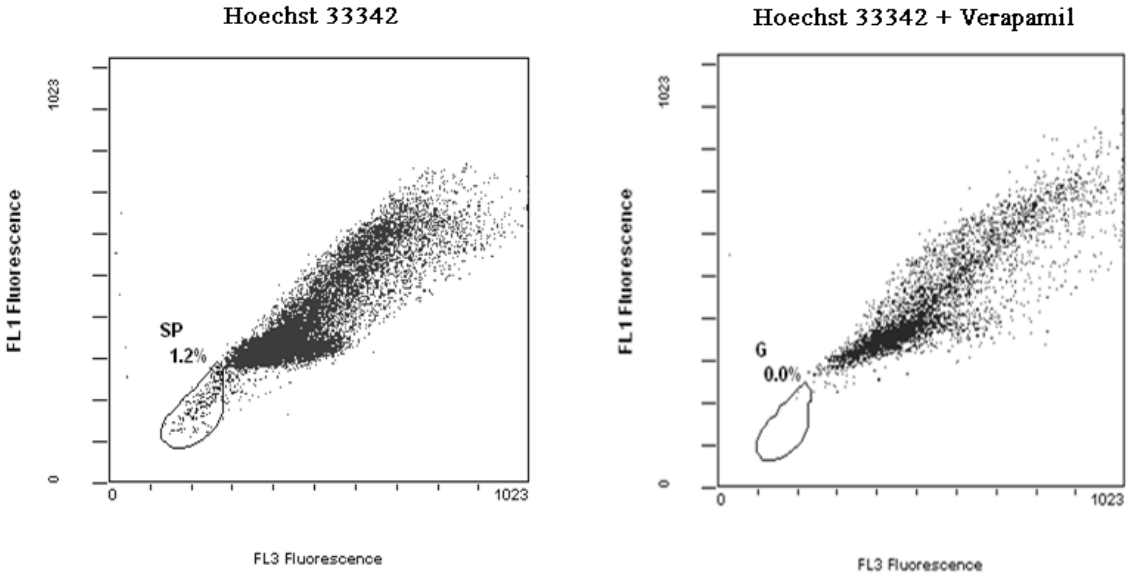

Figure 8.5. Identification of arterial SP cells.

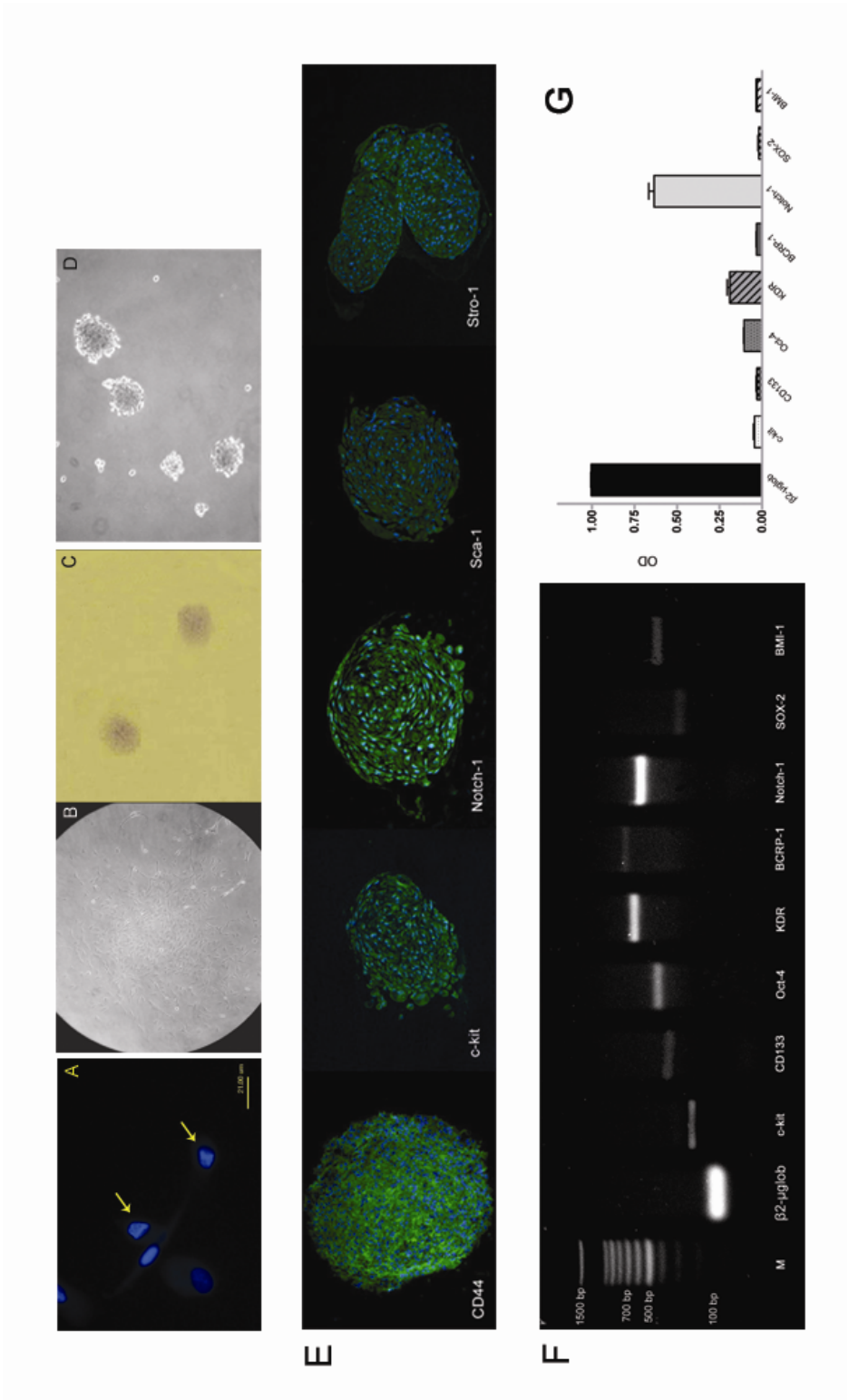

Figure 8.6. Stemness characteristics of VW-MSCs.

well; the outer cell layer had unexpectedly smooth surfaces with randomly spaced long and thin floating filopodia (**Fig. 8.7**).

SEM analysis demonstrated that the spheres were round with diameters ranging from 60 to 100 micron; on expanding TEM observations, outer cells showed cell membrane protrusions corresponding to surface blebbing (**Fig. 8.8**).

8.4 Multilineage differentiation potential

Cells were cultured under specific experimental conditions and exhibited a similar ability to differentiate along mesenchymal lineages.

All VW-MSCs showed high adipogenic potential, though this ability was more intense when VW-MSCs were isolated from thoracic aortas and femoral

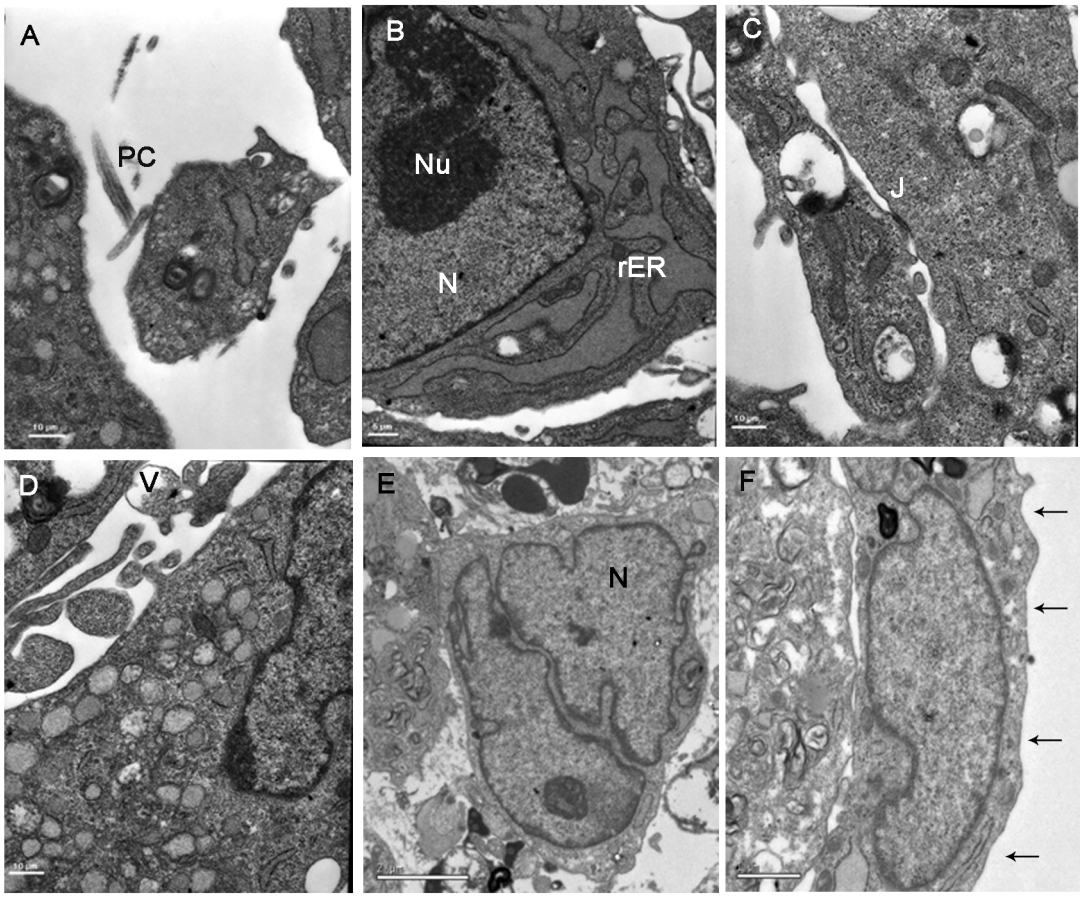

Figure 8.7. TEM analysis of spheres.Abbreviation: primary cilium (PC); nucleus (N) nucleoli (Nu), rough Endoplasmic Reticulum (rER); primitive intercellular junction (J); extracellular membrane vesicles (V).

