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VASCULAR WALL STEM CELLS. SELECTION AND CONDITIONING OF PROGENITORS USEFUL FOR CELL THERAPY. A PATHOLOGICAL CASE STUDY

Tesi di Dottorato

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ABBREVIATIONS

***Ab*:** Alcian blue

Abs: Antibodies

ALCAM :activated leucocyte adhesion molecule

***APC*:** Allophycocyanin

ASCs: Adult Stem Cells

***ASMA*:** Alpha Smooth Muscle Actin

BCRP1: Breast Cancer Resistance Protein

BDMA: Benzyldimetylammina

BDNF: Brain-Derived Neurotrophic Factor

***bFGF* :** basic Fibroblast Growth Factor

BM-HSCs: Bone Marrow- Hematopoietic Stem Cells

BMI-1: B lymphoma Mo-MLV insertion region 1

BM-MSCs: Bone Marrow derived Mesenchymal Stem Cells

***BMP*:** Bone Morphogenetic Protein

***BSA*: Bovine** Serum Albumine

CD: Cluster of differentiation

CEPs: Circulating endothelial progenitors

***CFU-f*:** Colony forming unit-fibroblasts

***CVCs*:** Calcifying vascular cells

***DAB*:** Diaminobenzidine

***DAPI*:** 4‟, 6-diamidino-2-phenyl indole

DMEM: Dulbecco's Modified Eagle's Medium

***EBs*:** Embryonic Bodies

***ECM*:** Extracellulaire Matrix

ECs: Endothelial Cells

***EPCs*:** Endothelial Progenitors Cells

ESCs: Embryonic Stem Cells

***FACS*:** Fluorescence activated cell sorting

***FBS*:** Fetal Bovine Serum

***FGF*: Fibroblast** Growth Factor

***FITC*:** Fluorescein isothiocyanate

***flk1*:** Fetal liver kinase 1

GDNF: Glial cell line-Derived Neurotrophic Factor

***GPI*:** Glycophosphatidylinositol

***GVD*:** Graft-Vs-host Disease

H&E: Hematoxylin and Eosin

hESCs: human Embryonic Stem Cells

HFSCs: Hair Follicle Stem Cells

***HITA*:** Human Internal Thoracic Aortas

***HLA*:** Human Leukocyte Antigen

H-MSCs: Homograft-Mesenchymal Stem Cells

***HPCs*:** Hematopoietic Progenitor Cells

***HSCs*:** Hematopoietic Stem Cells

***HUVEC*:** Human Umbilical Vein Endothelial Cells

hVPCs: human Vascular Progenitor Cells

IBMX: IsoButhyl-Methyl Xanthine

ICM: Inner Cell Mass

***IGF*:** Insulin-like Growth Factor

***IHC*:** Immunohistochemistry

IL-8: Interleukin 8

iPSCS: induced Pluripotent Stem Cells

***ISCs*:** Intestinal Stem Cells

IVF: In Vitro Fertilization

***KDR*:** Kinase insert domain receptor

***LM*:** Light microscopy

LV: Lateral Ventricle

***moAbs*:** Monoclonal Antibodies

MEFs: Mouse Embryonic Fibroblsts

mESCs: mouse Embryonic Stem Cells

MHC**:** Major Histocompatibility Complex

MSC: Marrow Stromal Cell

MSCs**:** Mesenchymal stem cells

NSCs: Neuronal Stem Cells

o.n.**:** over night

OCT4**:** Octamer-4

P: Passage

pAbs: polyclonal Antibodies

PBS**:** Phosphate Buffered Saline

PC5**:** Phycoerythrin-Cyanin 5.1

PCR: Polymerase Chain Reaction

PDGF-BB**:** Platelet-Derived Growth Factor beta

PE**:** PhycoErythrin

PECAM-1**:** Platelet Endothelial Cell Adhesion Molecule-1

RT: Reverse Transcriptase

rER: rough endoplasmic reticulum

rt**:** room temperature

RT-PCR**:** Reverse Transcriptase- Polymerase Chain Reaction

Sca1**:** Stem Cell Antigen-1

SGZ**:** SubGranular Zone

SH2**:** Src Homology 2

SH3**:** Src Homology 3

SH4**:** Src homology 4

SMCs**:** Smooth muscle cells

SmGM2: Smooth muscle Growth Medium

SOX2**:** SRY (sex determining region Y)-box 2

SP**:** Side Population

SSCs: Somatic Stem Cells

***SSEA-1*:** Stage-Specific Emrbyonic Antigen-1

***SSEA-3*:** Stage-Specific Embryonic Antigen-3

SSEA-4: Stage-Specific Embryonic Antigen-4

STAT 3: Signal Transducer and Activator of Transcription 3

***STAT 5*:** Signal Transducer and Activator of Transcription 5

***SVZ*:** SubVentricular Zone

TA: Transit Amplyfing

TBS: Tris Baffered Saline

***Tel*:** Transcription factor translocation Ets leukemia

***TEM*:** Transmission Electron Microscopy

TGF-α: Transforming Growth Factor, alpha

TGF-β: Transforming Growth Factor βeta

TGF-β1: Transforming Growth Factor βeta class 1

TGF-β3: Transforming Growth Factor βeta class 3

***tie2*:** Tyrosine kinase with immunoglobulin-like and EGF-like domains 2

UV: UltraViolet

***VEGF*:** Vascular endothelial growth factor

***VEGF-R1 or Flt1*:** Vascular endothelial growth factor receptor 1 or fms-like tyrosine-kinase 1

***VEGF-R2 or KDR*:** Vascular endothelial growth factor receptor 2 or kinase-insert domain receptor

VWCs: Vascular Wall Cells

VW-EPCs: Vascular Wall- Endothelial Progenitor Cells

***vWF*:** von Willebrand Factor

VW-MSCs: Vascular Wall- Mesenchymal Stem Cells

INTRODUCTION

1. VASCULAR SYSTEM

Vascular system is a close, double and complete system that carry the blood in every human body district; it contain the heart, the central organ and propeller of blood circulation, and blood vessels, divided into artery, vein, capillary in which the blood flow. Vascular system begins with the aorta, the main and largest artery, that through several forks give origin to minor arteries with a decreasing diameter (from 25 mm in aorta to 0,2 mm in arterioles) that reach organs and tissues. Depending on the caliber and on the quantity in the thickness of the vessel wall of elastic fibers and muscle fibrous cells we can distingue elastic and muscular arteries and arterioles and they are a transporter of oxygenated blood from the heart to all the peripheral tissues of the organism. Arterioles ramify in lower caliber arterial vessels becoming thinner near organs and forming nets know as capillary plexus that is regarded as a bridge between arteries and veins, which are involved in the exchange of substances between blood and tissues. Thanks to their small diameter (5-10 μm), their thin wall with a single layer of endothelial cells and the low hydrostatic pressure with which blood flow into, capillaries can easily exchange breathing gases, nutrients, enzymes, hormones and waste products. After the release of the necessary substances and building-up of deoxygenated blood rich of carbon dioxide the blood pass from the capillaries to very small veins (venules) that carry this blood from periphery to the heart. Those small veins merge each other forming a decreasing number of veins with an increasing caliber till they flow in the cave vein that reaches the heart. Vein's characteristics are the quite thin wall, they are not really elastic comparing to same caliber artery even if they have a similar structural organization. Major caliber veins contain special valves, known as swallow nest that impede the blood ebb, contributing to the regulation of blood flow in a centripetal way.

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 *S 200*9).

1.1 BLOOD VESSELS: HISTOLOGY and CLASSFICATION

All arterial system wall is constituted by 3 concentric laminas: Internal laminae (intimal), Medial Laminae (mesoartery) and External Laminae (adventitia). Between those laminae, as bounds, there are 2 other laminae of elastic tissue, called inner elastic and outer laminae. *(Fig.1)*

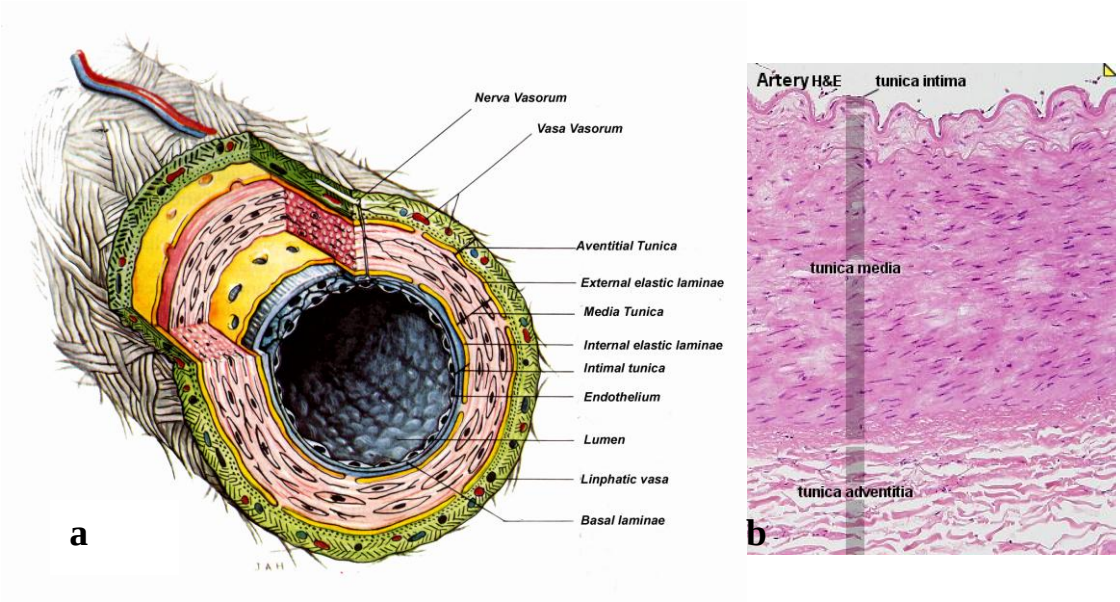


Fig.1: The three concentric laminas of artery wall: intima, media, and adventitial layer. a) Schematic representation; b) Histology (H&E)

Intimal Tunica

The Intimal tunica is the most internal layer, in close contact with the flowing blood; it is constituted by an endothelial and a sub-endothelial layer. The endothelial layer presents a single line of endothelial cells that are overlooking to the longitudinal axis of the vessel. Those cells are present in the whole vascular system and in the heart. The endothelial cell leans on a meagre layer of loose connective tissue, or sub-endothelial laminae, with mesenchymal cell interpose in it, fibroblast end rare smooth muscle cells. The intimal tunica acts as an interface with the blood and guarantee the regolation of the transport of material and blood through tissues.

Medial Tunica

The medial Tunica is the intermedial layer between the intimal and adventitia, separated by the internal and external elastic laminae. In the large is the thickest tunica, that mainly presents the vessel, and permit a taxonomic placement. Medial tunica contains in essence smooth muscle cells in a matrix rich in elastic fibers, collagen and proteoglican; those components are always present in the vessel wall, the amount of each component is different according to the area in which they are. Medial tunica smooth muscle cells are roundly arranged in the vessel lumen.

The activation of the sympathetic permit both the twitch of the vessel, reducing his diameter, (vasoconstriction) and the relax of the vessel, increasing his diameter, (vasodilatation); those actions leads to changing in both blood pressure and bloodstream through the vessel. Medial tunica separate from the adventitial tunica by an aggregate of elastic fibers called external elastic laminae. 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).

Adventitial Tunica

The adventitial tunica is placed externally to the external elastic laminae, it is a loose connective tissue, rich in collagen fibers sprawling orientated, few smooth muscle and inflammatory cells, and adipocytes. It can be considered a connective sheath, with a containing role; it glides into the surrounding connective tissue securing steadily blood vessels. In large and medium caliber vessels the adventitial tunica can be well-developed to hold in his thickness both vasa vasorum (small vessels drizzling and feeding vascular wall) and nerva nervorum (vegetative sympathetic fibers controlling mainly the smooth muscle fibers of the medial tunica).

From the heart to peripheral capillaries blood flow through a network of arteries with a decreasing diameter; by their size and structural characteristics arteries are distinguished in large caliber elastic artery, medium caliber muscular artery and small caliber artery different by contractility and elasticity. Different composition gives also different characteristic in the conduction and distribution suitable with the anatomic area in which they are. Both kind of artery present the same wall structure with the 3 layers, the difference between elastic and muscular arteries depend on the amount of elastic tissue in the medial tunica (*Fig.2*).

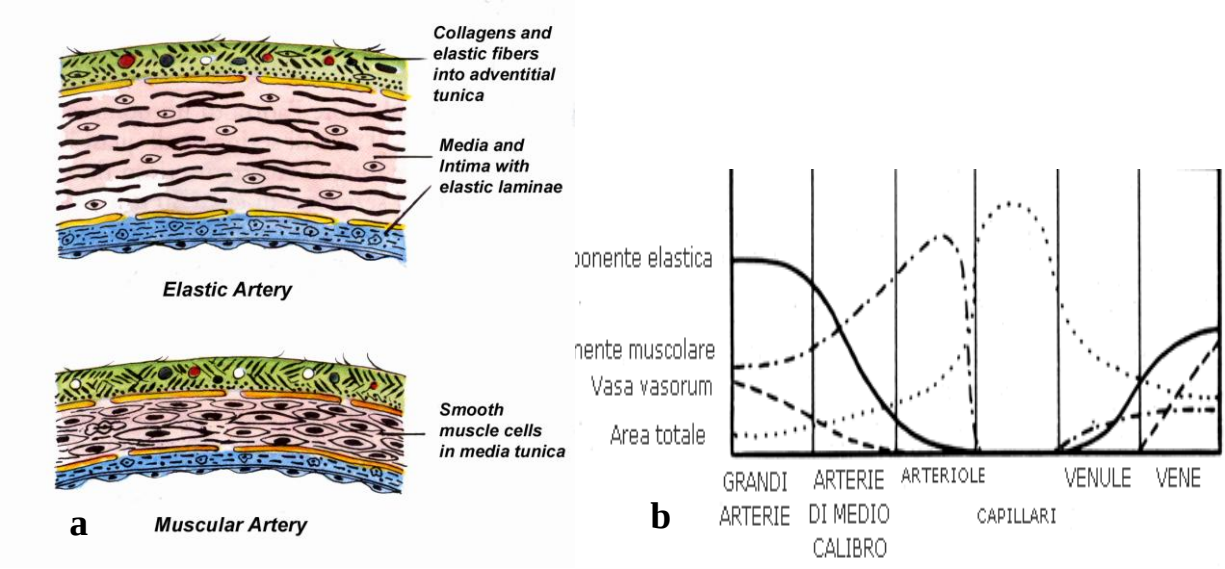


Fig.2: Differences between elastic and muscular arteries. a) Schematic representation; b) Modulation of elastic and muscular components depending on the parietal thickness

Large arteries or elastic arteries or conduction arteries: large vessels that carry the blood from the heart to the muscular arteries. Examples of elastic arteries are pulmonary trunks and aortic arch and their principal branches (such as pulmonary artery, common carotyd, subclavian and iliac common).The vessel wall, characterized by numerous elastic fibers, is thin and presents an ample lumen with a diameter over 2,5 cm. Medial tunica is rich of elastic fibers (40-70 concentric elastic laminae) extremely extending, able to accumulate energy impressed on the haematic mass by ventricular systols and release it slowly during diastole to the blood flowing toward the periphery. The aim is convert the intermittent haematic flow deriving from the heart into a continuous (laminar) flow, necessary to the peripheral district (capillary) in which there will be the exchanges.

Medium caliber arteries or muscular arteries or distribution arteries: receive blood from elastic arteries, from which originate, and release it to

skeletal muscles and internal organs too. Classic examples of muscular arteries are external carotid artery, bronchial arteries, femoral arteries and mesentheric arteries. Those arteries present variable diameters, from 2,5 to 7 mm, with a large lumen and a strong not elastic wall, that modify itself to become mainly a leiomuscular component, in fact they must be able to help with its contractions the blood to flow where heart force is not sufficient to let the blood going on. Medial tunica presents 40-70 layers of concentric smooth muscle cells that, wrapping in a concentric way as opposed to the vessel axis, form a kind of muff around it. There are no elastic lamellae, with the exception of the elastic layers, the internal and the external one.

Small caliber arteries or arterioles*:* are artery branches that arrive to the capillary, its diameter is less than 0,5mm. Rich in muscular tissue, those small arteries are characterized by a gradual loss of elastic tissue, elastic internal and external laminae appear as fragmented, and it is difficult to discern all layers. They present also a small lumen and a thick and contractile vessel wall that regulates and control flow resistance (*Martini 2003*).

2. STEM CELLS

Stem cell research is one of the most fascinating areas of contemporary biology since the cells are "parent" of each organ, tissue and organism cell type, due to their, innate properties to renew themselves for an indefinite period, known as “immortal” because they are able to develop into specialized cells of various body tissues in early life and growth. These specific characteristics make cells stem very promising for the treatment of severely debilitating degenerative diseases and could be interesting for many applications in tissue engineering, cells therapy and drug screening. The biology of stem cells is important in understanding the ontogeny but also to maintain homeostasis by replacing damage cells in adult organism.

2.1 DEFINITION and CLASSIFICATION

Stem cells differ from other cells type in the body and depending on their origin; they can be distinguished in three important characteristics (Fig.3) (*National Institute of Health 2002; Ulloa-Montoya et al., 2005*):

Stem cells are capable to self-dividing and self-renew: they are able to renew themselves through numerous cycles of cell division for long periods maintaining the undifferentiated state. During early development, stem cell division is symmetrical i.e. each cell divides itself to give rise to two unspecialized daughter cells, like the parent, each one with the same potential. In the later development, the cell divides itself asymmetrically with of the daughter cells will maintain a stem characteristic and the second will be a more differentiated cell.

Stem cells are unspecialized: they haven't acquired yet any tissue-specific structures that allow them to perform specialized functions like the ability to become any cell type.

Stem cells are able to give rise to specialized cells: under special conditions, unspecialized cells begin to "differentiate" into specialized cell type and to develop into specific tissues and organs. Each step of the differentiation is trigger by internal signals (cell's genes) and external signals such as cytokines and chemokines secreted by other cells, physical contact with adjacent cells and other molecules in the microenvironment.

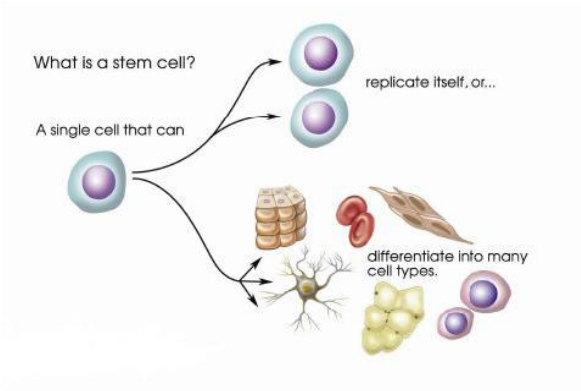


Fig.3: Definition of "stem cells"

Depending on differentiation potential, stem cells are classified in: totipotent, pluripotent, multipotent and unipotent. All human beings start their lives from a single cell called a "zygote", produced from the fusion of an egg and a sperm cell. The zygote is defined the *totipotent* stem cell and can differentiate into embryonic and extra-embryonic cell types constructing a complete, viable, organism. The zygote, after a series of multiple successive divisions, in about 5 days, gives rise to cave spherical agglomerate known as blastocyst. Embryonic Stem Cells, originate as inner mass cells within a blastocyst, are capable to differentiate into cells

from all three germinal layers: endoderm, mesoderm and ectoderm and to generate all tissues present into adult organism thank to their characteristic *pluripotency*. The Adult Stem Cells or Somatic Stem Cells, typically programmed to give rise to different cell types of the tissue of derivating, are called *multipotent* cell and can give rise only to certain cell types. The main role of adult stem cells in a living organism is to maintain and to repair the tissue in which they are found. The *unipotent* stem cells have the capacity to differentiate into only one type of cell or tissue, which is a lower potential. Despite their limited differentiation potential, unipotent cells still have therapeutic potential to treat injuries and diseases. We can assert that a stem cell with its characteristic open a new research for its possible therapeutic for degenerative diseases like Alzheimer disease, Parkinson disease, stroke, cardiopathy, diabete and cancer (*Fig.4*).

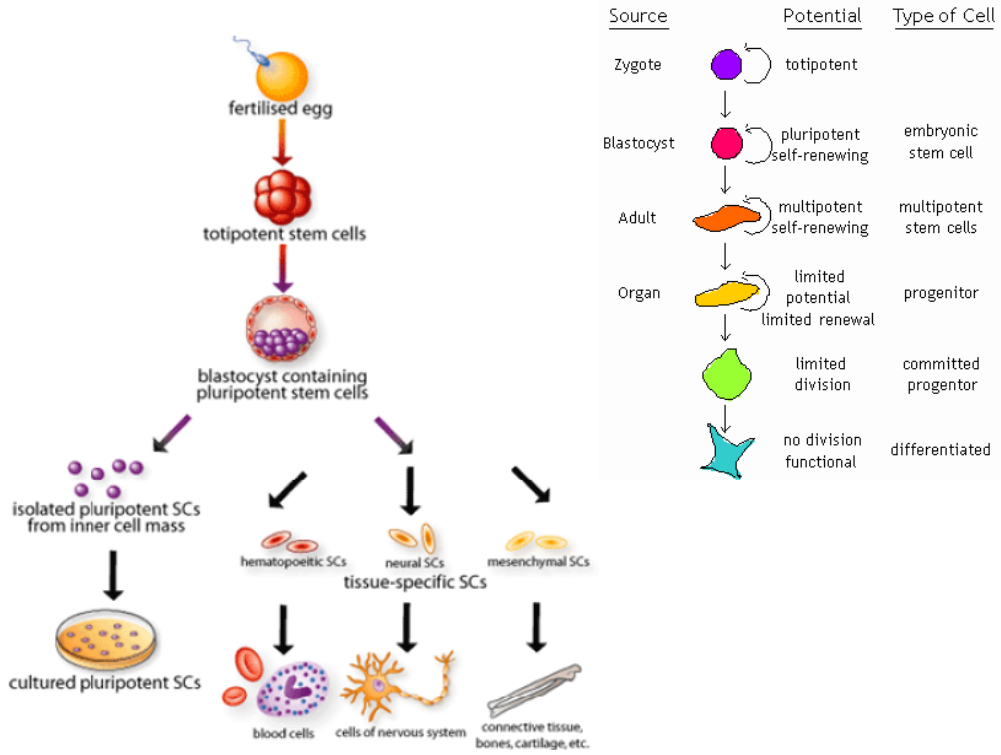


Fig.4: Classification and destiny of "stem cells"

2.2 EMBRYONIC STEM CELLS

Embryonic Stem Cells (ESCs) derived from totipotent cells of the early mammalian embryo and are capable of unlimited and undifferentiated proliferation in vitro. These stem cells obtained from the epiblast tissue of the inner cell mass (ICM) of a blastocysts, of a 4-5 days old embryos, consisting of 50-150 cells (*Fox news*) (*Fig.5*).

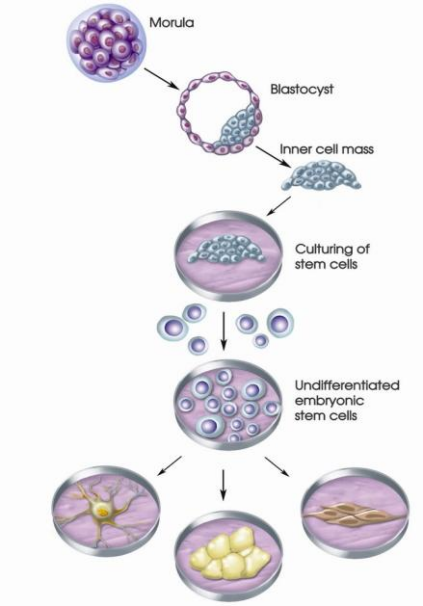


Fig.5: Schematic representation to obtain ESCs

ESCs are the best characterized of all the stem cells; they are pluripotent thank of the ability to differentiate into cells deriving from the three main germ lines: ectoderm (neurons, skin, etc), mesoderm (muscle and bone) and endoderm (Hepatocytes, pancreatic beta cells) and not contributing to the extra-embryonic membranes (*Fig.6*). We can assert that sufficiently and necessarily stimulate for a specific cell type, they can develop into almost all cell types of the adult body. ESCs are immortal, have been maintained in culture for several doublings, and are able to maintain a normal karyotype (*Amit et al., 2003*).

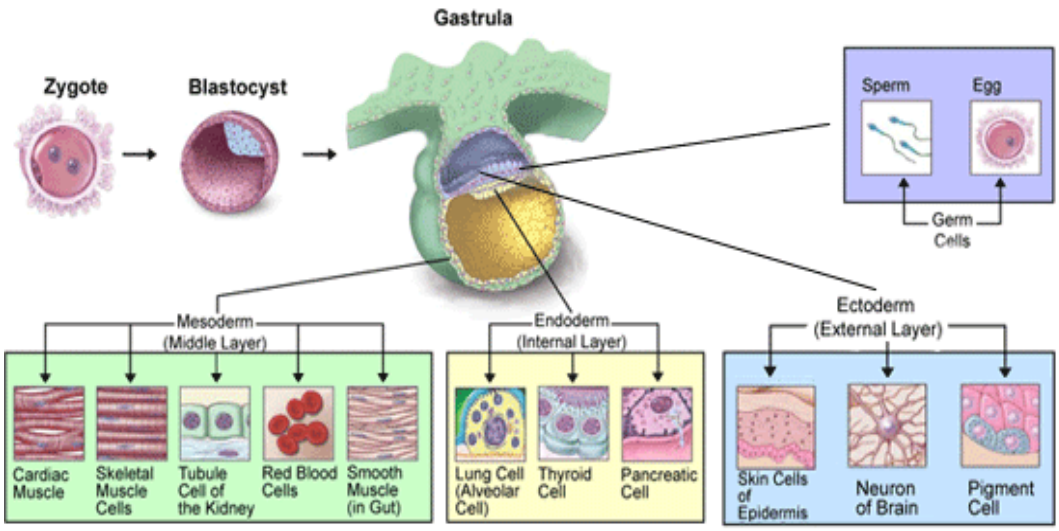


Fig.6: Pluripotency of ESCs

The mouse embryos ESCs have been isolated and cultured since the 1980 by various groups of researchers (*Evans et al., 1981, Axelrod 1984, Wobus et al, 1984, Doetschman et al., 1985*). These pioneers established that murine embryonic stem cells line could differentiate into several different cell types (*Thompson et al., 1995, 1998*) and when injected into a pre-implantation embryo, can produce functional differentiated progeny in all tissue and organ (*Smith et al., 2001*).

In 1995 and 1998, ESCs were isolated from primate and human (*Amit et al., 2003*). human Embryonic Stem Cells (hESCs) should be able to give the same results of mouse Embryonic Stem Cells (mESCs), but for ethical reasons it cannot be demonstrated. ESCs, as their name suggests, derive from embryos produced with in vitro fertilization (IVF) for clinical purpose and then donated for research purposes with informed consent of the donors. 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) form the core regulatory network that ensures the suppression of gene that lead to differentiation and the maintance 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 antigens Tra-1-60 and Tra-1-81. The undifferentiated state of hESCs was maintained in culture condition, they grown on a feeder layer of mouse embryonic fibroblasts (MEFs) and require the presence of basic Fibroblast Growth Factor (bFGF or FGF-2)(*National Institutes of Health*). The differentiation potential was tested in vitro by suspension culturing that form three-dimensional mature cells aggregate derived by ectodermal, endodermal e mesodermal line called embryoid bodies (EBs)(*Shamblott et al., 1998*); their following injection into immunodeficient mice, develop teratomas that typically contain a mixture of many, differentiated or partly differentiated, cell types of the ectoderm, mesoderm and endoderm layers (*Wobus et al., 1984; Reubinoff et al., 2000*) this could be a disadvantage for cell therapy. ESCs remain a potentially source for regenerative medicine and tissue replacement after injury or disease; but considering the ethical issue, the possible development of teratomas and highly immunogenic potential has prompted researchers to study the Adult Stem Cells or Somatic Stem Cells.

2.3 ADULT STEM CELLS or SOMATIC STEM CELLS

The term Adult Stem Cells (ASCs), also known as Somatic Stem Cells (SSCs), refers to any stem cell which is found in a developed organism, in children as well as adults. ASCs seems to be an undifferentiated cell that can renew itself and differentiate in almost the total of the major specialized cell types related to the tissue or organ (*Gardner, 2002*); some of them are indeed lineage-restricted (multipotent) and are generally referred to their tissutal origin (i.e. adipose-derived stem cell,

etc).(*Barrilleaux et al., 2006, Gimble et al., 2007*). The primary roles of ASCs in a living organism are to maintain and to repair the tissue in which they reside; they are also presents in several different tissues including bone marrow, blood, brain, peripheral blood, blood vessels, skin, teeth, heart, liver, skeletal muscle, testis and ovarian epithelium and those findings led us to believe that ASCs can be used for transplantation-based therapies. In fact, the use of ASCs in research and therapy is not as controversial as ESCs, because the production of these cells does not require the destruction of an embryo.

ASCs research of began in 1960, when some researchers discovered that the bone marrow contains two population of stem cells: *HEMATOPOIETIC STEM CELLS* (HSCs) forming all types of blood cells in the body (*Islam, 1985*) and *MESENCHYMAL STEM CELLS* (MSCs) discovered subsequently and they can generate bone, cartilage, fat and cell that support the formation of blood and fibrous connective tissue. (*Friedenstein et al., 1974*) (*Fig.7*)

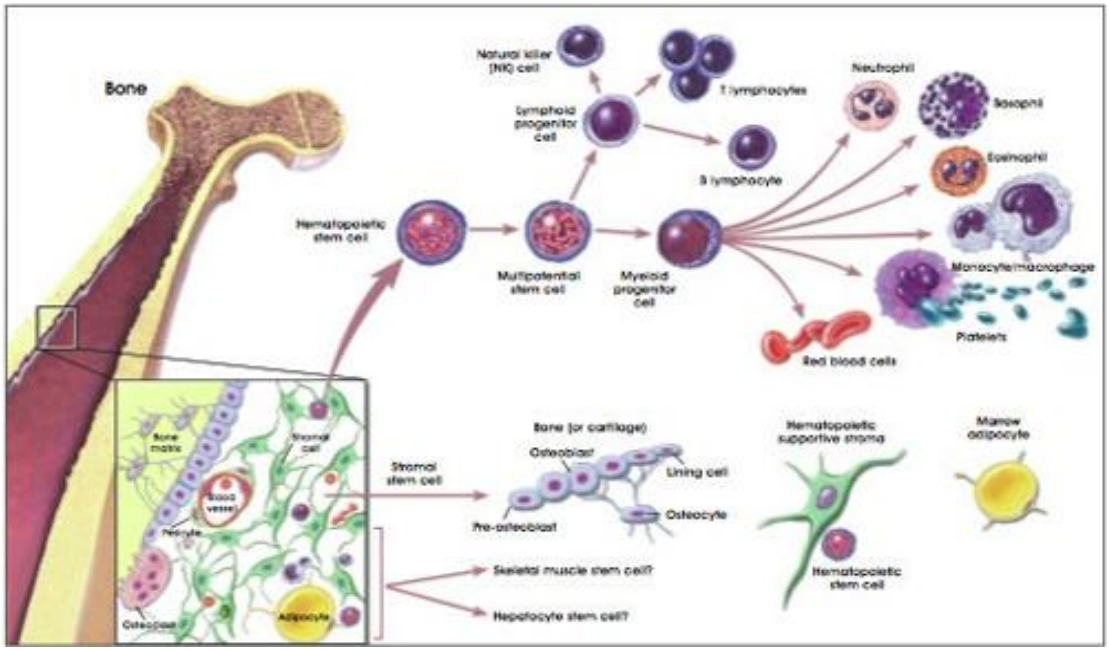


Fig.7: Adult Stem Cells in the bone marrow

The HSCs are the best characterized multipotent stem cells that give rise to all the blood cell types including myeloid and lymphoid lineages and contain cells with long-term and short-term regeneration capacities and committed multipotent, oligopotent, and unipotent progenitors. The HSCs were isolated from bone marrow in the mouse (*Spangrude et al., 1988*) and in murine transplantation experiments it has been demonstrated that one single HSCs reconstitute the entire mouse blood tissue (*Smith et al., 1991*). Subsequently, HSCs were found and isolated also in adult human tissues like bone marrow, umbilical cord blood, placenta and peripheral blood by the expression of cell surface marker $CD34^{+}/CD38^{-}$. The role of HSCs is to differentiate continuously into multiple lineage of different blood cell type and replicate themselves thanks to their self-renewal ability to prevent the depletion of the stem cells pool in bone marrow (*Huang et al., 2007*).

HSCs have been studied by scientists for many years, and they were the first stem cells from the bone marrow to be used successfully in therapies. In fact BM-HSCs have been used for decades to treat blood cancer (i.e. leukemia) and other blood disorders such as aplastic anemia, thalassemia, etc (*Ulloa-Montoya et al., 2005*) and more recently, to treat breast cancer and coronary artery disease. The HSCs are mostly quiescent cells and are about the ≈ 0.05% of the bone marrow components and through intrinsic and extrinsic signals, they proliferate and differentiate into progenitor cells which eventually give arise to terminally differentiate cells in the peripheral blood. External environmental signals must integrate with intrinsic molecular mechanisms to control HSCs fate. In particular, several transcription factors are implicated in the regulation of self renewal such as transcription factor translocation Ets leukemia (tel)(*Hock et al., 2004*), Hox4 (*Sauvageau et al., 2004*), signal transducer and activator of transcription 5 (Stat5) (*Kato et al., 2005*), Stat3 (*Chung et al., 2006*). In addition, it was demonstrated that several proteins involved in the

modulation of gene expression are necessary to regulate HSCs self-renewal such as B lymphoma Mo-MLV insertion region 1 (BMI-1),which together leading to the repression of transcriptional activity through the maintaining of epigenetic memory (*Iwama et al., 2004*). On the other hand, the environment signals implicated in the regulation of HSCs and self-renewal are the transduction pathways of Notch (plays an important role in cell fate for maintaining HSCs in the undifferentiated state (*Varnum-Finney et al., 2000, Calvi et al., 2003*), WNT (*Duncan et al., 2005)* and bone morphogenetic protein (BMP).

A number of other adult stem cells have been studied even if they weren't as well characterized as HSC. Neural Stem Cells (NSCs) that give arise to neurons, astrocytes and oligodendrocytes (*Gage 2000*). Mesenchymal Stem Cells (MSCs) that differentiate into fibroblasts, osteoblasts, chondrablasts, adipocytes and skeletal muscle. (*Pittenger et al., 1999, Prockop 1997, Friedestein 1982*). Other stem cells have been identified, including gastrointestinal stem cells (*Potten 1998*), epidermal stem cells (*Watt 1998*) and hepatic stem cells(also called oval cells)(*Alison et al., 1998*). The idea that stem cell are committed cells has been recently challenged by several bizarre and unexpected findings. Several experiments have suggested that certain adult stem cells types are pluripotent and can transdifferentiate into different cell types not only of the origin tissue but also other tissue i.e. hematopoietic stem cells may differentiate into three major types of brain cells (neurons, oligodendrocytes and astrocytes) (*Krause et al., 2001*); bone marrow stromal stem cells into cardiac muscle cells (*Ferrari et al.,1998*) and endothelium (*Rafii et al., 1994*; *Asahara et al., 1997*), and brain stem cells into blood cells (*Bjornson et al., 1999*) and dental pulp stem cells into neural tissue (*Shen et al., 2003*). This reported phenomenon is called *plasticity* (*Fig.8*).