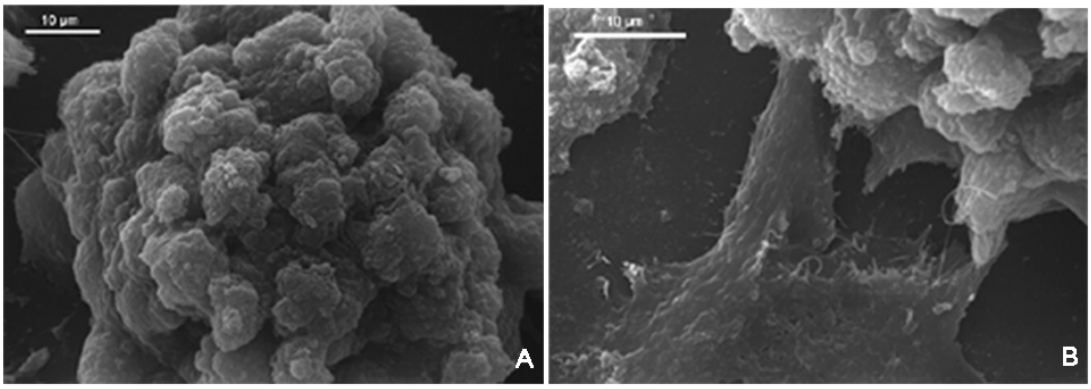

Figure 8.8. SEM analysis of spheres.

arteries. VW-MSCs treated with adipogenic media showed the presence of multiple cytoplasm vacuoles that increased in size and number with the time of induction. Conversely, uninduced cells retained their morphology and did not display significant cytoplasm vacuoles. As seen in **Fig. 8.9**, vacuoles stained intensely with the lipid dye Oil Red O. By TEM, VW-MSCs contained multiple, confluent lipid droplets in the cytoplasm and small dense mitochondria, besides an elevated endocytic activity.

VW-MSCs also showed a good capacity for chondrogenic differentiation; light microscopy on high-density cell cultures revealed that cells were embedded in an abundant extracellular matrix; immunohistochemistry revealed the presence of human type II collagen which is typically found in joint cartilage. Ultrastructural analysis confirmed the presence of abundant extracellular matrix containing mature collagen fibers and proteoglycan particles in induced cells exclusively. Unlike adipogenesis and chondrogenesis, the osteogenic differentiation potential was not so efficient. At light microscopy, von Kossa staining showed a calcium-rich mineralized matrix but the staining was less diffusely distributed than expected. However, TEM revealed multiple foci of electron dense fibrillary deposits which were decorated with needle-shaped hydroxyapatite crystals. Overall these results were illustrated in **Fig. 8.9**.

Regarding the leiomyogenic differentiation potential, gene expression analysis showed increased levels of alpha-Smooth Muscle Actin (ASMA) and High-Caldesmon (H-CALD) genes compared to controls. Again, stimulation with TGF-β1 and PDGF-BB caused de novo expression of the smooth muscle specific marker calponin 2 (CALP2). By comparing smooth muscle marker expression levels no significant difference was seen among VW-MSCs deriving from various arterial sources. TEM of induced cells showed a myofibroblast phenotype; the cells were spindle-shaped and contained abundant peripherally arranged

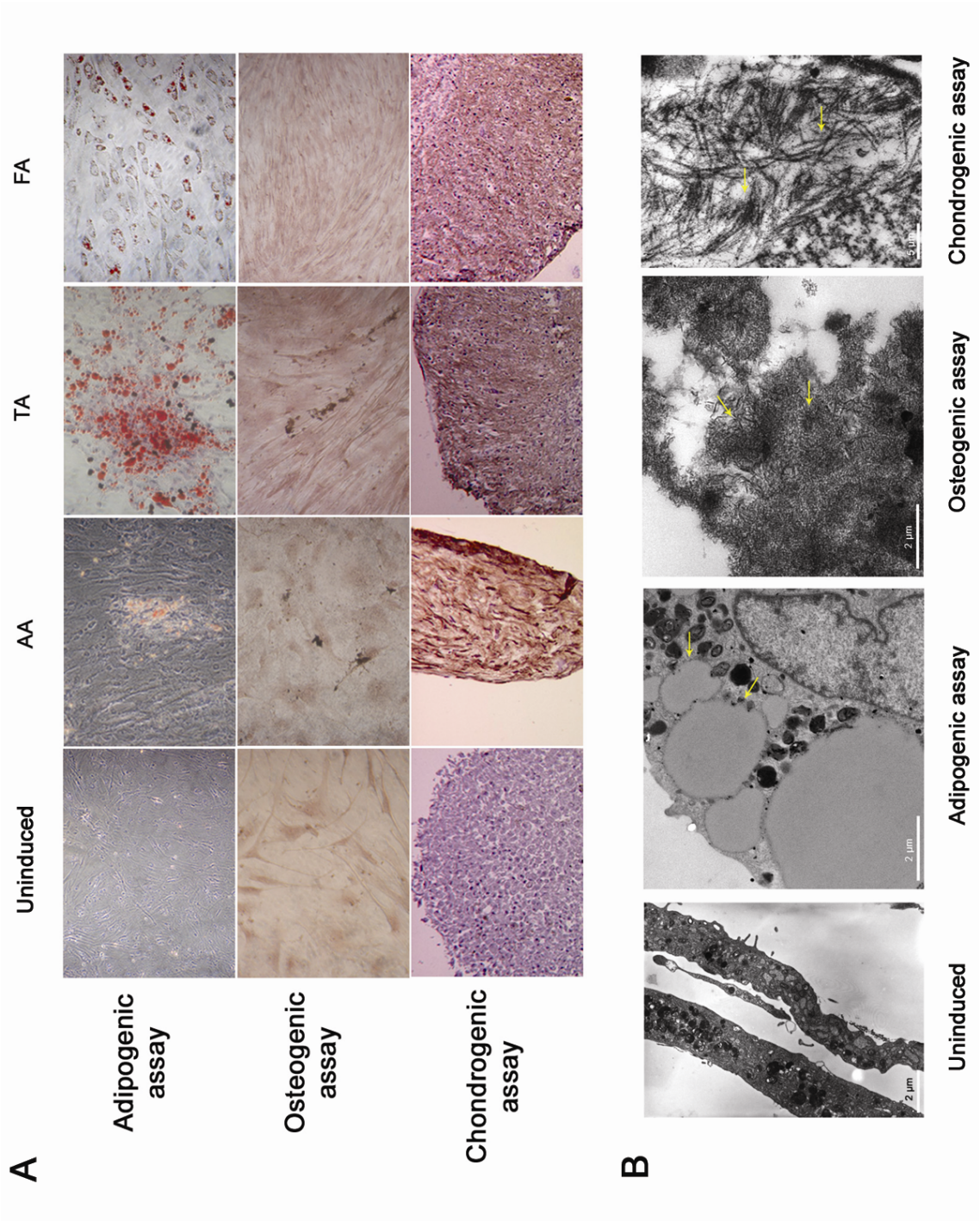

Figure 8.9. Multilineage potential of VW-MSCs.

contractile filaments along with profiles of rough endoplasmic reticulum; well-organized fibronexus junctions were also seen; control cells, in contrast, maintained an undifferentiated mesenchymal phenotype (**Fig. 8.10**). Immunofluorescence analysis on induced and uninduced cells confirmed RT-PCR results; in fact, smooth muscle specific proteins were up-regulated after leiomyogenesis induction (**Fig. 8.11**).