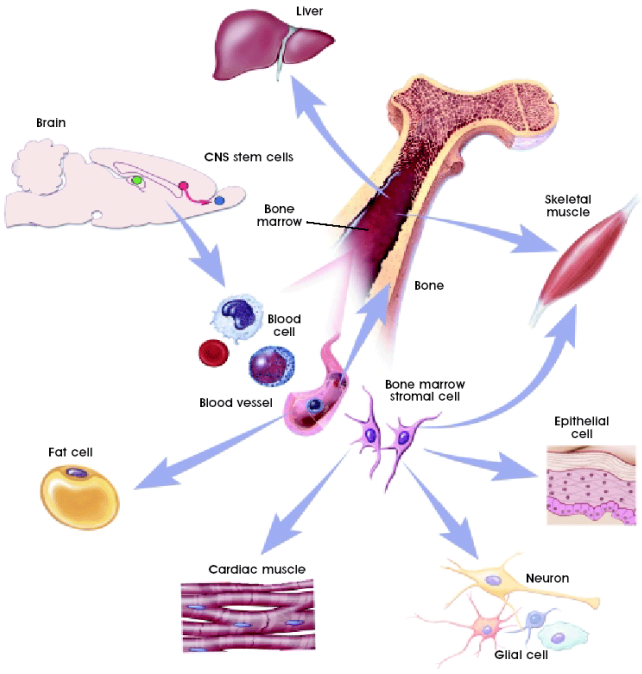


Fig 8: Plasticity of Adult Stem Cells

It has also recently demonstrated that certain adult cell types can be "reprogrammed" into other cell types using an in vivo well-controlled genetic modification process. For example, pancreatic beta cells, the insulin-producing cells that are lost or damaged in diabetes, could possibly be created by other reprogramming pancreatic cells (*Zhou et al., 2008*). In addition, beside the reprogram of the cell to become a specific cell type, it is now possible to reprogram adult somatic cells, through the introduction of embryonic genes, to become like embryonic stem cells a process called induced pluripotent stem cells (iPSCs).

Adult stem cells offer a hope in the future for cell therapy to treat diseases like Parkinson, Alzheimer, stroke, heart disease, diabetes and rheumatoid arthritis. In addition, using patient's own cells it can be overcome the immunological compatibility issue. But ASCs are rare in mature tissue and it's still a challenge and expanding methods in culture, have not yet been worked out. This is an important problem because for stem cell

replacement therapies it's necessary a large numbers of cells. However, ESCs have been found to be better for both differentiation potential and ability to divide themselves in culture, but their production meets strong resistance and limitations of ethical issues.

2.4 MESENCHYMAL STEM CELL

The adult bone marrow contains not only the hematopoietic stem cells, but also 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. The original term "colony forming unit-fibroblast (CFU-F)" or "marrow stromal fibroblasts (MSF)" (*Castro-Malaspina et al., 1980, Piersma et al., 1985, Kuznetsov et al., 1997*) has been gradually abandoned and replaced by different and still indistinct denominations like "marrow stromal cells (MSCs)" (*Prockop et al., 1997*), "mesenchymal stem cells (MSCs)" (*Caplan et al., 1994*), or mesenchymal progenitor cells (*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*). 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, 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*). Initially, peripheral blood was the first sources studied for its easy availability. Only one research's group isolated mononuclear cells population by adherence, morphology, phenotype and differentiation potential typical characteristics of MSCs; these cells were isolated from over 100 samples of peripheral blood of healthy volunteers, but these data haven't never been confirmed (*Zvaifler et al., 2000*). Alternative sources are the fetal membrane a fetal discarded tissue and above all they don't generate ethical conflicts. Moreover, MSCs have high efficient recovery with no intrusive procedures (*Alviano et al., 2007*); the umbilical cord blood is rich in MSCs, because during embryonic development the embryonic hematopoiesis change site, from the yolk sac, the initial site, to liver and then to bone marrow with a consequent migration of HSCs and MSCs; but an interesting source could be the adult dental pulp and adipose tissue, very accessible tissue research. Many studies showed the presence of stem cells in dental pulp (*Gronthos et al., 2000, Pierdomenico et al., 2005*) and adipose tissue (*De Ugarte et al., 2003, Zuk et al., 2002*) possessing stem cell-like qualities, including a good self-renewal and multilineage differentiation (adipogenic and osteogenic) abilities. This source could still be a fascinating subject of study and a valuable therapeutic tool.

In bone marrow, MSCs are important components of the HSCs niche. In fact, all niche's components like stroma, stromal cells (endothelial cells, adipocytes, macrophages, reticular cells, fibroblasts and osteoprogenitors) and cellular microenvironment, where MSCs are presumed to exist, ensure the survival and growth of HSCs (*Koller et al., 1997, Strobel et al., 1986, Tavassoli et al., 1982*). MSCs have been defined by their plastic adherent growth and subsequent expansion under specific culture conditions, by a panel of non-specific surface antigens and by their in vitro e in vivo differentiation potential (*Javazon et al., 2004*). The gold standard assay utilized to identify MSC is CFU-F assay which identifies adherent, spindle-shaped cells that proliferate to form colonies (*Friedenstein et al., 1970*). MSCs, with their fibroblast-like morphology, were initially isolated as the plastic adherent fraction of bone marrow (*Friedenstein et al., 1970*). A Percoll density gradient was used to eliminate unwanted cell types, present in the bone marrow aspirate, and the present MSCs were a small percentage (estimated at about 0.001-0.01%). The general procedure used to isolate mononuclear cells is a gradient centrifugation and the following seeding on culture plates in medium with fetal bovine serum (FBS). After attachment of the adherent cell fraction, the medium is removed to eliminate non-adherents cell, the adherents cell are so expanded in a limited number of passages (*Ulloa-Montoya et al., 2005*). Although there aren't 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 this antibody SH2 identifies an epitope of endoglin (CD105), the Transforming growth factor beta (TGFβ) receptor III present on endothelial cells, erythroblasts, monocytes, and connective tissue stromal cells and 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 were 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(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(recognizes a family of proteins known as the leukocyte common antigen exclusively expressed on the surface of almost all haemato-lymphoid 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, osteoblasts and chondrocytes (*Dennis et al., 2002*) which reinforce the functional role of MSCs. In addition, the expression of Stro-1 distinguishes two cultured population of MSCs with different homing and HSC-supportive capacities (*Bensidhoum et al., 2004*). 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's 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, 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*) (*Fig.9*) and showing also a high degree of plasticity (*D'Ippolito et al., 2004, Zhao et al., 2002*).

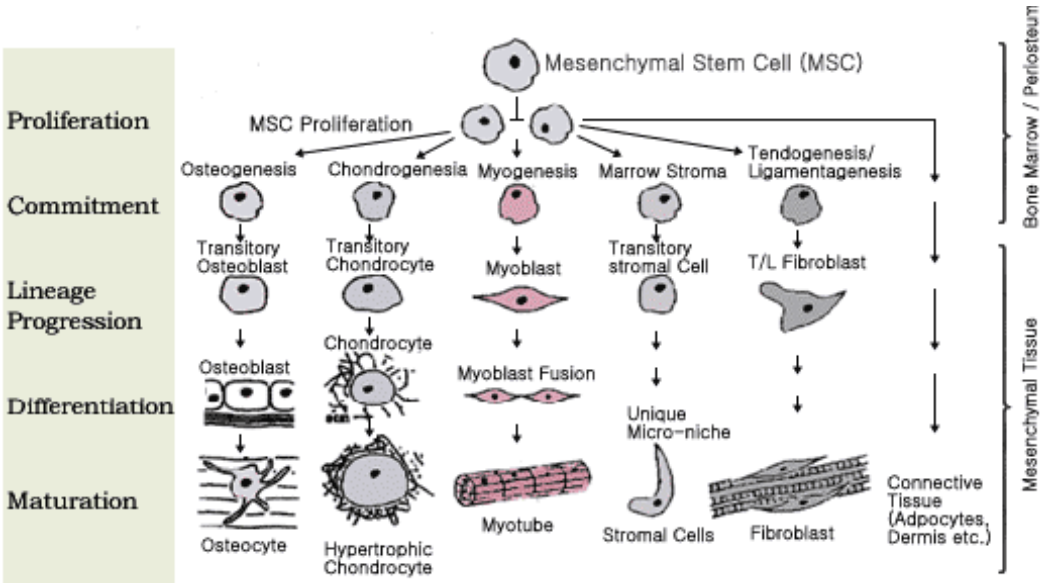


Fig.9: The mesengenic process of MSCs

Furthermore, MSCs can commit to a particular differentiation pathway by their self-renewal and proliferation abilities and the micro-environmental in which they are. The lineage-committed cell progresses through several stages of maturation process 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*).

Individual colonies derived from single MSC precursor have also been reported to be heterogeneous for their multilineage differentiation potential (*Friedenstein et al., 1970*). 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 tri-lineage(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 are a pool of cells that include MSCs and different subpopulations at different state of differentiation. During in vitro culture, were isolated all or a subset of these MSCs. 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*) (*Fig.10*).

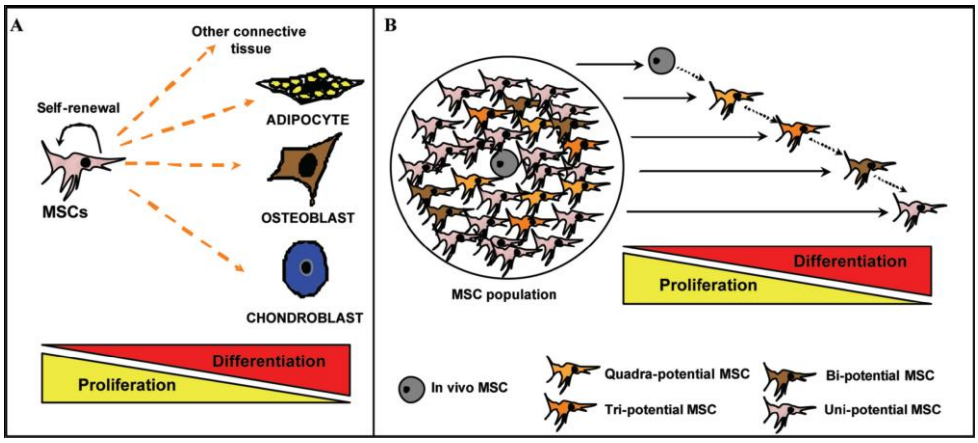


Fig.10: Models of MSCs differentiation; A) capacity to differentiate into all connective tissue cell types; B) MSCs population that with different differentiation potentials (*Baksh et al., 2004*)

For 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 (*Fig.11*). 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 (*Zimmet et al., 2005*) 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*).

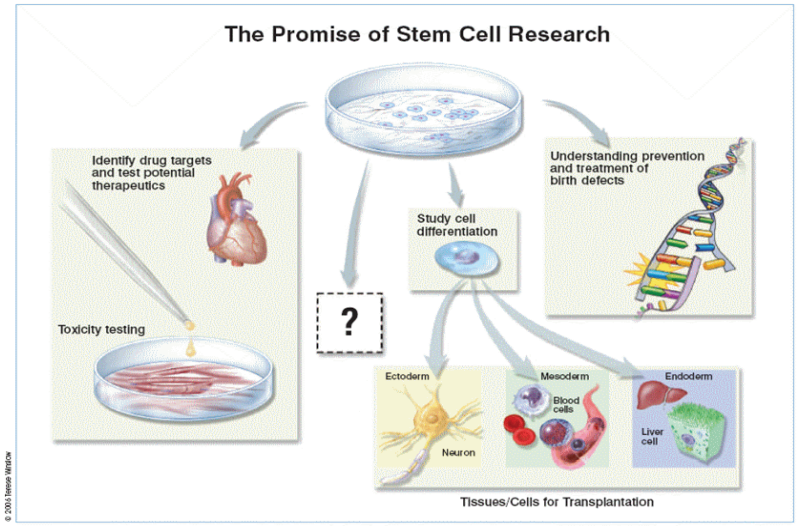


Fig.11: Potential uses of Adult Stem Cells in regenerative medicine, cells therapy and drug screening.

3. STEM CELLS NICHE

It is well know that self renewal and pluripotentiallity 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. Microenvironment are constituted by adult tissue-specific stem cell but also other kind of cells characterized by the present of adhesive molecules and extracellular matrix proteins, that have an important role in the space localization and organization, in fact it has also regulating functions to determinate stem cell differentiation into various kind of mature cells, replacing cells lost due to natural cell death (apoptosis) or injury, safeguards against excessive stem cell production that could lead to cancer and to preserve a sufficient stock of stem cell for the future, infact, stem cells must periodically activate to produce progenitor or transit amplifying (TA) cells that are committed to produce mature cell lineages (*Fig.12*).

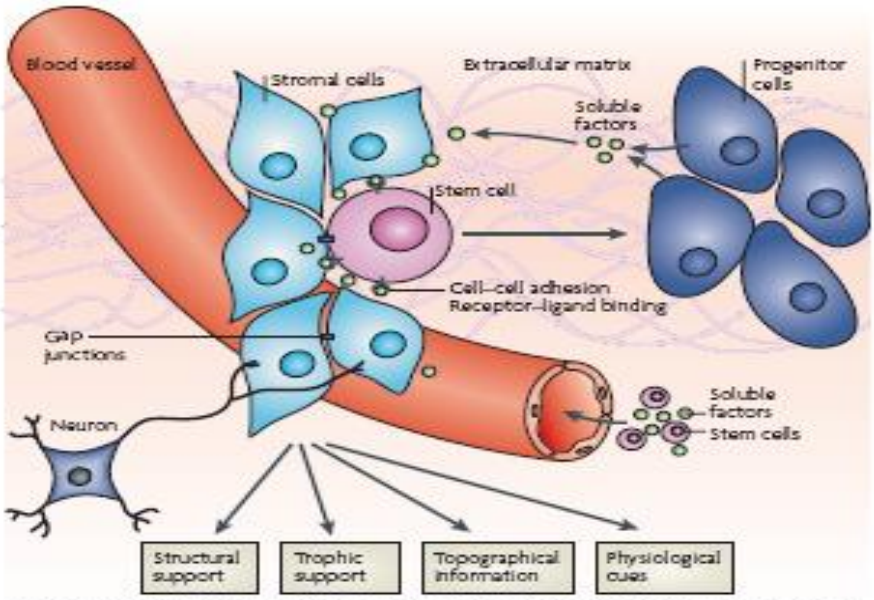


Fig.12: Stem cells niches

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 suggest the presence of a "stem cell niche". The idea of specialistic areas in the microenvironment seems to be supported by the production of grown factor in outline compartment (*Gordon MY 2008*). Stomal cells releases locally grown factors that bind extracellular matrix structures, in this way target cells recognized them by specific receptor (*Gordon MY 2008*). This mechanism permit to localize high concentration of specific grown 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. A recent hypothesis is that the regulating marrow stimulus is placed in a 3D organization to establish the "stem niches". Niche can be defined as a spatial microstructure in which houses adult stem cells, which start self renewal activity interacting with external stimuli. Niche characteristics are here resumed: the number of stem cells in a niche is well-regulated; stem status depends on the interaction with other histologically different cells; stimuli produced by niches supply the molecular base for the interaction between cells, and allow the transduction of activator and inhibitor signals, presiding over the expansion of stem compartment and into a niche also a not stem cell can reach pseudo-stem characteristics.

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.

This hypothesis was supported by several researchers that during several studies identified different niches in different anatomic district.

Human stem cells niches known fill today are*:*

3.1 HEMATOPOIETIC STEM CELLS NICHE

The bone marrow contains multiple stem cell types, including HSCs and mesenchymal MSCs which have the ability to self-renew and to differentiate into cartilage, bone and adipose tissues at the single cell level (*Pittenger et al., 1999*). HSCs are the best characterized adult stem cell population. Single HSCs are multipotent, highly self-renewing, and cycle with slow kinetics. In adult bone marrow, a part of these HSCs are known to reside in two different niches, an "endosteal" niche and a "perivascular" niche. The endosteal niche it seems to maintain HSC in a long term quiescence whereas perivascular niche it seems to maintain the quiescence of HSCs for a shorter period, supporting HSC's proliferation, favoring myeloid and megakaryocytic lineage differentiation and mediating HSC's circulation (*Perry et al., 2007*). In the endosteal niche, HSCs are associated with a subset of osteoblasts that line the inner surface of trabecular bone cavities and giving arise to progenitors that migrate to blood vessels in the middle of bone marrow cavity where they mature and differentiate (*Nilsson et al., 2001, Gong 1978, Heissig et al., 2002*) (*Fig.13a*). Studies have shown that the position of osteoblast cells is a for the begin of the process as previously described (*Zhang et al., 2003*).The role of osteoblasts a support for HSC growth was suggested through in vitro coculture experiments (*Taichman et al. 1994, 2000*), and simultaneously using genetic mutant mouse models (*Zhang et al., 2003, Calvi et al., 2003, Arai et al., 2004*) was identified osteoblastic cells as a key component of the HSC niche. Endosteal osteoblasts are thought to provide a several factors that regulate HSC number and function (*Zhang et al., 2003, Calvi et al., 2003).* A possible mechanism by which osteoblasts would regulate the number of HSCs is through secretion of osteopontin that is a bone matrix glycoprotein that seems to maintain HSC in quiescence form and to

regulate HSC proliferation in a negative way. An increasing number of molecular studies point to the existence of a complex paracrine signaling network at the interface between the niche osteoblast and the adjacent HSCs; Kit ligand is expressed by osteoblasts and is able to activate Kit on HSCs surface. Notch signaling plays an important role in cell fate to maintain HSCs in an undifferentiated state (*Calvi et al., 2003, Duncan et al., 2005, Varnum-Finney et al., 2000*). Another important interaction is between the ligand angiopoietin-1 a the osteoblast surface and the receptor Tie-2 (Tyrosine kinase with immunoglobulin-like and EGF-like domains 2) expressed on HSCs, which has been shown to modulate HSC quiescence (*Arai et al., 2004) (Fig.13b).*

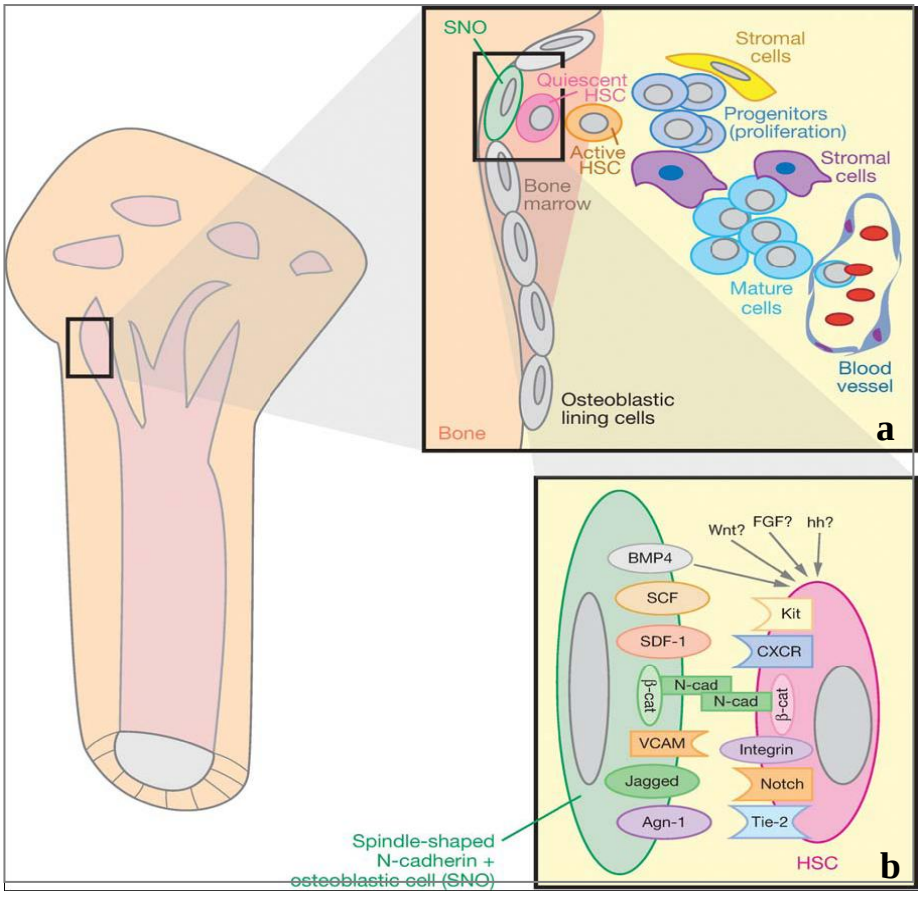


Fig.13: The Hematopoietic Stem Cells (HSCs) niche in the bone marrow. a) Schematic diagram of HSCs and niche cellular components in the bone marrow; b) Extrinsic signaling pathways that regulate proliferation and differentiation of HSCs (*Li et al., 2005)*

3.2 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 (*Fig.14a*).

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*). The multipotentiality of single HFSCs has been shown by using cells expanded in vitro. An in situ tracking method has shown that progenitors in the hair follicle contribute to single lineages and possess limited self-renewal potential, suggesting that it may be possible to measure various lineage potentials rigorously when and how they segregate after HFSCs activation (*Legue et al., 2005*).

The molecular analysis epithelial stem cells has revealed the following features: 1) the expression of adhesion molecules known to be involved in stem cell-niche interaction, 2) the presence of growth inhibition factors such as TGFβ/BMP molecules and cell cycle inhibitors, and 3) Wnt pathway including receptors and inhibitors such as Dkk, sFRP, and WIF is important in the hair follicle niche. Taken together, these molecular features indicate that the epithelial stem cell niche is a growth- and differentiation-restricted environment (*Tumbar et al. 2004*) (*Fig.14b*).

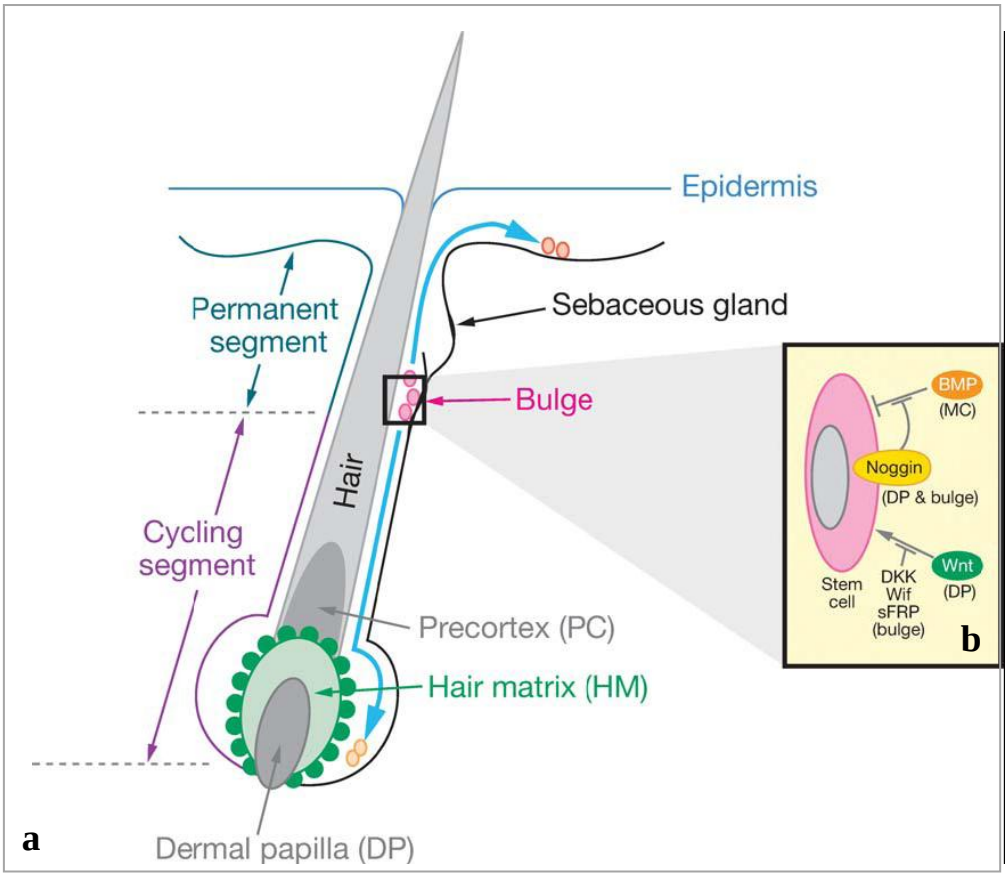


Fig.14: The Epidermal Stem Cells (ESCs) niche. a) Schematic diagram of the major types and spatial orientations of HSCs that make up the hair follicle; b) Interactive signaling pathways that mediate HFSC proliferation (*Li et al., 2005)*

3.3 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, et al., 1995, Winton et al., 2000*) (*Fig.15a*).

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 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 in the promotion of ISC activation/self-renewal; in contrast, BMP signaling restricts ISC activation/self-renewal and crypt cell fate (*Haramis et al. 2004, He et al. 2004*) (*Fig.15b*).

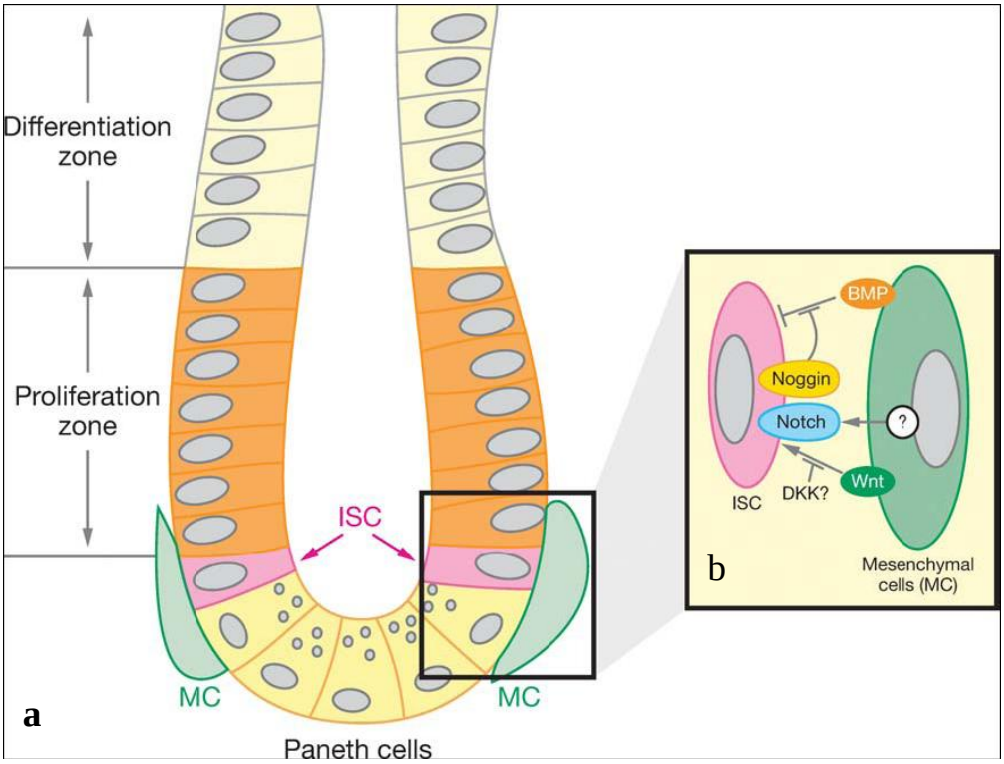


Fig.15: The Intestinal Stem Cells (ISCs) niche. A) Schematic diagram of the major types and spatial orientations of cells found within the crypt niche and the villus. B) Interactive signaling pathways that mediate ISC proliferation (*Li et al., 200)5*

3.4 NEURAL STEM CELLS NICHE

In adult life, neurogenesis is possible in specific brain area where were identified and characterized neuronal niches.

Neural stem cells (NSC) were identified in 1990 and then isolated from various regions in the adult brain and peripheral nervous system (*Alvarez-Buylla et al., 1990*).The *subventricular zone (SVZ)* and the *subgranular*

zone (SGZ) of the hippocampus region are the primary and well-characterized germinal regions in which NSCs reside and support neurogenesis in the adult brain (*Doetsch et al. 1999, 2003, Lois et al. 1993*)

SUBVENTRICULAR ZONE (SVZ) is a single layer of multi-ciliated ependymal cells separates the SVZ from the lateral ventricle (LV). There are four main cell types in the SVZ: neuroblasts (Type A cells), SVZ astrocytes (Type B cells), immature precursors (Type C cells) and ependymal cells (*Doetsch et al., 1997*). In this region, the SVZ astrocytes, located adjacent to the ependymal cells, have stem cell features and give rise to TA precursor C cells. Infact, immature cells, deriving from SVZ astrocytes, are precursors of a group of neuroblasts which differentiate into neurons and migrate toward the olfactory bulb and other regions. SVZ astrocytes can also generate oligodendrocytes (*Doetsch 2003*, *Mirescu et al., 2003, Temple 2001*). Another noteworthy feature of this region is a specialized basal lamina, which extends from blood vessels in SVZ region and terminates in small bulbs adjacent to ependymal cells, and contacts all SVZ cell types. In SVZ are also present blood vessels and endothelial cells that lining the blood vessels and these are likely a source of signals for adult neurogenesis (*Fig.16a*).

SUBGRANULAR ZONE (SGZ) is the germinal layer between the dentate gyrus and the hilus in the hippocampus and it is responsible for the generation of dentate gyrus granule neurons (*Palmer et al. 1997*). In the SGV region, neurogenesis occurs in foci closely associated with bloods vessels (*Palmer et al. 2000)*. As in the SVZ, SGZ astrocytes acts as a stem cell; are the primary precursors of neurons and generate daughter cells that further produce granule neurons (*Fig.16b*).

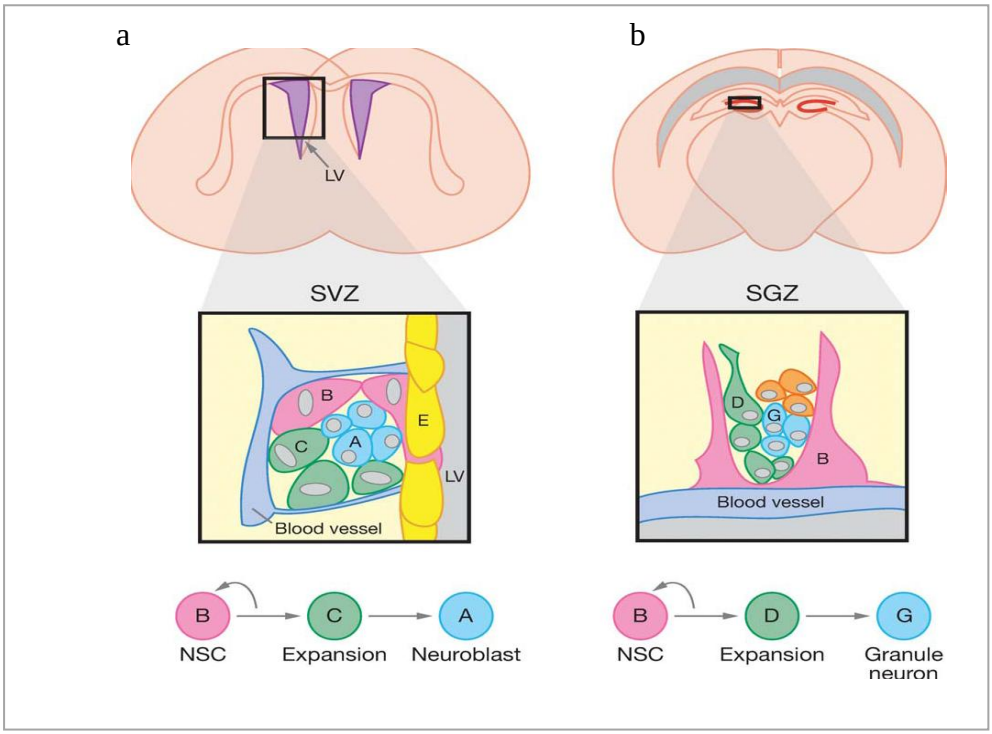


Fig.16: The neural stem cell (NSC) niche. a) The subventricular zone (SVZ). Astrocytes (B) lining the ependymal cells (E) function as NSCs; they give rise to transient amplifying cells (C) (green), which further produce neuroblast cells (A); b) The subgranular zone (SGZ). Astrocytes (B) directly attach to the blood vessel and receive signals from the endothelial cells that direct NSCs to undergo self renewal, proliferation (D), and differentiation (G) (*Li et al., 2005*)

In both regions, endothelial cells and the specialized basal lamina are essentials components of the NSC niche. The ECs provide attachment for SVZ and SGZ astrocytes and generate a variety of signals controlling stem cell self-renewal and lineage commitment (*Doetsch 2003*, Shen *et al. 2004*). In fact, angiogenesis and neurogenesis may be co-regulated and reciprocally signaled. Both are stimulated by the same factors, including bFGF, VEGF, insulin-like growth factor (IGF-1) and TGF-a; ECs secrete well-known mitogens, differentiation and survival neuronal factors like bFGF, IGF-1, VEGF, PDGF, IL8 and brain derived neurotrophic factor (BDNF) (*Palmer et al. 2000, Jin et al., 2002*, *Louissaint et al., 2000*).

3.5 VASCULAR NICHE

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. This zone of vascular wall serves a niche containing the vascular wall-endothelial progenitor cells (VW-EPC) resident, which are capable of forming capillary sprout in arterial ring assay in vitro, outside the bone marrow, even if Ingram's group reported the existence of VW-EPCs but did not furnished the exact location within the vascular wall (*Ingram et al., 2005*) but also MSCs and probably also vascular wall hematopoietic progenitor cells (HPCs) (*Fig.17*).

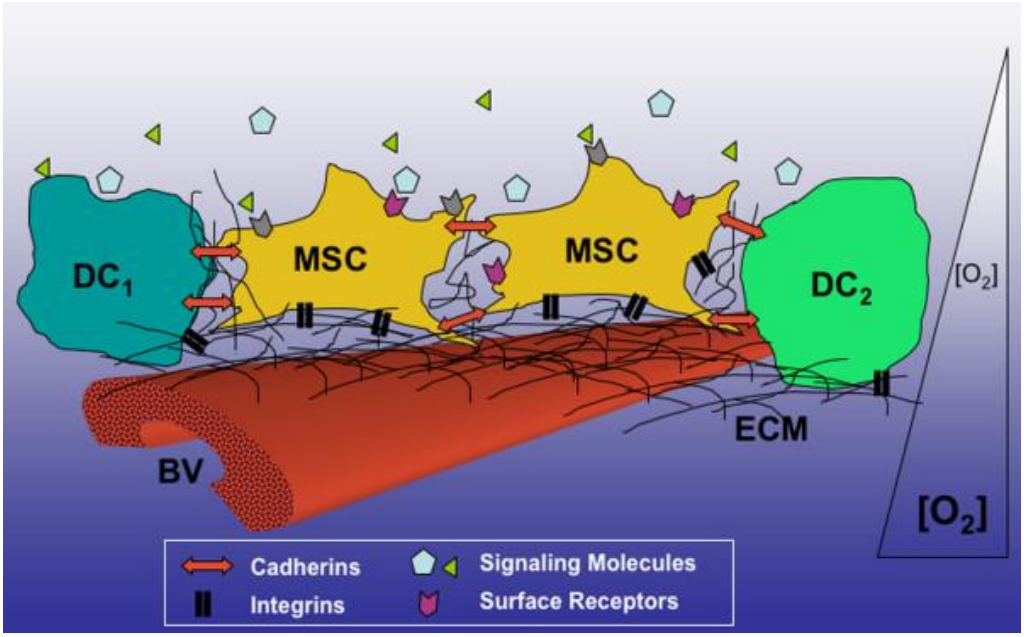


Fig.17: Vascular niche

Moreover, this area could correspond to a vascular wall stem cell niche identified as special microenvironment in a strategic location at the interface between the media and adventitial layers, physiologically limited and specialized, an unexpectedly elevated cell proliferation under normal conditions, selective localization of cells expressing the stem cell surface molecules c-kit, in which stem cells and multipotent stromal cells stay; both cytotypes could contribute in maintaining post-natal vascular homeostasis replacing old or damaged elements/items (*Pacilli et al., 2009*).