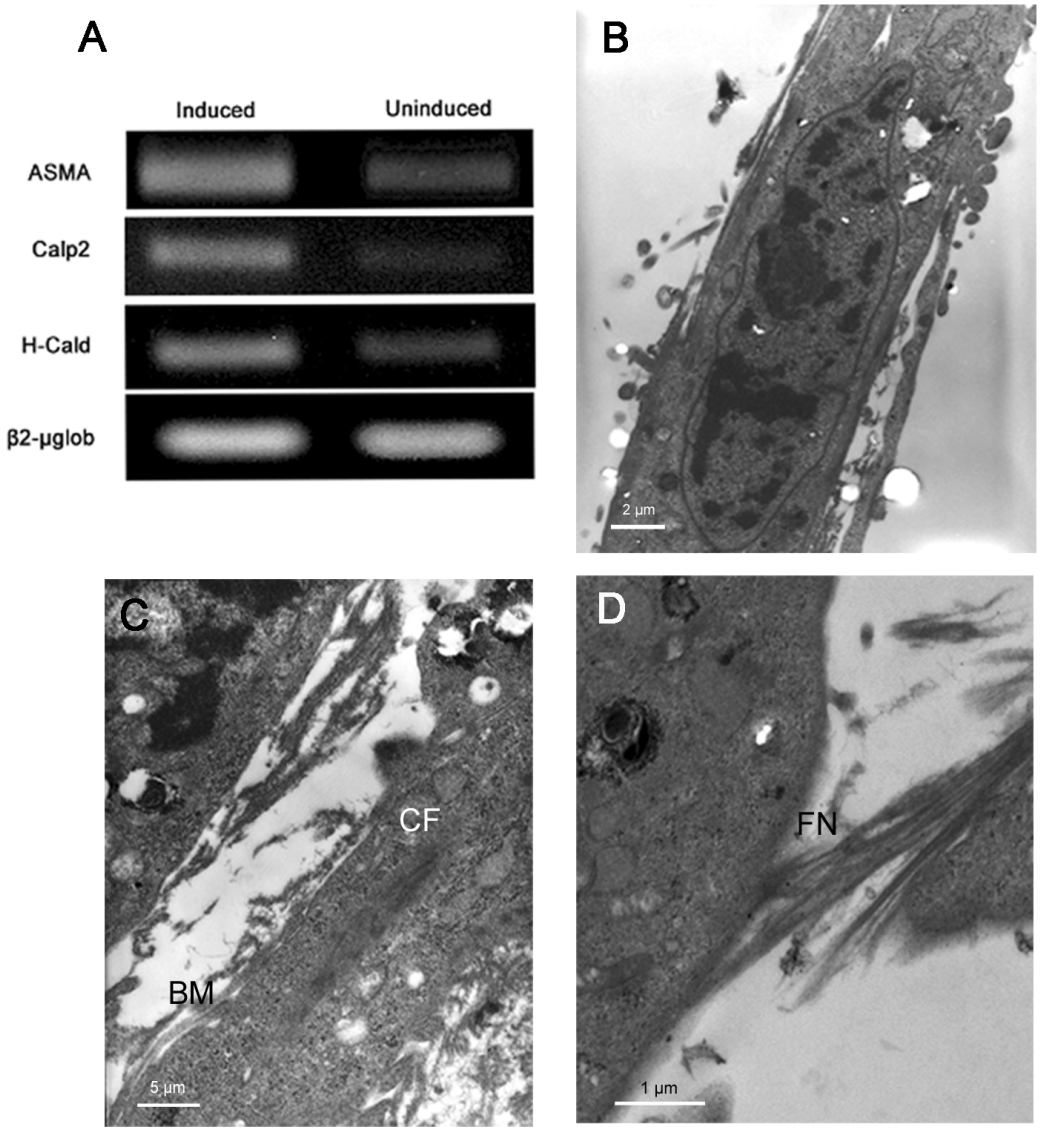

Figure 8.10. Leiomyogenic differentiation of VW-MSCs. Abbreviations: Basal Membrane (BM), Contractile Filaments (CF), Fibronexus (FN).

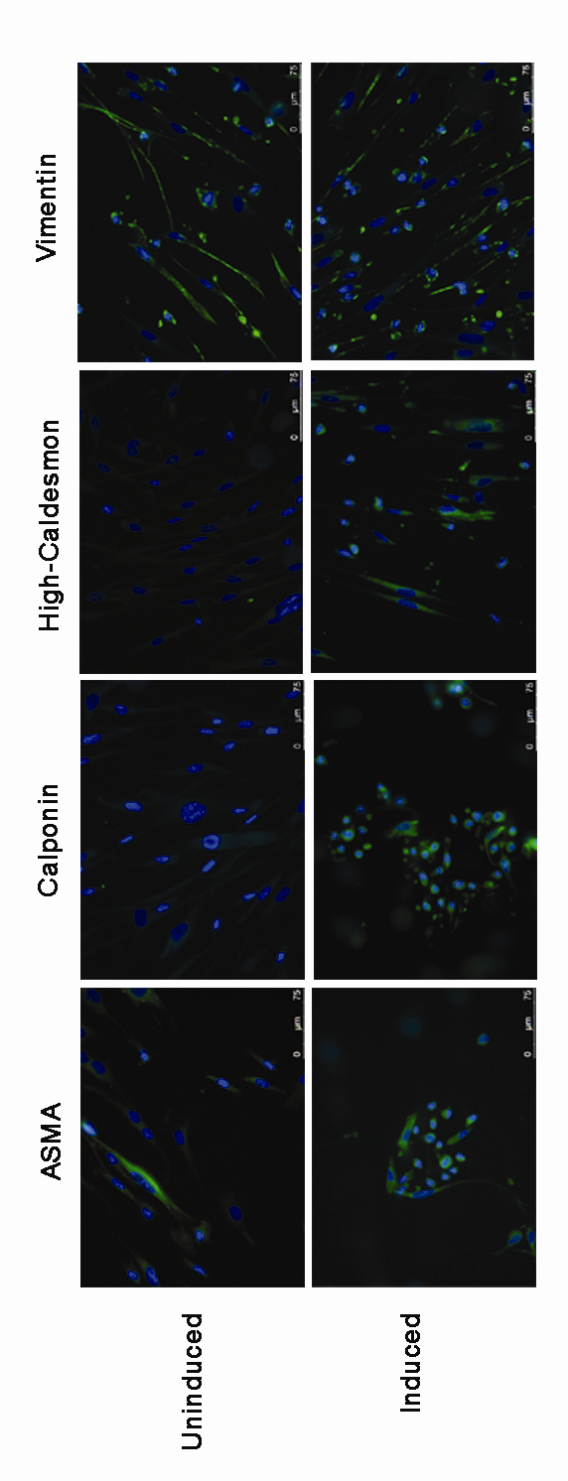

Figure 8.11. Immunofluorescent analysis of leiomyogenesis.

Role of vascular progenitor cells in therapeutic angiogenesis

8.5 Blood flow recovery

The hind limb ischemia was successfully reached and the intramuscular injection of VW-MSCs did not cause neither adverse reaction or weight loss in treated animals. Eco Doppler analysis showed that in this pivotal study cellular administration did not significantly accelerate blood flow recovery in treated animals compared to control one; however, as we can see in **Fig. 8.12**, we could assist to

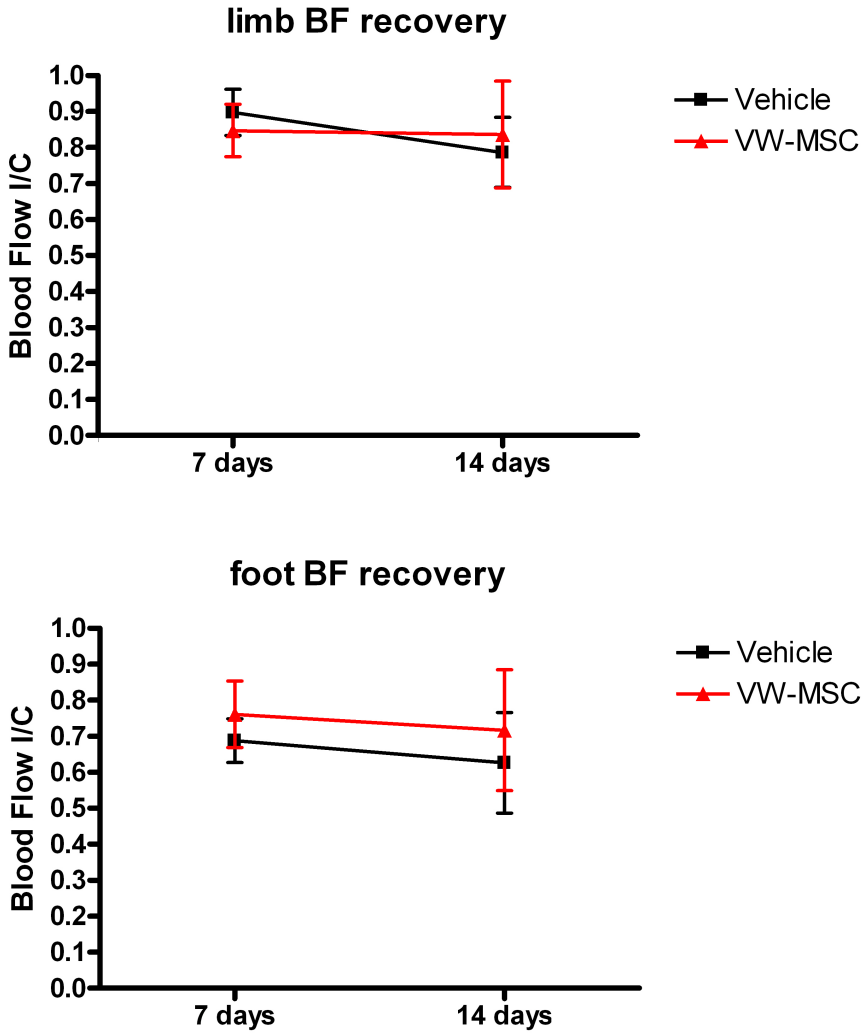

Figure 8.12. Blood flow recovery analysis.

a slight ameliorating limb blood flow at 14 days in treated animals compared to 7 days postischemia whereas control animals showed a decline in limb blood perfusion. On the contrary, both at 7 days and at 14 days after hind limb ischemia we observed higher levels of foot perfusion supported by cellular injection compared to untreated group (**Fig. 8.12**).

8.6 Capillary density and Hypoxia

Eco color Doppler results were in line with those obtained with immunohistochemistry analysis of capillary density and hypoxia conditions of adductors and gastrocnemii muscles. Indeed, we found that capillary density was higher in VW-MSC-injected than in vehicle-injected muscles and this difference was statistically significant in adductors. We also evaluated hypoxic condition in muscle tissues by counting HIF-1α positive cells and fold change data reported in **Fig. 8.13** showed a remarkable reduction in hypoxic status presumably due to VW-MSCs contribute.