4. VASCULAR WALL RESIDENT STEM CELLS

In growing body several evidence suggests a close relation between hematopoiesis and vasculogenesis in vertebrates. During early embryogenesis, hematopoietic and endothelial lineages derive from aggregates of mesodermal cells that subsequently mature and form blood islands in the extra-embryonic yolk sac. These blood island consists of an inner core of blood cells and an external layer of endothelial cells (ECs)(Sabin 1920, Murray 1932) and the simultaneous presence of these 2 kind of cells has led to the hypothesis that they originate from a common precursor called hemangioblast (Murray 1932).The formation of the blood islands in the yolk sac marks the begin of the vascularization in the developing embryo. Two different processes contribute to the formation of the vascular system. The first process, vasculogenesis, requires the differentiation of endothelial cells from hemangioblast and their subsequent organization into a primary capillary plexus (Risau et al., 1988, 1995) and it is restricted to early embryogenesis. The second process, angiogenesis, results in the formation of new vessels by sprouting from preexisting blood vessels (Folkman 1992, 1995) and occurs both during development and postnatal life. However, studies have showed that postnatal angiogenesis may occur by recruitment of bone marrow and peripheral blood and of endothelial progenitors cells (EPC), with property of embryonal angioblst, involved in the new blood vessel formation in response to various stimuli (Asahara et al., 1999a). Once mobilized from bone marrow and released into the circulating blood (CEPs), these progenitors are supposed to participate in physiological and pathological arterial wall remodeling during their lifetime (Carmeliet 2003). In fact, experimental evidences support the use of EPCs in angiogenic therapies or as biomarkers to assess cardiovascular disease risk (Rafii et al., 2003, Vasa et al., 2001, Kalka et

al., 2000, Kawamoto et al., 2001, Tepper et al., 2002). Although, EPC residing in the site of ischemia contribute, as has been demonstrated, to the formation of new vessels (Hristov et al., 2003). Recently, another group of vascular progenitor cells has been found abundant vascular in the adventitia of ApoE-deficient mice, in particular in the aortic root; these progenitors containing stem cell markers e.g. Stem cell antigen-1 (Sca-1+), c-kit+ CD34+ and fetal liver kinase-1 (flk-1+) but not SSEA-1+, contributed to experimental atherosclerosis and did not originate from the bone marrow (Hu et al., 2004). Moreover, the pool of mature human endothelial cells seams to contain a subpopulation of EPCs allegedly organized in a completely hierarchical manner with different clonogenic and proliferative potentials. Although various studies have identified other peripheral sources of EPCs including skeletal muscle (Majka et al., 2003), spleen (Wassmann et al., 2006), liver (Aicher et al., 2007), fat (Planat-Benard et al., 2004) and adventitial of the arterial wall, the best characterized source remains the bone marrow.

Most of the knowledge about resident vascular progenitors comes from animal models. Tintut et al. first demonstrated that MSCs were also present in the adult animal vessels. They described and isolated a subpopulation of vascular cells with lineage plasticity and self-renewal capacity also known as Calcifying Vascular Cells (CVCs). This CVCs expressed surface markers commonly expressed by BM-MSCs like CD44 and CD29 while were absent hematopoietic antigens such as CD14 and CD45; these cells showed not only osteoblastic potential but also chondrogenic, leiomyogenic and stromogenic lineages and limited adipogenic potential under specific experimental conditions (Tintut et al., 2003). In 2006, it has been demonstrated the presence of resident precursors of endothelial and smooth muscle cells in healthy arteries of adult mices defined “side population” (SP) using flow cytometry method. The researchers selected the

progenitors in the medial tunica layer of thoracic and abdominal aortas, with phenotypic and functional progenitor cells properties, and then differentiated them with VEGF and TGF-beta1. This vascular plasticity suggests that the SP could allow a physiological renewal and could provide to a regeneration after injury by the rapid growth of new ESc and smooth muscle cells (SMCs) participating also in the homeostasis and remodeling processes (Sainz et al., 2006). Even though more recent studies provided informations about their presence and role in the human vascular wall.

Resident vascular progenitor cells were also found in the human fetal aorta as demonstrated by Invernici's study. This immature vascular progenitors cells residing in the human fetal aorta are able to give rise to endothelial and mural cells in response to vascular endothelial growth factor (VEGF) and plateled derived growth factor beta (PDGF-BB) and are also able to improve the neovascolarization in a murine models of peripheral ischemia (Inverinci et al., 2007). The absence of a CD45 marker suggests that human vascular progenitor cells (hVPCs) may not have a hematopoietic origin but may derive from immature mesenchymal stem cells residing in niches located in the fetal paraortic membrane or in the peripheral parenchym aorta wall (Alessandri et al., 2001).

A great contribution to assess the presence of EPCs in the adult vascular wall was provided by Zengin and his studies. His research group isolated vascular progenitors cells from the human internal thoracic artery wall (HITA), suggesting the existence of a "vasculogenic zone" as previously described, in the wall of large and mid-sized blood vessels, where might be found not only the VW-EPC but also a genuine hierarchy of resident stem cells including MSCs and probably resident vascular wall hematopoietic progenitor cells (HPCs) able to differentiate into ECs, local immune cells such as macrophage and HSCs as well as precursors of SMC, fibroblast and pericytes (Zengin et al., 2006). According with the hypothesis, several

experimental observations had shown that the same area in postnatal artery walls may contain MSCs with a multilineage differentiation. More recently, other groups found multipotent vascular resident MSCs in human vascular adventitia, pulmonary artery (Hoshino et al., 2008) and in the saphena vein (Covas et al., 2005). Our group described the presence of vascular wall resident mesenchymal stem cells (VW-MSCs) in normal human adult thoracic and femoral artery aortas from healthy and heart beating donors (Pasquinelli et al., 2007,2010). Cryopreserved thoracic aortas were harvested from heart-beating multi-organ donors and was evaluated the resistance of vascular wall cells (VWCs) to cryoinjury (Pasquinelli et al., 2006, Pacilli et al., 2009); the majority of VWCs, i.e. EC and SMCs were irreversibly injured by cryopreservation procedures. Interestingly, the few stress-resistant cells in the cryopreserved arterial wall which survived after 4 days of organ culture were localized near to the vasa vasorum, vascular structure CD34+, at the border between media and adventitia layers according to the vasculogenic zone previously described by Zengin; this cells were able to induce angiogenesis in ex vivo culture experiments. Furthermore, in the vasculogenic area, previously described by Zengin in HITA, was identified a double population CD34+ and c-kit+ with putative stem cells characteristics; immunohistochemical tissue analysis confirmed the presence of an analogous well-developed plexus of CD34+ small vessels in the aorta and in the femoral artery as well. Single immunostaining on serial sections supported the presence of CD45+ inflammatory cells that correspond to the resident CD34+/CD31- endothelial progenitors reported in HITA. Beside its assumed vasculogenic post-natal ability, this area could act as strategic for the recruitment of blood circulating cells. Again, additional experiments have also revealed the presence of CD68+ and S-100+ perivascular cells that represent resident macrophages and dendritic cells. In similar samples, we isolated a

cell population with analogous properties with bone marrow derived-MSCs, demonstrating a strong angiogenic ability (Pasquinelli et al., 2007) and adipogenic, osteogenic, chondrogenic and leyomiogenic potentials. These VW-MSCs could be actively involved in the remodeling of the wall in both normal and development of ectopic tissue like cartilage, marrow and fat tissue in pathological conditions like vascular disease and atherosclerosis.

5. HOMOGRAFT

The use of vascular prosthesis for the hemodynamic restore and recover and anatomic re-vascularization in surgery techniques for ateropathy is a serious problem that lead to a great scientific interest. The first experiment to rebuild a damaged artery was in 1774 (*Callow 1982*), in this year a medicine student from Paris, LeConte, used as a vascular prosthesis a duck feather segment; this approach was a failure because of the insurgence of infection loci, from that moment vascular research start to develop different kind of prosthesis. In the last XIX century, Masini in 1895 and Nitze in 1897 had the idea to invaginate glass or avory cylinder in vessels that need to be repaired (*Callow 1982*). At the beginning of the XX century the use of homolog and natural prosthesis put the bases for the modern concepts of vascular surgery. The homograft is a homologous prosthesis that offer the possibility to substitute arterial segments with transplanted homologous venous segments. The term homograft in literature was first mentioned by Carrel (*Carrel 1912*), who positioned homologous and xenologous prosthesis in dogs; later Goyanes used the poplitea vein to reconstruct the poplitea artery during a anurysmectomy (*Goyanes 1906*); the very first clinic procedure with homologous artery for the aortic aneurism was tanks to Gross (*Gross et al., 1948*), Oudot (*Oudot 1953*) and Dubost (*Dubost et al., 1951*). Since then the use of homograft has been considered a possible surgical treatment for stenotic and aneurysmal diseases of the aortic-iliac-femoral vasculature (*Odero et al., 2001*). Unlikely those homografts lead to a structural degeneration provided with dilatations, rupture or thrombosis, becoming soon aneurysmal. Besides the low availability of autologous vein for their caliber, varicose and inflammation or previous sampling, lead the research to the development of new strategy to create synthetic vascular prosthesis and other therapeutic procedures less invasive. The introduction

in the ‘50s of synthetic prosthesis and their immediately encouraging short and long term results, clearly better than homografts one, bring to a temporary abandon of the last one. The introduction, in normal clinic techniques, of efficient blood anti-coagulative methods, of an adequate antibacterial therapy and the development of surgical techniques able for atherosclerosys treatment, after few years, in 1991, this technique was suggested again with completely different indications from those for which they had originally been proposed. France vascular surgeons of Pitié-Salpètriére of Paris proposed the vascular homograft in patients who had infected prosthesis, representing the 2-3% of cases who had vascular prosthesic surgery mainly in aortic district (*D'Addato et al., 1997*), because the natural resistance to bacterial colonization makes the homograft an ideal candidate for the treatment of this infection. In fact the surgical treatment of this pathology, for its not simple solution, it can’t be treat by reposition of a new prosthesis, because as a synthetic material (Vinyon N, Nylon, Teflon, Orlon e Dacron) could be a fertile ground for the regeneration of the infection and for the targeted antibiotic treatment, even in loco, can give some results in low microbic infections. Arterial homograft represents the surgical treatment of choice for this complication, because the use of homolog arteries guarantees the ill from the infection being able to permit an anatomic re-vascularization. Samples, from multi organ donors, were readily implanted or stocked at 4° in RPMI buffer (culture cells buffer) for not more than 30 days (*Bahnini et al., 1991, Kieffer et al., 1993, Koskas et al., 1996*) but the need of arterial segments used in normal clinic practice increased. So in 1990 were found in Europe the Cardiovascular Tissues Banks, structures responsible for the process of preservation, certification and distribution of tissues, offering the possibility to have homografts of various diameter or length in case autolog material will be not available or usable. Recently AOCP and abdominal

aortic aneurysm have been inserted in the pathologies treatable with this procedure. Often the homgrafts are unsatisfactory and the major inconvenient seems to be the immune reaction (caused by the resistance of cells to the cryopreservation process). Even in the existent literature, a very low interest has been given to the immune response of the receiver treated with vascular homograft caused by prosthesis infection, this because vessels are considered low antigenicity tissues. Moreover a lot of author, in clinical practice, don't respect\contemplate any compatibility between donor and receiver, neither for blood group (ABO compatibility) or eventual antibodies preexistent in the receiver serum (cross-match). It was documented that vascular wall cells, like endothelial ones and other like smooth muscle cells and fibroblast express both antigens of blood group (A, B, 0 groups) and major histocompatibility antigens, known as HLA (Human Leukocyte Antigens) able to active the immunological response rejection (*Pober et al. 1986*). In reality, some clinical studies reported late complications of the homografts referred to episodes of chronic reject (*Ruotolo et al., 1997*). In particular the same French school documented, in the 25% of treated cases, episodes of chronic reject like miointimal hyperplasia, necrosis of muscular tunic and adventitial inflammatory infiltration. In fact the first consequences of receiver's immunological reaction are preceded by a rapid disappearance of immunological endothelial cells, first target of the direct attack (cytotoxic). Later disappear gradually smooth muscle cells, attacked mainly by an antibody mechanism, that cause the thinner of the medial tunica. The last phase of the reject process is characterized by a miointimal proliferation, composed by the cells of the receivers (*Fig.18b*). Functionally, in large caliber vessels the necrosis of the media, its thinner and the substitution with fibrotic tissue that predispose the insurgence of the aneurysmal dilation. In the contrary, in small caliber vessels, the miointimal hyperplasia is responsible of a

decrease of the lumen. To conclude, arterial homograft are immunogenic and induce in the receiver a specific cellular and humoral immunological reaction responsible of reject episodes. For this reason, it is necessary to respect the compatibility of at least blood groups and perform the transplant without the present of preformed anti HLA antibodies in receiver serum (negative cross-match).

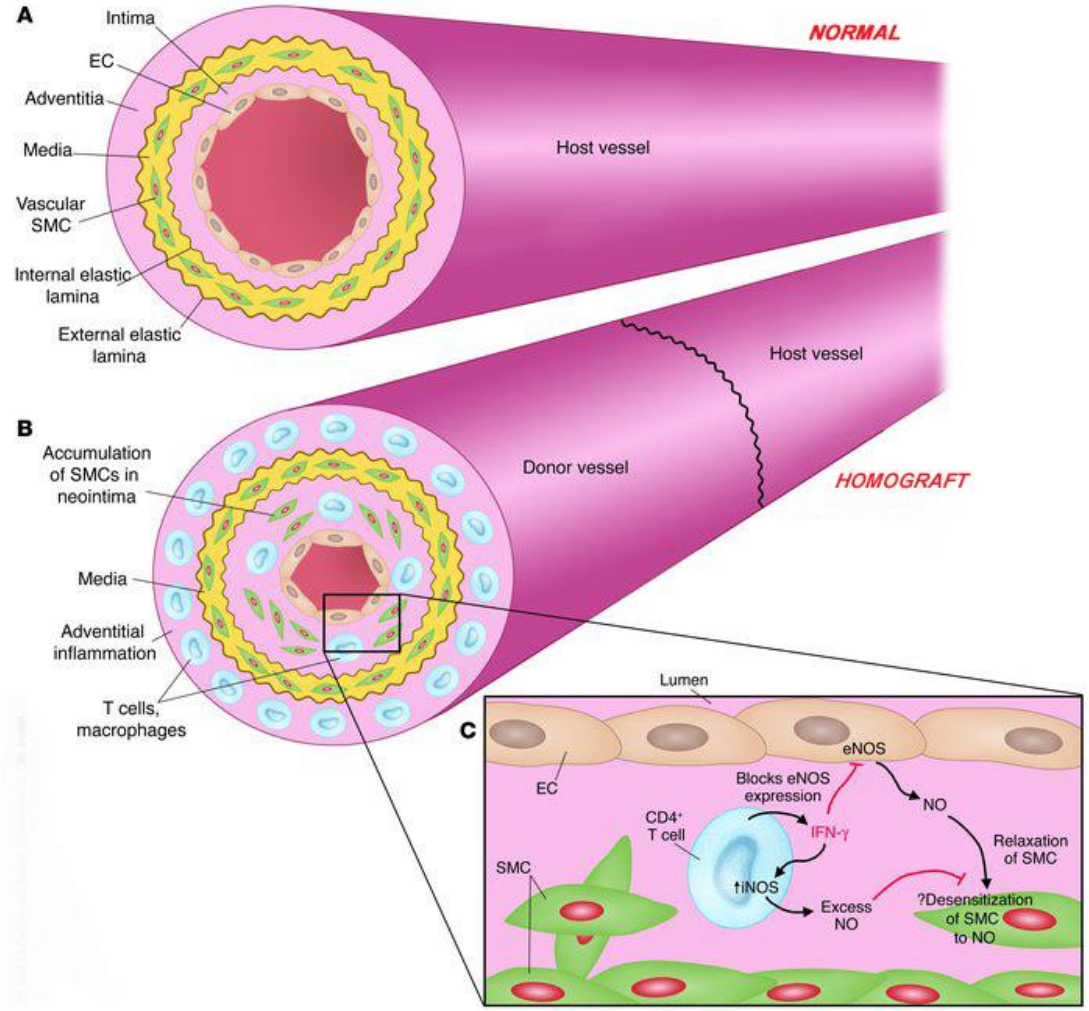


Fig.18: Schematic representation of vascular wall remodeling. a) Normal artery wall. b) Proliferation myointimal into vascular wall homograft. c) Signals pattern of wall remodeling.

EXPERIMENTAL DESIGN

6. AIM OF THE WORK

Stem cell research is one of the most fascinating field of contemporary biology, in particular, Adult Stem Cells that thank to their stem characteristic offer a possible therapeutic application in the regenerative medicine and tissue engineering. Adult Stem Cells reside in a "niche", a special microenvironment, present in different tissues including blood vessels and provide to maintain the homeostasis of the tissue.

Many studies demonstrated that the artery wall is a recipient and a source of Mesenchymal Stem Cells (MSCs) that can differentiate along multiple lineages, and can probably useful as a source of cells for postnatal vasculogenesis. In adult human wall, of large and mid-sized blood vessels, was identified a "vasculogenic zone" between the smooth muscle and the adventitial layers, containing a complete hierarchy of resident stem cells. In this hypothetical "vascular niche" could be reside MSCs capable of differentiating into ECs, local immune cells such as macrophage and HSCs as well as precursors of SMC, fibroblast and pericytes (*Zengin et al., 2006) (Fig.19).*

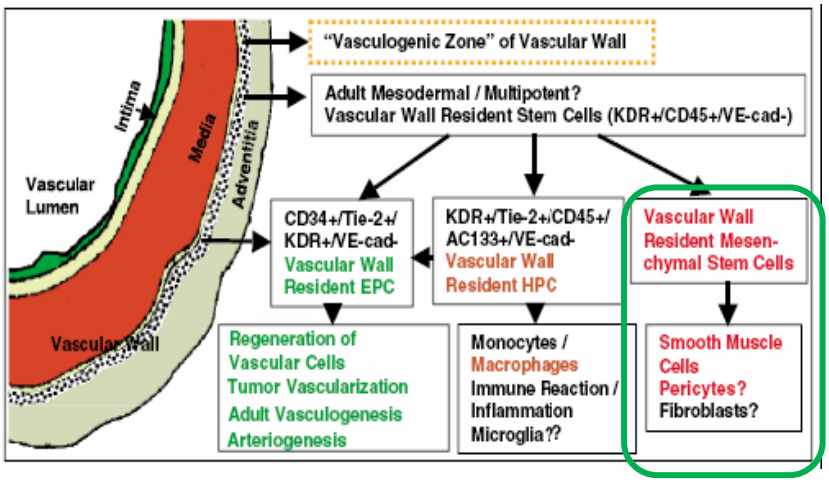


Fig.19: The " Vasculogenic zone" in the arterial wall (*Zengin et al., 2006*).

According with the hypothesis, several experimental observations shown that the same area in postnatal artery wall may contain MSCs with

multilineage differentiation. Recently, our group identified, in normal human adult thoracic aortas and femoral arteries from young and healthy heart-beating multi-organ donors, the vasculogenic zone previously described by Zengin using immunoistochemical staining (*Fig.20*); we also isolated and described the presence of resident MSCs (VW-MSCs) with angiogenic ability (*Pasquinelli et al., 2007*) and multilineage potential (*Pasquinelli et al. 2010*) that present features similar to the mesenchymal stromal cells in bone marrow.

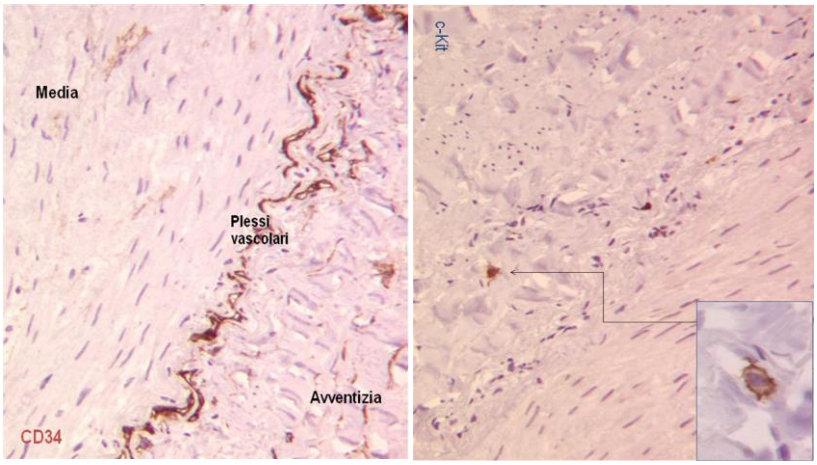


Fig.20: "Vasculogenic zone" at the border between adventitia e media layers. a) CD34 positive cells; b) C-kit positive cells (Pasquinelli et al., 2007 a).

The aim of this work was to identify and localize in situ, with immunoistochemical analysis, the adult stem cell populations that could be reside in the vasculogenic zone and to understand their role in the vascular wall remodeling in failed homograft. Subsequently, we isolated this cell population and tested *in vitro* their multilineage differentiation potential through immunohistochemical, immunofluorescence, RT-PCR and ultrastructural analysis. The study of VW-MSCs could elucidate the pathological mechanisms of vascular aneurysms and may be useful for the development of new therapeutic strategies. For all those experiments, we used pathological vascular wall homografts.

7. MATERIALS AND METHODS

7.1 ORGAN CULTURE

Organ culture of the vessel wall is a useful in vitro method to study vascular cell biology preserving the in vivo anatomic relationships; the complex-three-dimensional organization in a more natural microenvironment. Besides, the organ culture offers an intermediate model in which the cells are those naturally forming the tissue, with their cell-cell and cell-extracellular matrix relationship (Wilson 1996, Jeremy1997, Porter1996), morphology and functions of differentiated cells. (Yamawaki1999 e 2000, De Mey 1989).

This experimental procedure let study whether undifferentiated cells like putative resident vascular progenitors, are able to resist to chemical and physical stresses, to participate in vascular remodeling in a 3D model without any contributes of hematopoietic cells and whether these cells are able to develop inner/outer cell linings in long-term cultures without VEGF stimuli.

7.1.1 Organ culture procedure

A 12 cm-long fresh femoral artery was collected from a heart-beating donor at Cardiovascular Tissue Regional Bank of General Hospital S. Orsola - Malpighi of Bologna, and maintained for 72 hours in an antibiotic mixture (mefoxin 240mg/ml, lincomycin 120mg/ml, colimycin 100mg/ml, vancomycin50 mg/ml); after decontamination, random samples were processed for LM and TEM. The remaining tissue was washed in a sterile physiological solution, cut in 3x3 mm slices, and placed random into individual wells of 12-well culture plates containing basal medium DMEM supplemented with 5% FBS and antibiotic (100 U/ml penicillin and 100

µg/ml streptomycin) and cultured for up 70 days. Tissues were incubated at 37°C in an atmosphere of 95% air and equilibrated with 5% $C0_2$; culture medium was exchanged with fresh medium every 3 days. At intervals of seven days, samples were recovered in triplicates and cut into three additional segments for histological, immunohistochemical and ultrastructural analysis.

7.1.2 Histological analysis

At the end of the culture period, each femoral artery segment was removed from the culture dish and a ring of each organ culture segment of femoral artery was processed for histological analysis. Thereafter, sample was fixed by immersion in 10% formaldehyde solution, embedded in paraffin to obtain 3µm-thick vessel sections to use for histological staining. In the fields of histology, pathology, and cell biology, fixation is a chemical process by which biological tissues are preserved from decay, either through autolysis or putrefaction. The purpose of fixation is to preserve a biological sample as close to its natural state as possible; acts to disable intrinsic biomolecules, such as proteolytic enzymes, which would otherwise digest or damage the sample; protect the sample from extrinsic damage such as bacteria which might exist in a tissue sample or which might otherwise colonize the fixed tissue and preserve the morphology of the sample as it is processed for further analysis. Subsequently, sample fixed was washed and dehydrated by passing the tissue through a series of increasing alcohol concentrations to remove all traces of water. The block of tissue was transferred sequentially to 70%, 96%, and 100% alcohols for 3x5 minutes each. After dehydration, the tissue can be embedded in paraffin but first clear the tissue with xylol that is an intermediate fluid that is miscible with ethanol and paraffin. 3µm-thick homograft of artery wall

sections was obtained with microtome. For conventional histopathological analysis the sections were stained with hematoxylin & eosin (H&E) according to the histological procedures and observed with light micsroscope (LM). The staining method involves application of the basic dye hematoxylin, which colors basophilic structures with blue-purple hue, and alcohol-based acidic eosin Y, which colors eosinophilic structures bright pink. 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 magnificatio10X-20X analyzed using the Image-Pro Plus® 6 software (Media Cybernetics, Inc., Bethesda, MD, U*SA).*

7.1.3 Vascular wall remodeling: immunohistochemical analysis

At the end of the culture period, each femoral artery segment was removed from the culture dish, fixed in 10% buffered formalin and embedded in paraffin. Four-µm-thick sections were used for immunohistochemical studies to localize vascular wall-MSCs in situ involved intovascular wall remodeling using antibodies (Abs) directed against molecules commonly expressed by MSCs.The slides were processed according to the non-biotin-amplified method with kit reagents (NovoLink™ 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°C, 1atm for 21 minutes. After cooling and washing, the endogenous peroxidase activity of the tissue was quenched for 10 minutes at 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 Novocastra™ 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% BSA in PBS over night (o.n.) at 4°C. Vascular tissue sections were stained using moAbs against CD34 (1:80, clone QBEND-10, Dako Cytomation); CD31 (1:50, clone JC70A, Dako Cytomation); α-SMA (1:9000, clone 1A4, Sigma); Desmin (1:600, clone D33, Dako Cytomation); CD44 (1:100, clone G44-26, BD Pharmigen); CD90 (1:100, BD Pharmigen); CD105 (1:50, clone 266, BD Pharmigen); Vimentin (1:260, clone V9, Dako Cytomation); STRO-1 (1:100, R&D System); Mast Cell Tryptase (1:50, Dako Cytomation) and pAbs was used to reveal c-Kit (CD117, 1:200, Dako Cytomation). After washing, the slides were further incubated for 30 minutes at rt with Novocastra™ Post Primary Block to enhance penetration of the next polymer reagent, rinsed in TBS 1X and after that incubated with NovoLink™ Polymer for 30 minutes at rt. After washing, the enzyme activity was visualized with 3,3'-diaminobenzidine (DAB), as substrate, prepared from Novocastra™ DAB Chromogen and NovoLink™ 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 control consisted of omission of primary antibody from sections.

7.1.4 Ultrastructural analysis: TEM

Femoral organ culture samples were washed with phosphate-buffered 0,15 mol/L and fixed in 2.5% buffered glutaraldehyde and processed for TEM. Vascular tissue were rinsed in phosphate buffer, post-fixed in 1% buffered Osmium Tetroxide to preserve the lipid structures and cell membranes for 1 hour at rt. They were then washed, dehydrated in a graded series of ethanol from 70% to 100% (3x10 minutes each at rt) and immersed in propylene oxide (2x15 minutes at rt) and then embedded in epoxy resin (50% Araldite CY12 + 50% Araldite HY + 1,5% Benzyldimetylammina (BDMA) as accelerator; all purchased from TAAB, England). The semi-thin sections obtained with ultramicrotome were stained with Toluidine blue. Ultrathin sections were cut with diamond knives using an ultramicrotome, transferred to specimen support grids, counterstained with uranyl acetate and lead citrate and observed in a Philips 400T trasmission electron microscope.

7.2 PATHOLOGICAL CASES: HUMAN ARTERIAL HOMOGRAFT

In this study were analyzed small segments of pathological arterial walls (aneurysmal yolk of abdominal aortas and failed homograft of femoral artery) removed and harvested\ by the Vascular Surgery team of the S.Orsola-Malpighi General Hospital of Bologna during routine surgery.
Two posterior left femoral-tibial homograft segments were explanted from healthy male patients (mean age 70 years) after 3 year from their placement due to a preexisting aneurysmal dilation partially thrombotic.

7.3 HISTOLOGICAL ANALYSIS

A ring of homograft segment of femoral artery was processed for histological analysis. Briefly, sample was fixed by immersion in 10% formaldehyde solution, washed and dehydrated by passing the tissue through a series of increasing alcohol concentrations (70%, 96%, and 100%) and embedded in paraffin to obtain 3µm-thick homograft of artery wall section. This sections cut with microtome, stained with hematoxylin & eosin (H&E) for conventional histopathological analysis according to the histological procedures and observed with light micsroscope (LM). Images were digitalized through a video camera connected with light microscope; original images were taken at magnificatio10X-20X analyzed using the Image-Pro Plus® 6.

7.4 "VASCULOGENIC ZONE": IN SITU IMMUNOHISTOCHEMICAL ANALYSIS

Four µm-thick dewaxed homograft sections were used for immunohistochemical studies; to localize vascular wall-MSCs in situ was assessed using antibodies (Abs) directed against CD90, CD44, CD105 and CD166 antigens, molecules commonly expressed by MSCs. The slides were processed according to the non-biotin-amplified method with kit reagents (NovoLink^{TM} Polymer Detection System, Novocastra Laboratories Ltd., Newcastle Upon Tyne, United Kingdom). Details of the procedures here used were similar to reported elsewhere (see paragraph 7.1.3). Vascular tissue sections were stained using moAbs against CD44 (1:100, clone G44-26, BD Pharmigen); CD90 (1:100, BD Pharmigen); CD105 (1:80, clone 266, BD Pharmigen) and CD166 (1:100, clone 3A6, BD

Pharmigen). To detect the antigen/antibody reaction, the diaminodenzidine was used as chromogen. The immunohistochemical reaction was 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 and 20X. Negative control consisted of omission of primary antibody from sections.

7.5 ISOLATION AND CELL CULTURE

Fresh homografts of femoral artery were furnished by operating room of Vascular Surgery of the S.Orsola-Malpighi General Hospital of Bologna for the vascular stem cells isolation. Each fresh homograft segment, approximately 5-cm-long, was longitudinally cut, providing an exposed surface area measuring about 25-30 cm^2 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 Dulbecco's Modified Eagle Medium (DMEM) serum free (Lonza, Basel, Switzerland) for 30 minutes at rt (*Fig.21*).

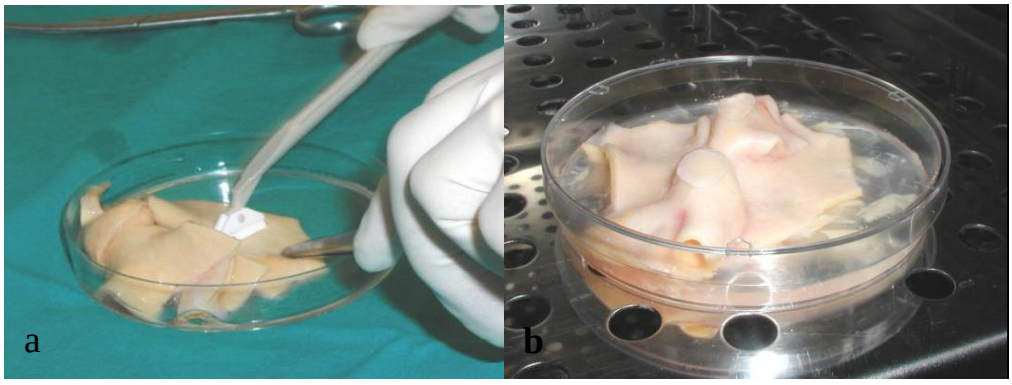


Fig.21: Isolation of stem cell in the vascular wall homograft. a) Mechanic scaper; b) Enzymatic digestion

Each minced tissue was subsequently incubated with the same concentration of Liberase type II at 37°C in 5% CO_2 humified atmosphere for other 30 min and subsequently at 37°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 $1x10^6/cm^2$ on collagen-I coated 12-well plates with DMEM supplemented with 20% heat inactivated fetal bovine serum (FBS; Lonza) and antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) and subsequently incubated at 37°C in a humified atmosphere with 5% CO_2. Nonadherent cells were removed after 72 hours by washing with PBS (Phosphate Buffer Saline). 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 cm^2 of surface area as passage 1(P_1), maintained in DMEM supplemented with 10% FBS and subcultured for up to P_{10}-P_{12} for expansion and *in vitro* analysis.

7.6 IMMUNOPHENOTYPING: FLOW CYTOMETRY ANALYSIS

The immunophenotype of each homograft-mesenchymal stem cells (H-MSCs) was investigated with flow cytometry that is able to analyze several thousand cells every second, in "real time," and can actively separate and isolate cells having specified phenotype. To detect surface antigen, cells taken at P_3-P_5 were washed twice with PBS containing 2% FBS and incubated for 20 minutes a rat using the following extensive conjugated monoclonal antibodies (moAbs) panel: anti-CD29-fluorescein isothiocyanate (FITC), anti-CD31-phycoerythrin (PE), anti-CD34-PE, anti-

CD146-PE, anti-CD44-FITC, anti-CD45-allophycocyanin (APC), anti-CD73-FITC, anti-CD90-phycoerythrin-cyanine 5 (PC5), anti-CD105-PE, anti-CD117-FITC (all from Beckman Coulter, Fullerton, CA, USA), anti-CD133-PE (Milteny Biotech, Bologna, Italy) von Willebrand Factor (vWF, clone F8/86, Dako Cytomation, Glostrup, Denmark), anti-KDR (Sigma), anti-NG2 (R&D System, Inc., MN, USA), anti-STRO-1 (clone STRO1, R&D System), anti-Notch-1 (clone C-20, Santa Cruz Biotecnology, Santa Cruz, CA, USA), anti-Oct-4 (clone C-10, Santa Cruz Biotecnology) and anti-Sca-1 (clone CT-6A/6E, Cederlane Laboratories). 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 for data acquisition (Beckman Coulter). Results were analyzed using the CXP Software (Beckman Coulter).