8.7 Engraftment and differentiation of transplanted VW-MSCs into ischemic tissues

The most important end point of cell therapy is the development of new vessels in order to supply to defective or occluded vessels. Thus, we evaluated the engrafment of human cells in pre-existent

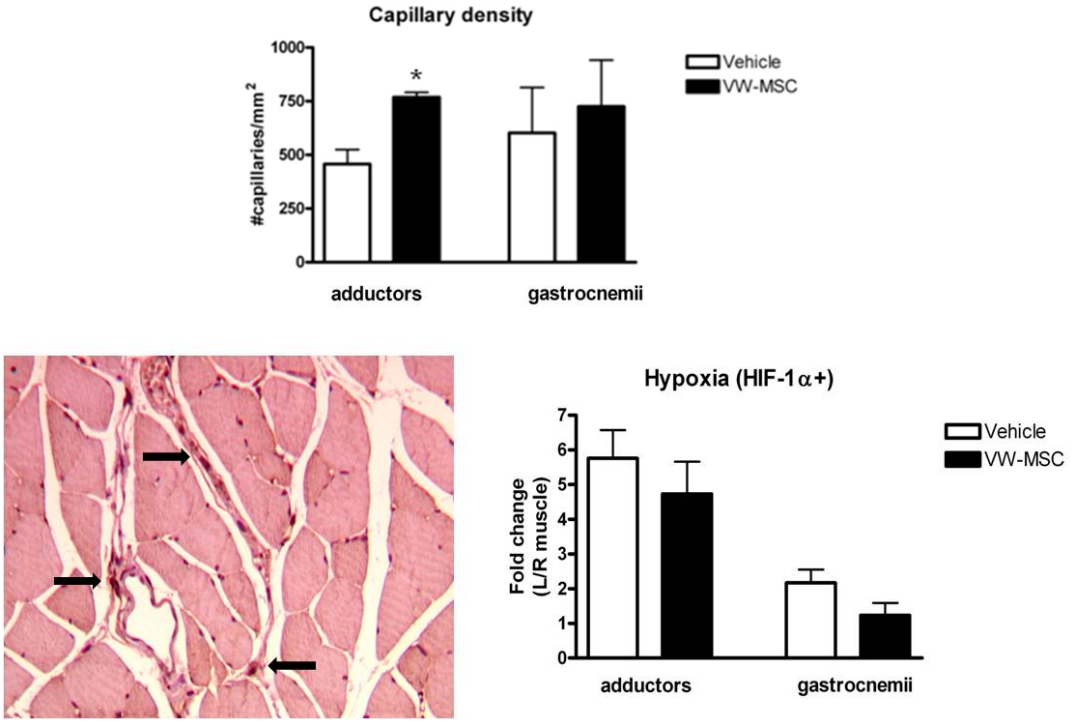

Figure 8.13. Evaluation of muscles capillary density and hypoxic condition.

vessels and the stem cells contribute in neoangiogenesis in ischemic muscles. We found that in adductors the 17 ± 1,6% of total vessels contain well integrated human cells, whereas in the gastrocnemii this percentage was lower 14 ± 6%. This result was expected because VW-MSCs were injected in adductors muscles, but contemporary we assisted to a migration of vascular cells in recalled by local stimuli produced in functionally compromised tissues. Furthermore we investigated the in vivo differentiation of transplanted cells in endothelial cells and we observed that the 16,5 ± 5% of total vessels contained human CD34 positive cells in the adductors muscles, whereas in the gastrocnemii the percentage was 20 ± 0,1%. We do not know whether these were functional vessels (**Fig. 8.14**).

8.8 Supporting role of transplanted cells

Numerous experimental evidences demonstrated that cellular therapy is not only efficient in term of neovascularisation, but also support local cells/tissues to repair their damages by synthesis of growth factors. We supposed that vascular progenitor cells could contribute in augmenting the VEGF availability and

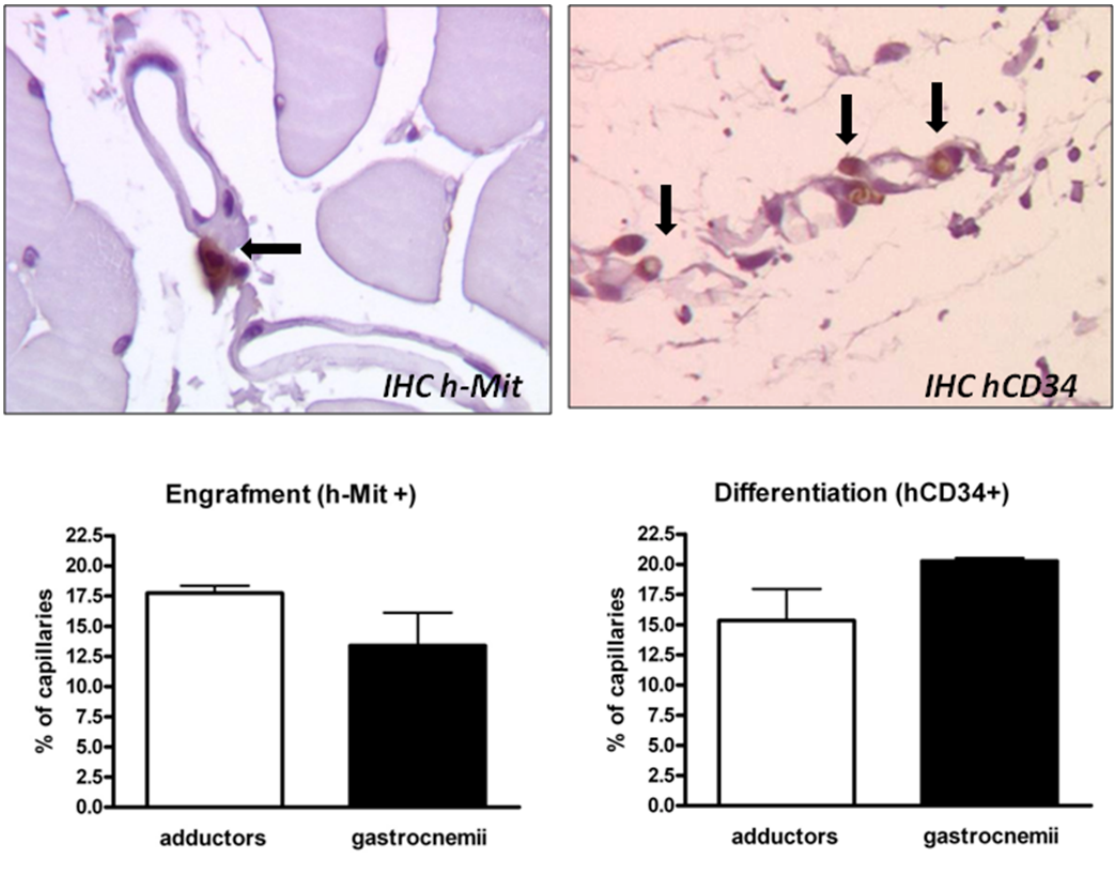

Figure 8.14. Engrafment and differentiation of transplanted human vascular cells.

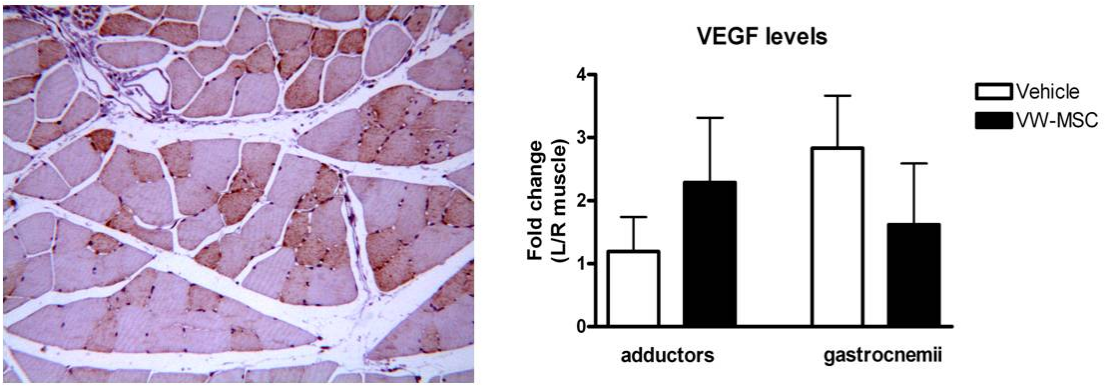

Figure 8.15. In situ evaluation of VEGF levels.

the muscle regeneration, thus we quantified this contribute evaluating muscle fibers synthesis of VEGF and Satellite Cells activation by counting PAX7 positive cells. We found that adductors and gastrocnemii muscles exhibited opposite behavior; indeed, the VEGF was much more expressed in VW-MSCs-injected adductors compared to vehicle-injected one and, at the same time, to gastrocnemii of treated animals. Conversely, gastrocnemii of control group had more VEGF level compared to the VW-MSCs-injected muscles. These results could explain the more elevated capillary density observed in adductors muscles compared to gastrocnemii; furthermore, the promoted angiogenesis may be due to higher level of hypoxia received in adductors after femoral artery ligation (**Fig. 8.15**).

As regarding the muscle regeneration, not surprisingly we observed that satellite cells were principally activated in vehicle–injected muscle; this was particularly true for adductors and less evident in gastrocnemii. It seems like that after ischemia the hypoxia condition, the reduced level of VEGF and the minor perfusion induced tissue damages resulting in untreated muscle regeneration; furthermore, adductors were more sensitive to the proximal femoral artery ligation than gastrocnemii (**Fig. 8.16**).

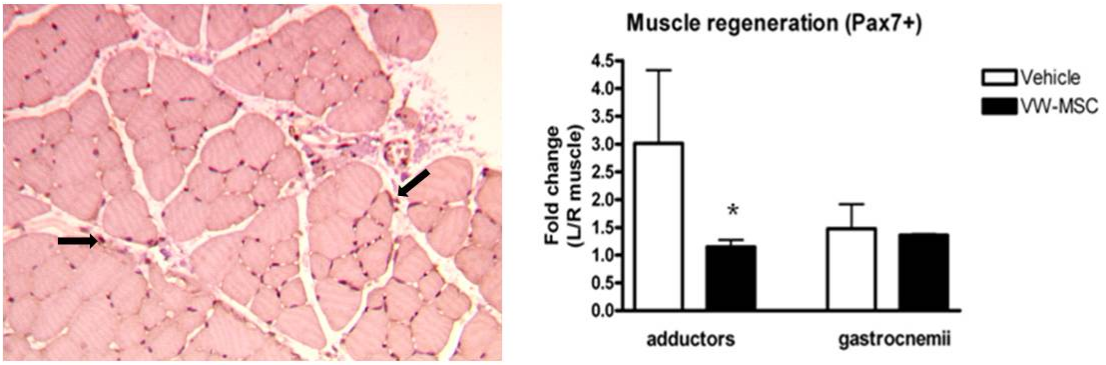

Figure 8.16. Skeletal muscle regeneration mediated by satellite cells activation.

Role of vascular progenitor cells in Mönckeberg sclerosis

8.9 Femoral arteries collection, classification and statistical analysis

From January 2005 to March 2010 we have collected a series of 111 human femoral arteries from healthy multiorgan donors ranging from 14 to 59 years old (middle age 36,5 years); 70 were male (63%) and 41 (37%) were female. On the whole, the 25,2% of arteries presented calcification in the tunica media (27% M and 32% F). Morphologically, the calcified vessels preserved their physiological structure, did not present inflammation elements (cells, endothelial rearrangements, ecc), but only focal ore more extended calcification especially in tunica media and sometimes in the inner elastic lamina containing osteoblasts, osteoclasts, chondroclasts, condroblasts (**Fig. 8.17**).

After the classification in calcified and non calcified vessels, the donors were divided in different sex and age classes: 10-19 years, 20-29 years, 30-39 years, 40-49 years and 50-59 years, and in different risk classes on the basis of own clinical status. Successively we performed correlation analysis and calculated the Odd Ratio (OR) between these different categories (**Tables 8.1** and **8.2**).

Our results showed that Mönckeberg sclerosis is more frequent in women (OR= 1,70), in young people (20-29 years; OR= 1,35) and older subjects (50-59 years; OR=1,97); furthermore, unlike atherosclerosis, we did not found any correlation between the presence of vascular calcification and diabetes, tabagism, and hypercholesterolemia, whilst we have noted a strong correlation with hypertension (OR= 1,68).

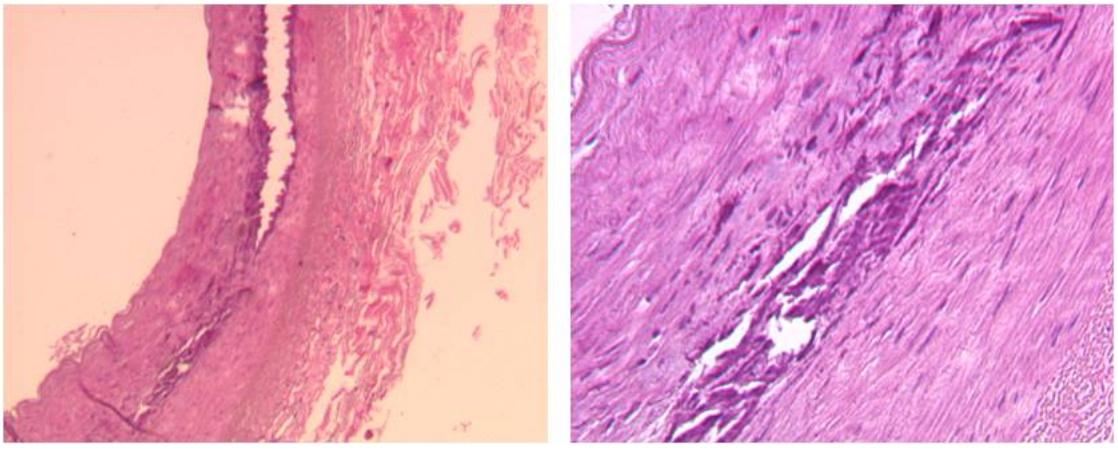

Figure 8.17. Hematoxylin and Eosin stain of calcified femoral artery.

Table 8.1. Patients classifications on the basis of age and arterial calcification.

Age (year)	**Calcified** (M:F)	**Non calcified** (M:F)
9-19	0 (0:0)	9 (6:3)
20-29	8 (4:4)	19 (13:6)
30-39	4 (3:1)	12 (10:2)
40-49	9 (5:4)	31 (18:13)
50-59	7 (3:4)	12 (8:3)

8.10 Isolation and flow cytometry characterization of calcifying cells

We succeded in isolation of VW-MSCs from one calcified vessel which were called cVW-MSCs. These cells were morphologically and immunophenotypically identical to the non calcified VW-MSCs (ncVW-MSCs); the cells were spindle-shaped and had a tendency to grow in a whorled pattern. cVW-MSCs population expressed typical mesenchymal antigens

Table 8.2. Patients classifications on the basis of risk factors and arterial calcification.

Risk Factors	**Calcified** (M:F)	**Non calcified** (M:F)
Diabets	0	4 (2:2)
Tabagism	7 (3:4)	28 (18:10)
Hypertension	2 (1:1)	4 (4:0)
Hypercolesterolemia	0	5 (3:2)

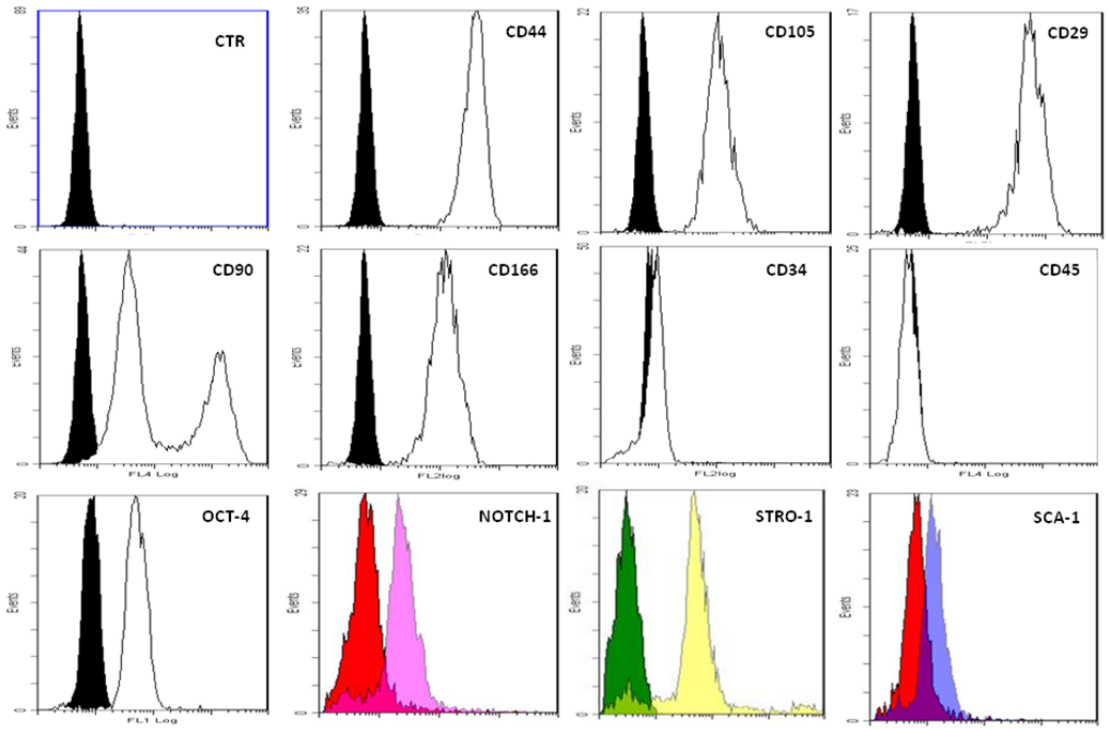

Figure 8.18. Flow cytometry of calcified VW-MSCs.

such as, CD44, CD105, CD90, CD29 and CD166; on the contrary hematopoietic (CD45) and endothelial (CD34) lineage markers were negative, whereas stemness markers, i.e., Sca-1, Oct-4, Notch-1 and Stro-1, were more intense (**Fig. 8.18**).

8.11 Osteogenic assay

At passage 3, cVW-MSCs were induced to osteogenic lineage; the differentiation was evaluated by von Kossa staining and ultrastructural analysis. Conversely to the ncVW-MSC, the osteogenic differentiation potential was very efficient; von Kossa staining revealed numerous and diffuse calcium deposits highlighted as brown spots in the **Fig. 8.19**.

The analysis in TEM showed that induced cells were able to produce mature osteoid matrix, precursor of trabecular bone, rich in ossification elements ranging from hydroxiapatite crystals to mature bone. Uninduced cells retain their mesenchymal characteristics, whilst ncVW-MSCs displayed a less intensive and early commitment revealed only by TEM and not by von Kossa staining (compare **Fig. 8.20** with **Fig. 8.9**).

8.12 Immunohistochemistry assay and statistical analysis

The immunohistochemical analysis of Osx positive cells on vascular tissue slides

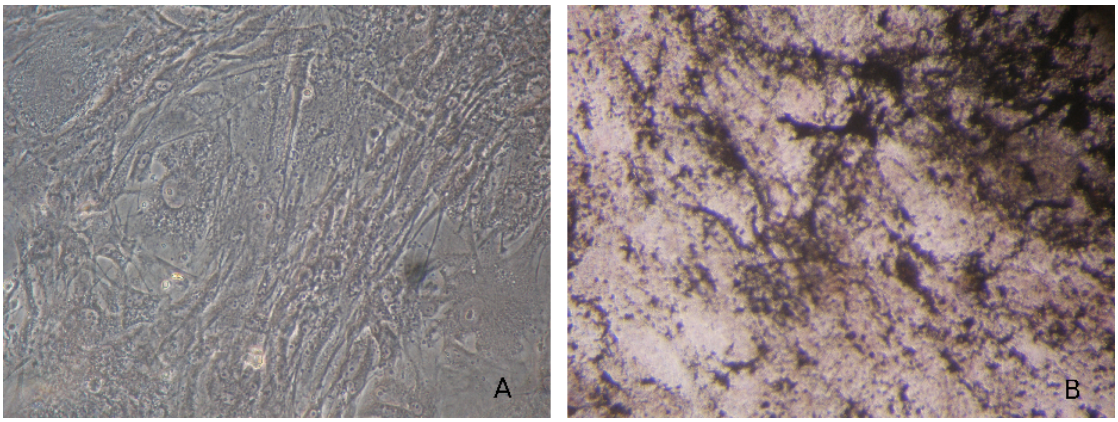

Figure 8.19. Von Kossa staining of c-VW-MSC. A) uninduced cells and B) induced cells.

permitted us to evaluate a possible differential distribution of osteoprogenitor cells along tunica media and adventitia and to quantify the cells in both tunica and in samples collected from donors with different clinical conditions. The Osx+ cells principally consist in smooth muscle cells and pericytes with rounded and small nuclei; no endothelial cells resulted positive to stain (**Fig. 8.21**).