7.7 IMMUNOFLUORESCENCE ANALYSIS

To detect, also, intracytoplasmatic antigens, aliquots of H-MSCs were stained by moAbs using an immunofluorescence staining. Cells taken at the same passage used in the flow cytometry analysis were plated at $1x10^3/cm^2$ in collagen biocoated slide chambers (BD Bioscence) and then cultured until near confluence. The slides were fixed in 2% paraformaldehyde in PBS for 6 minutes at rt. For investigate the expression of

intracytoplasmatic antigens, cells were permeabilized with 0,5%Tryton X-100 added during fixation. The slide chambers were incubated in a blocking solution containing 1% bovine serum albumin (BSA) for 30 minutes at rt in a wet chamber to reduce non-specific staining and then, remove the exceeding blocking solution from slide without washes. After that, the H-MSCs were labeled at 37°C for 45 minutes with anti-human mouse moAb diluited in 1% BSA in PBS as follows: anti calponin (CALP, 1:40, Dako Cytomation), anti H-caldesmon (CALD, 1:75, Dako Cytomation), anti PDGF-rβ (1:200, R&D System, Inc.), anti-α-Smooth Muscle Actin (α-SMA, 1:9000, clone 1A4, Sigma); anti-Desmin (1:600, clone D33, Dako Cytomation); anti-Vimentin (1:260, clone V9, Dako Cytomation,) and ki-67 (1:100, Novocastra).After repeated washing in PBS, the cells were incubated with polyclonal rabbit anti-mouse immunoglobulins FITC-conjugated (fluoresceine isothiocyanate, 1:250, Dako Cytomation) secondary antibodies in 1% BSA in PBS for 45 minutes at 37°C in the dark. Finally, after several rinses to remove the secondary antibodies unlabeled, the samples were coverslipped with Pro long anti-fade reagent with DAPI (Molecular probes, Milano, Italy). The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) using the kit citated above. Sample were observed under a Leica fully automated inverted fluorescence microscope DMI6000 B using a DAPI filter (Leica Microsystems, Milan, Italy) and Original images were taken at 10X. For negative control, the sections were processed omitting the primary antibody, and no signal was detected.

7.8 ULTRSTUCTURAL ANALYSIS: TRANSMISSION ELECTRON MICROSCOPY (TEM)

The subcellular characteristics of cells were investigated by transmission electron microscope (TEM). The P_4 H-MSCs isolated from pathological segment were washed with phosphate-buffered 0,15 mol/L and fixed in 2.5% buffered glutaraldehyde directly in culture plate for 10 minutes at rt. After scarper, the recovered cells were pelleted in an eppendorf tube, fixed again for 24 hours at 4°C and processed for ultrastructural analysis using the procedure as previously described (see paragraph 7.1.4). The semi-thin sections obtained were stained with Toluidine blue. Ultrathin sections were cut, transferred to specimen support grids, counterstained with uranyl acetate and lead citrate and observed in a Philips 400T trasmission electron microscope.

7.9 STEM CELL GENE EXPRESSION: RT-PCR

In previous studies (*Pasquinelli et al., 2007, 2010*), we had already shown that VW-MSCs isolated from multidistrict human vessels an constitutively expressed the embryonic stem cell marker Oct-4, molecules involved in stem cell critical regulatory pathways, i.e., c-kit, Notch-1, breast cancer resistance protein (BCRP-1), BMI-1, SOX-2, Oct-4, CD133 and KDR. Now, we investigated which regulation pathways are predominantly expressed in H-MSCs among them by reverse trascriptase-polymerase chain reaction (RT-PCR).

The PCR is a technique to amplify a piece of DNA generating thousands to millions of copies of a particular DNA sequence. The method relies on thermal cycling (20-40 cycles and each cycle typically consists of 2-3 discrete temperature steps), consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences

complementary to the target region along with a DNA polymerase (after which the method is named) are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified. PCR consists various steps (*Fig.22*):

- *Initialition step*
- *Denaturation step*
- *Annealing step*
- *Extension/Elongation step*
- *Final elongation*
- *Final hold*

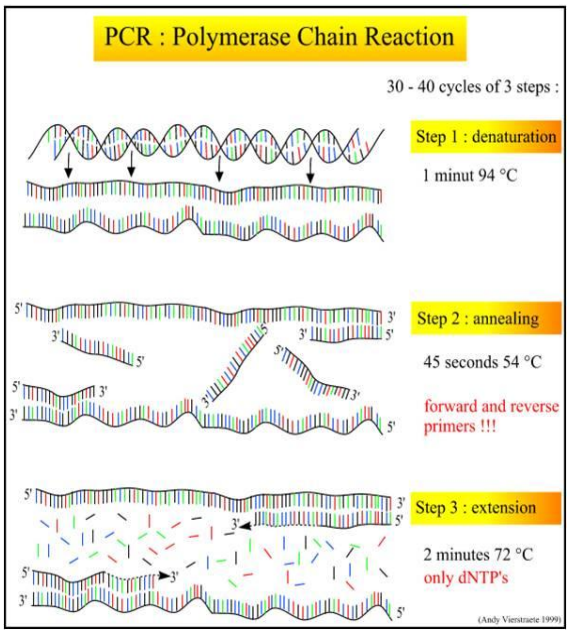


Fig.22: Schematic representation of various steps into Polymerase Chain Reaction

Briefly, total RNA was extracted from H-MSCs from P_4 using the RNA extracting reagent TRI reagent (Ambion Applied Biosystems, Austin, TX, USA) according to the manufacturer's instructions. The cells were homogenized in TRI Reagent solution, collected in an eppendorf and incubate for 5 minutes at rt. After that, the homogenate were centrifuged, incubated with 100 µl BCP 10 minutes at rt to generate the phase separation, recentrifuged again and the aqueous phase containing the RNA was transferred in a fresh tube. The RNA was precipitated by adding isopropyl alcohol, incubated at rt for 10 minutes, spinned to allowed RNA

to precipitate. After discard the supernatant, the gel-like bottom RNA was washed in 75% alcohol for washing, centrifuged, air-dried for 3-5 minutes, dissolved in DEPC water and quantified using Nanodrop.

The quality of RNA extract was evaluated measuring the A260/A280 ratio. 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.

Reverse trascriptase (RT) program was:

- annealing 25°C for 10 minutes
- extension 37°C for 120 minutes

PCR was performed with 1µl of cDNA which was amplified with specific primers and GoTaq® Hot Start Polymerase Promega in PCR reaction mix *PCR* program used was:

- denaturation step 94°C for 2 minutes
- denaturation step 94°C for 1 minutes
- annealing: variable foe each primer used for 1 min
- elongation/extension step 72 °C for 1 minutes
- final extension step 72°C for 5 minutes
- refrigeration step +4°C

Each cycle was repeated for 35-40 times. PCR primers are listed in table below (*Table1*) and were purchased from Invitrogen (Invitrogen Life Technologies, Inc.); 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 gel with 1x Tris-acetate-EDTA buffer, stained with ethidium bromide and photographed under ultraviolet light (UV).

GENE	Primers Sequence	Amplicon lenghth (bp)	Ta°C
Oct-4	REV 5'-CCACATCGGCCTGTGTATAT-3' FW 5'-CTCCTGGAGGGCCAGGAATC-3'	380	60
BMI-1	REV 5'-CATTGCTGCTGGGCATCGTAAG-3' FW5'GGAGACCAGCAAGTATTGTCCTTTTG-3'	369	62
BCRP-1	REV 5'-CTGAGCTATAGAGGCCTGGG-3' FW 5'-GTTTATCCGTGGTGTGTCTGG-3'	652	60
CD133	REV5'GTACAACACTACCAAGGACAAGGCGT3' 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	REV 5'-ATCTTCAAACCTCCATGATG-3' FW 5'-ACCCCCACTGAAAAAGATGA-3'	114	58

Table1: PCR primers sequence, annealing temperature and amplicon length for each gene used to quantify the stemness gene expression

7.10 PROLIFERATION ASSAY: ALAMAR BLUE

Cell proliferation was assessed by Alamar Blue™ assay (BioSource, Camarillo, CA), a nontoxic aqueous dye, formulated in order to quantify cell proliferation directly *in vivo.* In brief, the medium was removed from the H-MSC cells in culture at day 1, 4, 8, 10, 14, 18 and 21 and they were incubated at 37°C for 4 hours with 1 ml of Alamar Blue™ solution (10 % v/v in complete cultured medium) according to manufacturer's instructions. Alamar Blue™ reduction was quantified by measuring the fluorescence of 100 μl of incubated solution in triplicate from each sample (Ex 540/Em 590 nm) using a Wallac VICTOR^2 multiplate reader (Perkin Elmer, Milan,

Italy). Subsequently the cells were re-fed with fresh medium without dye. For continual assessment of cell proliferation, the Alamar Blue™ assay was performed every 2-3 days on the same cell population for up to 21 days. Unpaired t-test was used to evaluate statistical differences between mean values.

7.11 IN VITRO MULTILINEAGE CAPACITY DIFFERENTIATION

Since the cells were isolated from vascular wall homograft, as they showed a phenotype coherent with those described for mesenchymal stromal cells in bone marrow, we investigated their multipotency. In that regard, H-MSCs were induced to differentiate into angiogenic, osteogenic, adipogenic and chondrogenic (to check the attribute of mesenchymal stem cells) and also leyomiogenic, because smooth muscle cell is a cytotype present into vascular wall of blood vessels.

7.12 ANGIOGENIC DIFFERENTATION

To estimate the angiogenic capacity of H-MSCs induced, an analysis of capillary formation was performed according to Oswald (*Oswald et al., 2004*) using the in vitro angiogenesis assay kit (Chemicon, Temecula, CA). That is a convenient system for evaluation capillary-like structures formation. In fact, endothelial cells when cultured on ECMatrix rapidly align themselves and form hollow tube-like structures. Cells taken at P_4 were cultured until near confluence for 7 days in DMEM plus 2% FBS with and without 50 ng/ml VEGF (Sigma) as well as in DMEM plus 10% FBS (negative control).

Vascular endothelial growth factor (VEGF) is a sub-family of growth factors produced by cells that stimulates the growth of new blood vessels during embryonic development, after injury and collateral circulation to bypass blocked vessels. They are important signaling proteins involved in both vasculogenesis (the de novo formation of the embryonic circulatory system) and angiogenesis (the growth of blood vessels from pre-existing vasculature).

7.12.1 In vitro angiogenesis assay: Matrigel

Matrigel, a solid gel of basement proteins containing laminin, collagen type IV, various growth factors and proteolytic enzymes, was prepared according to the manufacturer's instructions. Briefly, 50µl of gel matrix solution were applied into one well of a 96-well plate and incubated for 30 minutes at 37°C to allow the Matrigel solution to solidify. H-MSCs were trypsinized and $5x10^3$ were suspended in 50 µl of DMEM containing or not containing VEGF, plated onto the gel matrix and incubated at 37°C 5% CO_2. Human umbilical vein endothelial cells (HUVEC) were used as a positive control. The formation of capillary-like structures was observed in a CKX41 Olympus (Tokyo, http://www.olympus-global.com) inverted microscope after 2, 4, 6, and 20 hours. Experiments were performed in triplicate.

7.12.2 Flow cytometry assay

In parallel experiments, the H-MSCs induced and control were analyzed at flow cytometry for the expression of endothelial antigens using the following anti-human moAbs: KDR-APC (R&D Systems), FLT-1 and vWF (DakoCytomation) and VEGF (Abcam) to detect the surface

expression of VEGF receptor 2 and the cytoplasmic expression of vWF. To demonstrate whether VEGF could prompt MSCs to differentiate into endothelium, cells were permeabilized with the IntraPrep Kit (Beckman Coulter) and subsequently incubated with vWF MoAb for 1 hour at rt. After two washes with PBS, cells were incubated with FITC anti-mouse IgG (Beckman Coulter) for 30 minutes at rt. Samples were then washed twice over and incubated for 20 minutes with normal mouse Ig (Sigma) to saturate free anti-mouse IgG sites. Analyses were performed as described above (see paragraph 7.6).

7.12.3 Immunofluorescence staining

The expression of endothelial antigens like vWF, VEGF and KDR and was visualize with immunofluorescence staining. The cells, conditioned as described above, were seeded (5,000 cells per cm2) on slide chamber, fixed and permeabilized in 2% paraformaldehyde in PBS and 0,1%Tryton X-100. After fixation, samples were stained with a moAb against KDR (1:500, Sigma) and polyclonal antibodies (pAbs) vWF (1:2000; Dako Cytomation) and VEGF (1:50, Abcam, Cambridge). Blocking, mounting, nucleus counterstaining and observations were performed as described above. For quantitative immunofluorescence analysis, digitalized images were acquired at 20 and 40x magnification (final magnification 200X-400X).

7.12.4 Ultrastructural analysis: TEM

The subcellular features of endothelium differentiation were evaluated with TEM. The undifferentiated and differentiate H-MSCs, cultivated in presence or in absence of VEGF for up to 7 days, were detached with treatment by 0.25% trypsin-EDTA, pelleted, fixed and subsequently

processed for ultrastructural analysis using the procedure as previously described (see paragraph 7.1.4).

7.13 ADIPOGENIC DIFFERENTATION

Adipogenesis was induced by plating homograft cells at a density of $2x10^4$ cells/cm^2 in a six-well plate and cultured until semi-confluence in basal medium. After 24 hours, the medium was then changed to DMEM supplemented with 10% FBS, 0.5 mmol/L isobutyl-methyl xanthine (IBMX), 200 µmol/L indomethacin, 1µmol/L dexamethasone and 20 µg/mL insulin (adipogenic induction medium; 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). Three complete cycles of induction/maintenance medium stimulated adipogenic differentiation, forming adipocytes. Control cells were assayed in the same manner, but DMEM plus 10% FBS was used instead of adipogenic induction/maintenance medium.

After for 3 weeks, morphological features of adipogenic differentiation were observed by inverted LM and TEM, whilst the cytoplasmic presence of lipid droplets was assessed by Oil Red O staining.

7.13.1 Oil Red O staining

After the three weeks of complete rotations of culture with induction/maintenance adipogenic medium, oil red O staining was performed. This protocol revels the cytoplasmatic presence of lipid droplets. Each well of vascular cells was fixed in 10% neutral buffered formalin, gently rinsed in 60% isopropanol for 5 minutes, and incubated for 30 minutes at rt with Oil Red O working solution to each wells. Cells

washed in 60% isopropanol again, lightly stained with Mayer's hematoxylin, rinsed with distilled water, mounted in aqueous mounting medium and viewed on phase contrast microscope.

7.13.2 Ultrastructural analysis: TEM

The H-MSCs were examinated for the presence of adipocytes by ultrastructural analysis. The cells washed with phosphate-buffered 0,15 mol/L and fixed in 2.5% buffered glutaraldehyde directly in culture plate for 10 minutes at rt. After scarper, the recovered cells were collected in an eppendorf tube, centrifugated for 10 minutes at 1800 rpm). The pellets were fixed again for 24 hours at 4°C and processed for ultrastructural analysis using the procedure as previously described (see paragraph 7.1.4).

7.14 OSTEOGENIC DIFFERENTATION

For osteogenic differentation, a 70% subconfluent culture of H-MSCs from P_4 was used. $5x10^3$ cells/cm^2 were plated in six well and one day after plating, the medium was replaced with fresh osteogenic induction medium DMEM supplemented with 15% FBS, 10 mmol/L β-glycerophosphate, 0.17 µmol/L ascorbic acid, and 0,1µmol/L dexamethasone (all Sigma) and cultured for 3-4 weeks replacing the medium every 2-3 days. Controls were cultured in basal medium (DMEM with 10% FBS). All experiment was followed by the morphological evaluation by phase contrast microscope. To demonstrate osteogenesis, the cells cultured were fixed and assessed by Von Kossa staining and TEM to identify calcium salt extracellular deposition.

7.14.1 von Kossa staining

Deposits of calcium or calcium salt were detected by von Kossa staining. This technique is not specific for the calcium ion itself. In this method, tissue sections are treated with a silver nitrate solution and the silver is deposited by replacing the calcium reduced by the strong light, and thereby visualized as metallic silver.

After removing the correspondent media from the wells, the cells plated were gently rinsed with PBS and fixed in 10% neutral buffered formalin. After that, cells were rinsed in distilled water and incubated in 1% silver nitrate solution under UV for 20 minutes at rt. Subsequently, several changes of distilled water are very important to wash silver nitrate out completely to prevent false positive. Samples were stained with a solution of 5% sodium thiosulfate to remove unreact silver for 5 minutes at rt, washed in distilled water, counterstained with 1% nuclear fast red solution, dehydrated trough graded alcohol and mounted using a permanent mounting medium (Canadian Balsam, Sigma).

7.14.2 Ultrastructural analysis: TEM

The calcium deposition in H-MSCs, after the osteogenic induction, was also analysed by ultrastructural analysis. The cells washed with phosphate-buffered 0,15 mol/L and fixed in 2.5% buffered glutaraldehyde directly in culture plate for 10 minutes at rt. After scarper, the recovered cells were collected in an eppendorf tube, centrifugated and pellets were fixed again for 24 hours at 4°C and after processed for ultrastructural analysis using the procedure as previously described (see paragraph 7.1.4).

7.15 CHONDROGENIC DIFFERENTATION

To induce chondrogenic differentiation, aliquots of $2,5x10^5$ cells were collected, centrifugated and gently pelleted in 15 ml polypropylene conical tubes (Corning) in 0,5 ml of DMEM medium. After 24 hours, the basal medium was then changed to chondrogenic differentiation medium (Lonza) containing DMEM, 2,5 µl dexamethasone, 5 µl ascorbate, 5 µl ITS+ supplement, 5 µl sodium pyruvate, 5 µl proline, 0,5 µl Gentamicin/Ampicillin 1000, 10 µl L-Glutamine supplemented with 10 ng/ml TGFβ3 (all product were from Lonza). Loosen the caps of the tubes containing pellets one half turn to allow the gas exchange and incubated at 37°C in humified atmosphere 5% CO_2 and cultured for three weeks in complete by feeding the cell pellets twice a week by completely replacing the medium taking care not to distrupt the pellets. Controls were cultured in chondrogenic differentiation medium without TGFβ3. To verify H-MSCs' chondrogenic differentiation potential, pellets were fixed and processed for histological and immunohistochemical staining and TEM analysis to evaluate proteoglycans and collagen type 2 synthesis.