We have manually counted nuclei and calculated the percentage of nuclear positivity on the total number of nuclei and successively we performed a statistical analysis of our results and compared the data obtained from samples of subject affected or not by hypertension. As reported in **Fig. 8.22**, we found that in calcified group, the Osx levels are higher in hypertension condition than in the

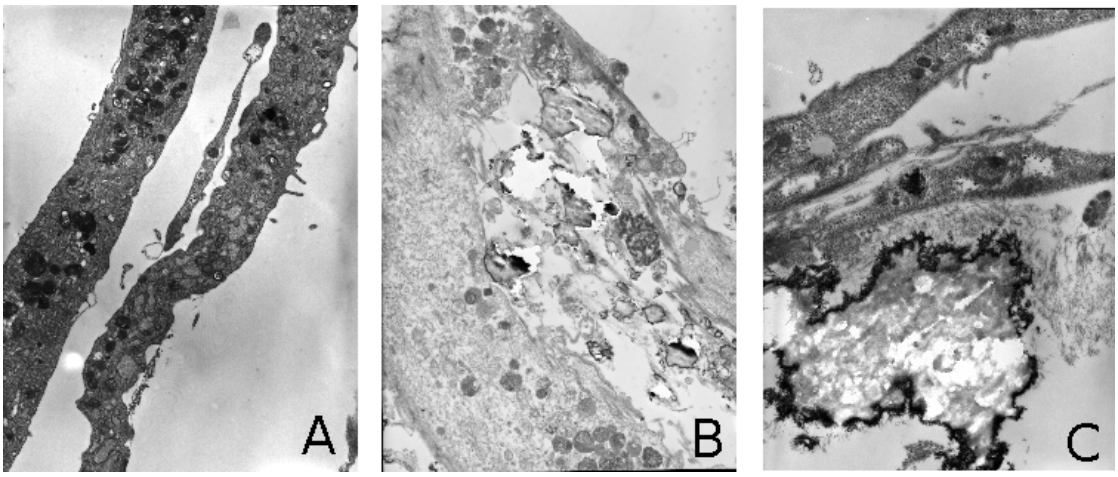

Figure 8.20. TEM analysis of osteogenic commitment of c-VW-MSC. A) uninduced cells and B) and C) induced cells.

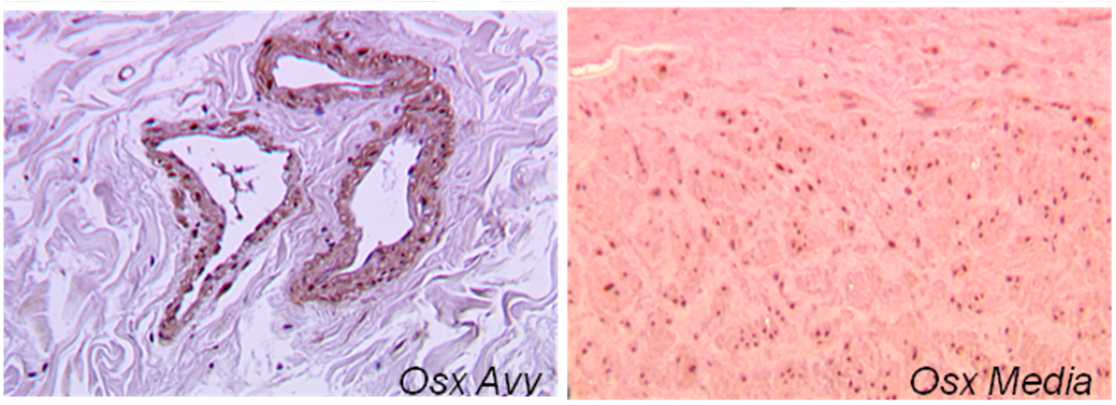

Figure 8.21. In situ analysis of Osterix positive cells.

absence of risk factors (P<0,01); conversely, in non calcified group there are not significant differences about Osx expression; these results are in line with those obtained from correlation analysis showing that in hypertension the vessels are more prone to calcification.

Successively we evaluated the Osx positivity separately both in tunica media and in tunica adventitia in order to investigate the different localization of calcifying cells. In calcified vessels of subjects with no risk factors we did not observe significant differences of Osx expression between the two tunica, though there are more positive cells in the adventitia than in the media; conversely, in hypertension we found higher levels of Osx expression in the tunica media compared to the adventitia. These results

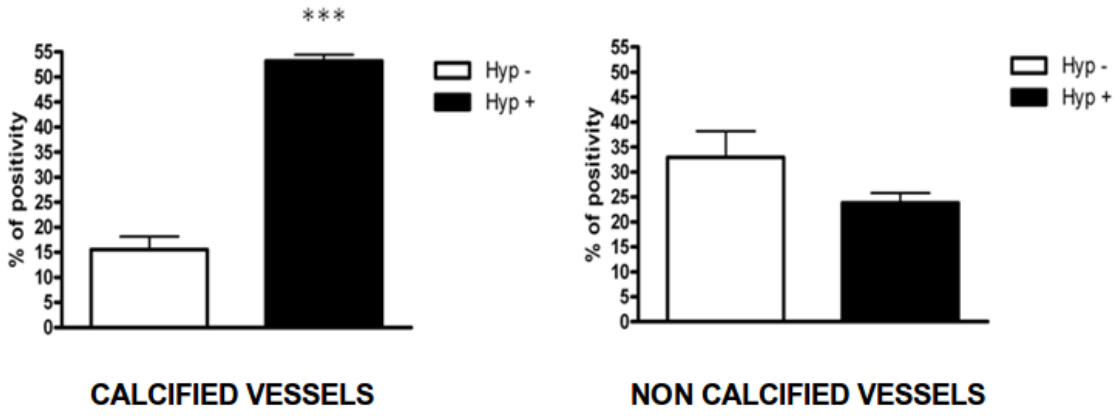

Figure 8.22. Quantification analysis of Osx positive cells in femoral arteries harvested from subjects affected by hypertension (Hyp +) or non exposed to risk factors (Hyp -).

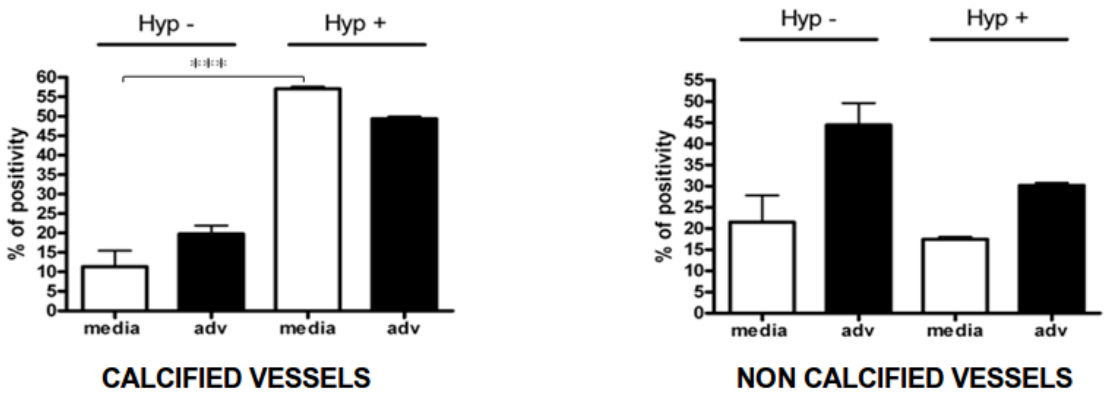

Figure 8.23. Quantification analysis of Osx positive cells in tunica media and adventitia of femoral arteries harvested from subjects affected by hypertension (Hyp +) or non exposed to risk factors (Hyp -).

support the hypothesis that vascular calcifying cells reside in the adventitia and in response to pro osteogenic stimuli migrate toward the media where they differentiate in osteoblasts and produce osteoid matrix. Not surprisingly, in non calcified vessels and in both conditions we found that Osx positive cells principally reside in the tunica adventitia; they are potentially able to produce osteoid matrix, but inert until osteogenic induction (**Fig. 8.23**).

Chapter 9

Discussion

Vessel wall progenitors are responsible for the tissue integrity and homeostasis during postnatal life. Responses to various stimuli and signals are synergic and involve cells which reside within all the vascular wall components. In the intima, ECs function is strictly dependent on the interaction with blood circulating cells which contain subsets of EPCs; these progenitors help resident ECs in lining repair. The interface between media and adventitia has vasculogenic properties and possibly a niche-like function which could explain the existence of resident MSCs in large and medium-sized vessels. The media layer contains cells of different embryological origin displaying heterogeneous properties in terms of multipotency, migration and proliferation ability. Thus each subpopulation may participate in tissue regeneration in a different manner. Again, morphological analysis reflects the same pattern of heterogeneity. Different vascular segments display variability as disease progresses and are susceptible to atherosclerosis and inflammatory diseases in a different way. Moreover, some vascular diseases arise in specific vascular districts: examples include vasculitis, inflammatory aneurysms and diabetic angiopathy. Aortic homograft transplantation in canine models co-validates this hypothesis: patterns of disease progression are strongly influenced by intrinsic differences in the arterial wall cells and are not environment-dependent; as a result hemodynamic flow patterns, alone, cannot trigger the development of atherosclerotic lesions *(Haimovici et al, 1964 and 1971).*

Apart from progenitors, adult arteries of animals and human may contain cells with characteristics of ancestral stem cells. Clues supporting this hypothesis come from studies on cells recovered from arterial segments. Examples include the presence of a "side population" at flow cytometry *(Sainz et al, 2006)*, the expression of the Sca-1 stemness molecule *(Hu et al, 2004)* and c-kit receptor *(Pasquinelli et al 2007 a)*, the ability to form spheroid colonies "in vitro" *(Howson et al, 2005).* However, a

cell able to give rise to a complete hierarchy of vascular progenitors still remains to be isolated from the adult vascular wall. Conceivably a model could be proposed in which a cell type, normally involved in physiological vascular homeostasis, might also act as a reservoir of undifferentiated cells ready to supply the cellular demands of the tissue they belong to, acquiring local phenotypic characteristics, or to mobilize in the circulating blood in response to vascular injury stimuli. MSCs would be good candidates for supplying this reserve function. This possibility is supported by a recent paper by a Brazilian research group *(da Silva Meirelles et al, 2006)*. MSC populations originating from brain, spleen, liver, kidney glomeruli, lung, bone marrow, muscle, thymus and pancreas present a similar morphology and, to a certain extent, surface marker profile. However, these cells differ in adipogenic and osteogenic potential, probably due to the influence of the local microenvironment from which they come. This may reflect the importance of the niche in establishing the phenotype of the stem cells it interacts with. The wide distribution of MSCs and their similar characteristics suggests a closer relationship among them. A few reports have hypothesized that, at least in animal fetal tissues, MSCs may derive from mesangioblast-like perivascular cells; therefore it is conceivable that tissue specific MSCs actually derive from the vasculature. To test this hypothesis the authors studied MSCs obtained from the aorta, the vena cava and kidney glomerulus capillaries. After comparing these populations with the MSCs obtained from more conventional sources, they concluded that the MSC compartment extends throughout the post-natal organism as a result of its perivascular location. However their precise native localization remains obscure. Recent observations identify the pericyte as the MSC "in situ" counterpart *(Crisan et al, 2008)*. However, when the perivascular microenvironment is studied in ultrastructural detail, two possible target cell populations can be associated with MSC potentialities: i) the pericyte, a myoid periendothelial cell which shares its basal lamina with that of ECs; ii) the perivascular fibroblasts originally identified by Joris and Majno as veiled cells in the rat coronary artery *(Joris et al, 1974)*. These cells lack any contractile filaments and are characterized by the presence of elaborate long extremely thin cytoplasmic processes. These differences could also explain why some MSC cell populations express markers of the pericyte lineage, e.g. NG2 and smooth muscle actin, whereas others maintain a more restricted mesenchymal phenotype. Although the precise in vivo MSC attribution remains to be established, it is conceivable that in case of necessity these cells may re-program giving rise to committed progenitors that gradually integrate into the specific tissues. This model does not exclude the possible existence of other tissue-specific stem cells; however it highlights that a portion of apparent post-natal stem cell diversity might be attributed to local MSCs behaving as tissue-specific stem cells, and not to a different MSC population *(da Silva Meirelles et al, 2006)*. Besides the

vascular progenitors identification and in situ localization, their potential role in vascular wall homeostasis and disease is becoming recognized.

The upshot is that identification and characterization of the progenitors involved in physiological homeostasis and pathological vascular remodeling is an issue of great interest, one that may improve current knowledge of vascular diseases and may provide useful strategies that can be transferred to the fields of repair or regenerative medicine and tissue engineering as well.

In previous studies we furnished a detailed description of the "vasculogenic zone" *(Zengin et al, 2006)* of human thoracic aortas and femoral arteries *(Pasquinelli et al, 2007 a and Pacilli et al, 2009)* and we have also already documented the angiogenic properties of thoracic aorta-derived MSCs *(Pasquinelli et al, 2007 a)*. On the basis of our and other works we tried to elucidate stem cell features and multipotent abilities of multidistrict VW-MSCs, extending our observations to the aortic arch and femoral artery.

We therefore analyzed fresh elastic and muscular arteries from healthy multiorgan donors from which cells with a plastic-adherent growth and a fibroblast-like morphology were obtained. These cells had the typical antigen expression pattern of culture-expanded MSCs; they were positive for a mixture of stemness (Stro-1, Notch-1, Oct-4) and mesenchymal (CD44, CD90, CD105, CD73, CD29, CD166) markers, and never expressed hematopoietic or endothelial cell markers. Since VW-MSCs are recovered from entire human arterial segments, we evaluated the expression of stemness markers directly on tissue sections by immunohistochemistry. In agreement with the niche-like function of the vasculogenic zone here we found that Notch-1, Stro-1, Sca-1 and Oct-4 positive cells are located in this area. Postnatal perivascular stem cells are believed to be descendant of cells derived from the embryonic dorsal aorta called mesoangioblasts *(Minasi et al, 2002)*; the presence of Oct-4 positive cells in adult multidistrict vascular segments reinforces this hypothesis.

However, phenotype characterization is not per se sufficient to give to a cell population the property of stemness; thus we explored other features common to all stem cells. It is well known that the ABC transporters and breast cancer resistance protein (ABCG2/BCRP), are highly expressed in a population of primitive stem cells, the side population. SP cells were originally discovered in bone marrow by their capacity to exclude rhodamine 123 and Hoechst dye 33342; however, extensive research also revealed their presence in other non hematopoietic tissues*(Huls et al, 2009)*. Hoechst positive-SP cells have been also found in healthy arteries of the media of adult mice *(Sainz et al, 2006)*. Here we show that human VW-MSCs collected from different vascular segments contain a subset of ancestral cells with SP phenotype.

Furthermore, we seeded cells in low attachment plates to establish whether they were capable of suspension growth. In these conditions, neural and cancer stem cells prove able to form colonies

and VW-MSCs did so efficiently. The stemness of cells forming spheres was confirmed by immunofluorescence and molecular analysis while electron microscopy determined their undifferentiated condition. Comparing immunofluorescence analysis and RNA expression, we observed that cell spheroids expressed Notch-1, Stro-1, c-kit, and Oct-4; Notch-1 was the most expressed gene, thus suggesting a major role in regulating the stemness and the undifferentiated status of VW-MSCs. To explain the lower expression level of Oct-4, we hypothesized that a subset of VW-MSCs had more ancestral characteristics; this is consonant with results provided by SP cells, clonal and bell-shaped nuclei analysis. As expected, TEM showed that VW-MSCs are undifferentiated mesenchymal cells. Intercellular junctions were rare and always of the primitive type; primary cilia were also present and probably influence the balance of stem and progenitor cell proliferation versus differentiation *(Dubreuil et al, 2007)*. One interesting characteristic of more ancestral cells is their clonogenic ability; accordingly, the clonogenic potential of single VW-MSCs was assessed in limited dilution experiments and a percentage of 0.36% cells displayed this ability. According to recent reports *(Kroschinsky et al, 2008; Gostjeva et al, 2006)*, we investigated the nuclear morphology of VW-MSCs as additional evidence of stemness. Regardless of the vascular source, the 0.30 ± 0.05 % of VW-MSCs showed bell-shaped nuclei. Taken together these results document the stemness and embryonic-like quality of VW-MSC populations.

MSCs are considered a subset of postnatal perivascular stem cells deriving from multipotent mesenchymal precursors, i.e., mesoangioblasts, which are localized in the embryonic dorsal aorta *(Minasi et al, 2002)*. Also human MSCs deriving from pulmonary artery *(Hoshino et al, 2008)* and saphena vein *(Covas et al, 2005)* are multipotent. Consequently, the in vitro mesengenic property of MSCs deriving from additional human vascular sources was investigated; VW-MSCs from different vascular segments were able to commit towards adipogenic, osteogenic, chondrogenic and leiomyogenic lineages.

As for adipogenesis, we observed a progressive increase in cytoplasmic lipid vacuoles in all populations; however, the extent of adipogenesis was higher in VW-MSCs deriving from thoracic aortas and femoral arteries than was observed in assays performed on cells deriving from aortic arches. Although we have no explanation for this difference, we may assume that it has a relationship with the embryological origin of the ascending aorta which is under the control of neuroectodermal progenitors *(Pacilli et al, 2009)*.

VW-MSCs exhibit a good propensity for chondrogenic but less for osteogenic commitment. Markers of cartilaginous differentiation such as type II collagen were detected with immunohistochemical and ultrastructural assays. The results from von Kossa staining suggested a failed commitment; however, TEM revealed an ossification corresponded to a stage preceding the formation of the trabecular bone.

After three weeks of induction VW-MSCs did in fact synthesize an osteoid matrix enriched with multiple hydroxiapatite crystals. Presumably, the exposure time was not sufficient to promote VW-MSCs to complete ossification because of their low innate propensity for osteogenesis. When stimulated with leiomyogenic media, VW-MSCs increased their levels of ASMA and h-CALD transcripts and proteins and showed de novo expression of CALP, all specific markers of human smooth muscle cells. These results were confirmed by TEM which demonstrated contractile filaments in induced VW-MSCs. The concomitant presence of rough endoplasmic reticulum and well-organized fibronexus allowed us to categorize the differentiating cells as myofibroblasts. According to Eyden *(Eyden et al, 2009)*, electron microscopy detection of cell-to-matrix junctions enables us to identify myofibroblasts and to distinguigh them from other ASMA-expressing cytotypes, including pericytes. This observation suggests that, in the vascular wall, myofibroblasts derive not only from smooth muscle cell-to-myofibroblast transition *(Hinz et al, 2007)* but also from local resident mesenchymal cells, VW-MSCs being the possible source. This is consistent with the concept that vascular smooth muscle cells are phenotypically heterogeneous *(Hao et al, 2006)*, and that cells involved in remodeling injured arteries apparently include smooth muscle cells from the media as well as adventitial fibroblasts *(Zalewski et al, 2002)*.

We confirmed stem cell properties and proangiogenic ability of VW-MSCs also in a immunodeficient murine model of hindlimb ischemia. After femoral artery ligation we intramuscularly injected TA-MSCs or vehicle in adductors of hischemic left limb; the right controlateral muscles acted as internal control. We chosed TA-MSCs because their angiogenic ability was well characterized in in vitro recent study *(Pasquinelli et al, 2007 a)*. Eco color Doppler analysis for Blood Flow recovery showed that cellular administration did not significantly accelerate blood flow recovery in treated animals compared to control group; however, it induced a slight ameliorating limb blood flow at 14 days in treated animals compared to 7 days postischemia and to vehicle-injected animals which showed a decline in limb blood perfusion. As regarding foot perfusion both at 7 days and 14 days after hind limb ischemia we observed higher levels of blood flow in VW-MSCs injected group compared to untreated one. Although there were not a significant recover of blood perfusion, the analysis of adductors and gastrocnemii explanted post-mortem revealed an important role of human vascular cells in reducing critical ischemia consequences such as limb amputation. In general, adductors were more sensitive to ischemia and cell therapy compared to gastrocnemii; this is probably due to the fact that femoral ligation was performed in proximal region which irrorates this muscle.

Indeed, we found that capillary density and VEGF level were higher in VW-MSC-injected than in vehicle-injected muscles and this differences were statistically significant. As results hypoxic

conditions were attenuated by vascular progenitor cells which are able to engraft the preexistent vessels and to originate new capillaries by differentiating into mature endothelial cells. Furthermore, unlike treated muscles, the vehicle-injected adductors were not able to adapt themselves to impaired oxygen flux; as consequence, we observed an activation of skeletal muscle stem cells called satellite cells (Pax7 positive), in order to preserve the architectural and functional integrity of the tissue. In gastrocnemii we received only moderate hypoxic conditions and consequently we did not found elevated level of VEGF or more abundant capillaries in VW-MSCs injected muscles than the vehicle injected muscles. However there was a reduction of hypoxia in treated muscles compared to the untreated one presumably due to the vascular progenitor contribute in neoangiogenesis as documented by human CD34 evaluation; furthermore this adaptation was sufficient for good tissue function which did not require satellite cells activation.

Overall, these data suggest that VW-MSCs have the capacity to incorporate in the host tissue, to localize around the capillaries as natural mural components and to support muscle function and perfusion. These results and those from other studies *(Invernici et al, 2007; Campagnolo et al, 2010)* permit us to think about clinical perspective regarding vascular progenitor uses; it is possible to speculate that, in general, VW-MSCs might have potential therapeutic application in vascular pathologies such as diabetic retinopathy that are characterized by dysfunction of perivascular support cells *(Hammes et al, 2002)*.

In summary, our studies confirms the recent acquisition that the MSC compartment is more widely distributed than previously thought; it further highlights that MSCs are resident in arteries of both elastic and muscular architecture. Changes in the immunophenotype and plasticity are presumably correlated to the specific *in vivo* vascular location and may also be a consequence of their embryologic origin. This suggests that MSC functional roles are at least partially organ and tissue specific; this intrinsic and innate characteristic could explain the different behavior of vascular district in terms of preferentially onset of vascular pathologies.

The arterial wall, indeed, is one of the most frequent location of pathological insults in humans; the balance between injury and repair is altered by acute and chronic stresses, the atherosclerosis being the major disease of the arterial wall; vasculitis, aneurysmal dilatation, dysplasia and even neoplasms are additional examples of diseases that can affect irreversibly the arterial wall. Furthermore, pathological arteries may develop heterotopic mesodermal tissues which are apparently unrelated to the original composition of adult tissue; examples include the presence of cartilaginous metaplasia with endochondral ossification *(Qiao et al 2003)*; exuberance of fibroblastic spindle cells and fat tissue in inflammatory abdominal aortic aneurysms *(Tang et al, 2005)*. The mechanisms determining the development of arterial calcification, a feature of the atherosclerotic fibro-calcific

plaque, remain largely unexplored. Arterial calcification can be seen in other conditions such as idiopathic infantile arterial calcification *(Glatz et al, 2006)*, dialysis *(Ketteler et al, 2005)* and diabetic *(Hayden et al, 2005)* patients. The premature diagnosis of vascular calcification is an issue of great interest since it can be pursuit without clinical invasive techniques and is useful to prevent cardiovascular events; nevertheless, four forms of vascular calcification exist and not all are a consequence of a preexistent pathology of vascular wall *(Guzman et al, 2007)*.

Medial arterial calcification is also called Mönckeberg sclerosis and may be found in conjunction with atherosclerotic lesions or it may occur independently; the latest assumption is not commonly accepted, in fact many researchers retain that Mönckeberg sclerosis is only the final step of the atherosclerotic process and do not recognize its own identity *(McCullough et al, 2008; Fuchs et al, 1985)*; however, the experimental evidences of an individual pathology are numerous *(Hirsch et al, 1993; Rumberger et al, 1994; Proudfoot et al, 2001)*. Several models have been proposed to explain the origin of vascular ossification and postulated the active contribute of pluripotent arterial cells named calcifying vascular cells in response to vascular stimuli. Another hypothesis ascribes the onset of vascular calcification to an altered bioavailability of proteins inhibiting precipitation, chelation and sequester of ions. However, these findings indicate that vascular calcification is an active, regulated process under the control of cells expressing genes proper of osteoblasts; it seems likely that proposed models are not mutually exclusive and resident cells play a fundamental role in the pathological mechanism *(Doherty et al, 2004)*. To establish VW-MSCs contribute in Mönckeberg sclerosis onset, we studied a wide series of healthy femoral artery segments collected from January 2005 to march 2010 at Regional CardioVascular tissue bank in Bologna; on these samples we evaluated the frequency of Mönckeberg sclerosis and investigated a possible correlation with common atherosclerotic risk factors. We recovered 111 samples from multiorgan donors ranging from 14 to 59 years old (middle age 36,5 years); 70 were male (63%) and 41 (37%) were female. On the whole, the 25,2% of arteries presented calcification in the tunica media (27% M and 32% F) and this phenomenon is more frequent in women (OR= 1,70), in young people (20-29 years; OR= 1,35) and older subjects (50-59 years; OR= 1,97); this findings well fit with those reported by other groups in which were demonstrated that estrogen could promote vascular calcification increasing calcified nodule formation and augmenting the expression of osteogenic markers and that vascular ossification starts during the second and third decade of life *(Hirsh et al, 1993; Rumberger et al., 1994)*. Although diabetes, tabagism and hypercholesterolemia promote vascular osteogenesis and play an important role in transforming vascular cells into osteoblast-like cells *(Aronson et a, 2002; Kilaru et al, 2001)* we did not find any correlation between the onset of Mönckeberg sclerosis

and these risk factors. However, un unexpected results was that the pathology is related with hypertension (OR= 1,68). Actually we are not able to demonstrate if the hypertension is a cause or a consequence of vascular rigidity; more experiments have to be performed to explain it. These findings support the hypothesis that Mönckeberg sclerosis is more frequent than previously thought; furthermore, the vascular ossification is asymptomatic, affects young people and is unrelated to typical aspects of atherosclerosis; we did not find inflammatory cells, lipid core, foam cells, but only bone elements like osteoblasts, osteoclasts, chondroclasts, condroblasts and sometimes architecturally complete trabecular bone; moreover, the calcifications occur in subjects with normal calcium and phosphorous levels and this distinguishes it from calciphylaxis. Spontaneous bone formation is difficult to explain if we did not refer to a direct involvement of vascular cells. We have already found that VW-MSCs isolated from healthy vascular segments are able to differentiate in adipogenic, chondrogenic and leiomyogenic lineages but are less prone to osteogenesis *(Pasquinelli et al, 2010)*; since resident progenitor cells can also be recovered from calcified femoral arteries and they are probably the major players in spontaneous calcifications, we tested their osteogenic ability. VW-MSCs derived from calcified vessels (cVW-MSCs) exhibit the same immunophenotype of cells derived from non calcified counterpart (ncVW-MSCs) but showed a strong predisposition in in vitro osteogenesis as demonstrated by von Kossa staining and TEM analysis; moreover, as Calcified Vascular Cells (CVCs) described by Demer's group in the aorta *(Tintut et al, 2003)*, femoral artery derived cVW-MSCs express Stro-1 and Sca-1, two markers of multipotent cells able to differentiate in mineralizing osteoblasts. Successively, we investigated the in situ location of osteoprogenitor cells evaluating the expression of Osterix (Osx), a note master gene essential for differentiation of mesenchymal progenitors into terminally differentiated osteoblasts, on tissue sections of calcified and non calcified femoral artery. Complessively, we found that in calcified group, the Osx levels are higher in hypertension condition than in the absence of risk factors ($P<0,01$); conversely, in non calcified group there are not significant differences about Osx expression; these results are in line with those obtained from correlation analysis showing that in hypertension the vessels are more prone to calcification. Successively, we performed analysis in order to appreciate different distribution of Osx+ cells between tunica media and tunica adventitia and to compare the vessels collected from subjects without exposition to risk factors and subject affected by hypertension. Some researchers retain that osteoprogenitor cells are contiguous to the vasa vasorum where they are inactive; further to osteogenic physiological stimuli the cells migrate to the tunica media and start to produce bone matrix *(Esner et al, 2006)*. According to this hypothesis, we found that in calcified vessels of subjects with no risk factors we did not observe significant differences of Osx expression between the two tunica,

though there are more positive cells in the adventitia than in the media; conversely, in hypertension we found higher levels of Osx expression in the tunica media compared to the adventitia. Probably, the hemodynamic flux in hypertension condition actives numerous osteoprogenitor cells normally resident in adventitia that migrate in the tunica media where they start to produce osteoid matrix. Furthermore, in vitro osteogenesis assay performed on cVW-MSCs and ncVW-MSCs showed that induced ncVW-MSCs express higher level of Osx compared to cVW-MSCs; uninduced cells do not express this osteogenic factor; thus, Osx is a key factor in regulating vascular ossification and probably it is switched off in mature cells. On the basis of our results we cannot establish if hypertension is a cause or a consequence of vascular calcification, but we demonstrate unequivocally that the Mönckeberg sclerosis has an independent origin from atherosclerosis and presents peculiar aspects respect to other vascular diseases. The ectopic formation of bone tissue is an active and strictly regulated process in which VW-MSCs are directly involved and Osx is an early activator.

On the basis of our results, we retain that the feasibility of isolating MSCs from arterial samples collected from multiorgan donors in qualified tissue bank facilities and of storing them in good cell and tissue practice-certified cell factories could prompt use of them in cardiovascular research and clinical applications. Indeed, VW-MSCs can be considered the natural candidate for the realization of biosynthetic arterial substitutes and for cell therapy, especially in the case of vascular diseases where conventional treatments fail. Future studies establishing the *in vivo* regenerative and repair potential of VW-MSC in animal models of vascular diseases will no doubt seek answers to this still unexplored field.

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