7.15.1 Alcian Blue staining

To establish the extracellular release of sulfated proteoglycans, a component of chondrogenic extracellular matrix, Alcian Blue staining was performed. Varying the PH of the solution of Alcian Blue more information can be gained concerning the types of acid mucin present. After the induction period, pellets were formalin-fixed and paraffin embedded. The dewaxed sections were stained with 1% Alcian blue in 3% glacial acetic acid at pH 2.5 for 30 minutes at rt; after rinsing in distilled water, hematoxylin counterstaining, the sections were dehydrated, mounted

with permanent mounting medium (Canadian Balsam, Sigma) and viewed on LM.

7.15.2 Type II collagen: immunohistochemical analysis

To assess chondrogenic potential, immunohistochemical staining was performed with an anti-human collagen type II (Chemicon International, Millipore, Billerica, MA) and a non-biotin-amplified method (NovoLink™ Polymer Detection System, Novocastra Laboratories Ltd., Newcastle Upon Tyne, United Kingdom) to evaluate the expression of type II collagen, a specific markers of chondroblasts. Briefly, pellets were formalin-fixed and embedded in paraffin to obtain 3µm-thick sections. H&E staining was used for histological assessment. Additional dewaxed sections were digested with pepsin (0.5 mg/ml Tris-HCl, pH 2.0) for 15 minutes at rt; endogenous peroxidase activity was blocked using absolute methanol containing 1.5% H_2O_2. Sections were incubated with mouse moAb anti-human collagen type II (1:200, Chemicon Int) in 1% BSA-PBS in a wet chamber at 4°C o.n. The antigen-antibody reaction was revealed using the Novolink Polymer detection System according to the manufacturer's protocol. The signal was developed with diaminobenzidine cromogen and sections were counterstained with Gill's hematoxylin. Subsequently, the samples were dehydrated, coverslipped and viewed in a LM using the Image-Pro Plus® program. Negative control was obtained by omitting the primary moAb.

7.15.3 Ultrastructural analysis: TEM

The H-MSCs pellets were examinated by ultrastructural analysis for to detect the presence of collagen fibers and extracellular matrix containing proteoglycans. The cells was washed, fixed in 2.5% buffered

glutaraldehyde for 24 hours at 4°C and processed for ultrastructural analysis using the procedure as previously described (see paragraph 7.1.4).

7.16 LEIOMYOGENIC DIFFERENTATION

To induce leiomyogenic induction, $2x10^3$ H-MSCs at P_5 were seeded in a six-well plates (Corning) in Smooth muscle Growth Medium (SmGM-2; Lonza). The next day, the medium was replaced with induction medium containing SmGM-2 plus 10 ng/ml TGF-β1 (Sigma) and 5 ng/ml PDGF-BB (Sigma).

TGF-β1 is a multipotent cytokine that is involved in the regulation of angiogenesis (*Pepper, 1997*); has a protective role like control of vascular cell proliferation, maintenance of differentiation and increase its immunomodulatory and anti-inflammatory effect (*Mallat et al., 2002*). PDGF-BB is a potent mitogen for a wide range of cell types including fibroblast, smooth muscle and connective tissue; growth factors that regulate cell growth and division and are involved in leiomyogenic differentiation.

The medium was changed every 3 days and the induction period lasted for 2 weeks. Cell controls were assayed in the same manner using SmGM-2 without additional growth factors. To evaluate smooth muscle marker expression, immunofluorescence, RT-PCR and TEM analysis were performed.

7.16.1 Immunofluorescence analysis

To investigate whether the H-MSCs are able to differentiate into leiomyogenic lineages, evaluating the expression of smooth muscle specific

antigens, an immunofluorescence staining was used. H-MSCs were washed with PBS, fixed and permeabilized with 2% paraformaldehyde 0,1% Tryton X-100 in PBS for 4 minutes at rt. Background staining was blocked by incubating with blocking solution containing goat serum (1:10, Sigma-Aldrich) in 1% BSA in PBS for 30 minutes at rt in a wet chamber. Cells were incubated for 45 minutes at 37°C in a wet chamber with the following anti human mouse moAbs: α-SMA(1:9000, clone 1A4); H-Caldesmon (clone h-CD, 1:75); Calponin (clone CALP, 1: 40); Vimentin (1: 260, clone V9) all taken from Dako Cytomation). Antigen-antibody reactions were labeled with goat anti-mouse Alexa Fluor® 488 (1:250, Invitrogen Corporation, Camarillo, CA) in 1% BSA in PBS for 1 hour at 37°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.

7.16.2 ***Semiquantitative analysis: RT-PCR***

The expression of smooth muscle specific genes, such as α-SMA, calponin 2 and caldesmon, was evaluated by RT-PCR. Total RNA was extracted from induced and non induced cells and RT was performed as described above. The primers listed in table below (*Table2*) were selected for amplification using GoTaq Hot Start Polymerase (Promega Corporation, USA); for detection of β2-μglobulin, ASMA, Calponin, and Caldesmon, the following parameters were used: 94°C for 3 minutes and then 30 cycles of 40 seconds at 94°C for denaturation, 30 seconds at 60°C for annealing, 90 seconds at 72°C for polymerization, and 5 minutes at 72°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 UV.

GENE	Primers Sequence	Amplicon lenghth (bp)	Ta°C
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
β2-µglobulin	REV 5'-ATCTTCAAACCTCCATGATG-3' FW 5'-ACCCCCACTGAAAAAGATGA-3'	114	58

Table2: PCR primers sequence, annealing temperature and amplicon length for each gene used to quantify the smooth muscle gene expression

7.16.3 Ultrastructural analysis: TEM

TEM analysis of H-MSCs leiomyogenic differentiation was performed as described above. The cells washed with phosphate-buffered 0,15 mol/L and fixed in 2.5% buffered glutaraldehyde directly in culture plate for 10 minutes at rt. After scarper, the recovered cells were collected in an eppendorf tube, centrifugated for 10 min at 1800 rpm). The pellets were fixed again for 24 hours at 4°C and processed for ultrastructural analysis using the same procedure as previously described (see paragraph 7.1.4).

8. RESULTS

8.1 ORGAN CULTURE

To furnish a direct prove of VW-MSCs involvement in vascular remodeling, repair and in stress conditions, we optimize a new useful approach consisting in a long-term organ culture system, up to 70 days, which gives us the opportunity to learn the spontaneous vascular wall remodeling in a three-dimensional system whitout the influence of circulating blood components. The experiment was performed on healthy adult human femoral artery segment.

8.1.1 Organ culture histological analysis

The histological analysis performed on H&E stained sections was used to verify the overall response of arterial wall resident cells to culture conditions; particular attention was paid on evaluating the number of cells within each arterial layers and their contribution to parietal remodeling during organ culture. Samples seen at day 0 showed an architecture and cellularity comparable to that of normal arterial samples before culture and consequently were considered as control. Within each arterial layer, cells showed normal cytological details; endothelial and smooth muscle cells were easily recognizable; the adventitia layer contained a rich vascular plexus. Minimal cell changes were observed up 35 days of organ culture. At day 14 cells decreased in the intima whereas no significant difference was seen in the medial and adventitial layers. At day 21 the number of intimal cells increased and the media showed a consistent cell decrease; in the adventitia cellularity remained unchanged. Between 28 and 35 days there was an overall decrease in arterial wall cellularity and only a few spindle cells were seen in intima and media layers; adventitia contained no

cell at all. At 42 days the external surface of the adventitia and intima tunica were lined with a single layer of spindle cells, while media did not show significant changes (*Fig.23)*.

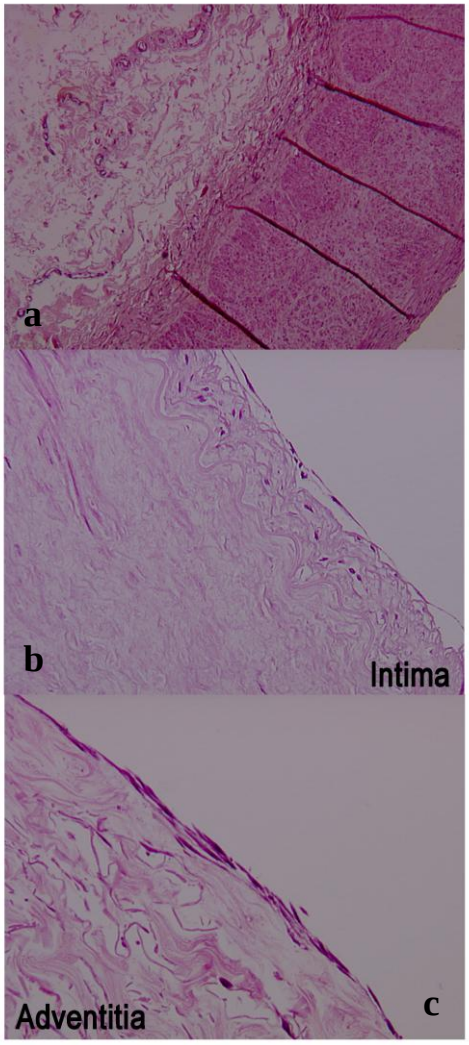


Fig.23: Histological analysis of organ culture. a) Organ culture time 0. b) Remodeling of intima tunica after 42 days of organ culture. c) Remodeling of adventitial tunica after 42 days of organ culture

At day 49 cell linings became more continuous and the adventitial thickness increased due to cell multilayering; these spindle cells also showed a tendency to migrate toward the external edge of the samples; on the contrary the inner portions of intima and media did not contain cells. These features remained unchanged from day 56 up to the end of the organ culture. Results are schematized in the figure below (*Fig.24*).

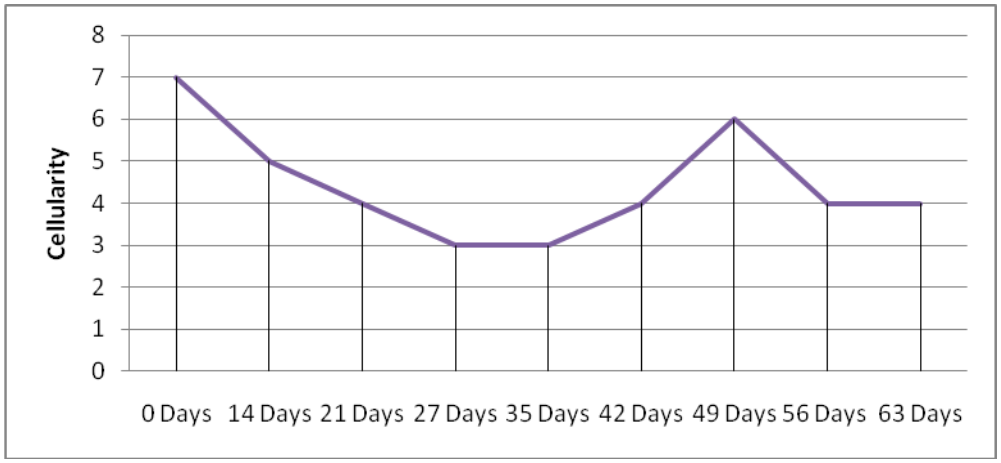


Fig.24: Schematic representation of organ culture vascular wall remodeling

8.1.2 Immunohistochemical analysis

The immunohistochemical analysis was performed to investigate cell immunophenotype changes during organ culture remodeling. Basically a panel of moAbs and pAbs against molecules expressed by MSC (CD105, CD90 and CD44), endothelial cells (CD34, CD31 and VWF), smooth muscle cells (ASMA, Vimentin and Desmin), stem cells (c-kit, Stro-1) and mast cells (Tryptase) was used.

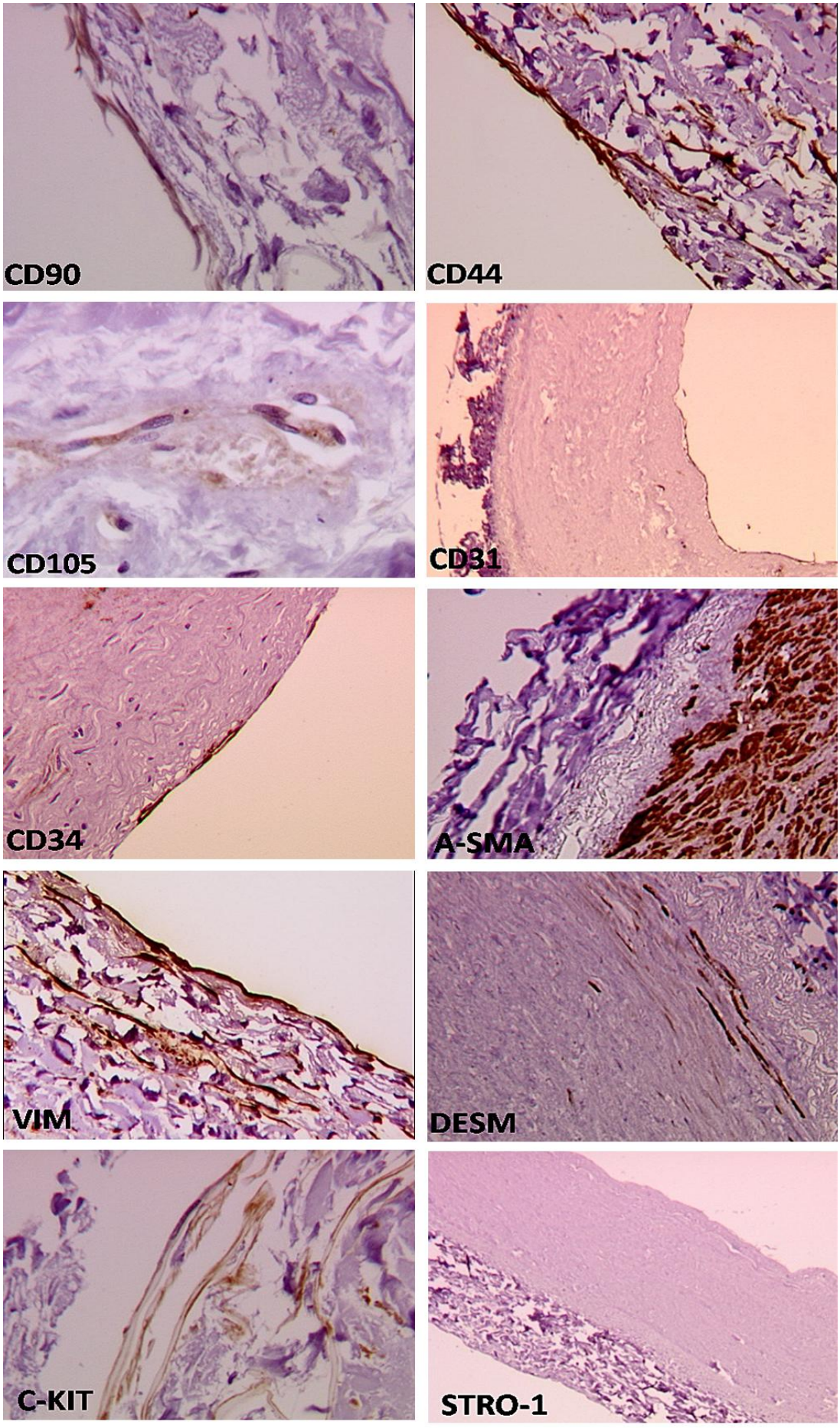
The cell immunophenotype of samples taken at day 0 was quite similar to that of normal femoral artery including the presence of small caliber $CD34^{+}/CD31^{-}$ vessels between the media and adventitia layers.

The most interesting results are those related to the observations carried out from 42 days of organ culture; in fact, spindle cells lining the adventitia surface showed a mesenchymal phenotype, i.e., $CD44^{+}$, $CD90^{low}$, $CD105^{low}$, $Vimentin^{+}$. On the contrary, spindle cells covering the intima expressed almost exclusively the endothelial cell molecule $CD31^{+}$ and a focal presence of CD34 expressing cells that did not exceed the 20% of the intimal lining cells; except for Vimentin all other investigated proteins were negative, including CD44, CD34, α-SMA, Desmin, CD90, CD105

and Tryptase). Tha vascular wall was intensly positive for Vimentin, α-SMA and Desmin.

To study the presence of uncommitted stem cells residents in the vascular wall of human femoral artery, further investigations were carried out using specific stem cell markers such as Stro-1 and c-Kit; while the bone-marrow stromal cells marker, Stro-1, was consistently negative scattered c-Kit positive cells were seen in the adventitia; these cells had a spindle morphology with long and elaborated cytoplasmic projections (*Fig.25*); in contiguous sections, $Tryptase^{+}$ cells was also found; these cells were located between media and adventitia layers and in the external edge, with a distribution pattern that was not superimposable with that of c-Kit expressing cells (data not show).

Fig.25: Immunohistochemical analysis of typical CD markers present on MSCs (CD90, CD44, CD105), endothelial markers (CD31, CD34), smooth muscle markers (ASMA, VIMENTIN, DESMIN) and stem cell markers (c-Kit, Stro-1) Magnificatio 10X-20X


CD90
CD44
CD105
CD31
CD34
A-SMA
VIM
DESM
C-KIT
STRO-1

8.1.3 Ultrastructural analysis: TEM

TEM observation showed that adventitia presents a heterogeneous population: cells directed toward the endothelial phenotype, myoid cells and mesenchymal stromal cells. Endothelial cells were characterized by intermediate filaments with a lassa distribution of vimentin, micropinocitic vesicles and tight junctions; in the cytoplasm there were also oval electrodense granules; probably immature bodies of Weibel-Palade (*Fig.26*). Moreover, spindle cells with typical ultrastructural characteristics of smooth muscle cells: abundant contractile actin filaments, solitary sub-plasmalemmal densities and basal lamina (*Fig.27*). At the end, we observed then cells with a high nucleus/cytoplasm rate with a large euchromatic nuclei and prominent nucleoli. In the cytoplasm, the rER cisternae were distended, some mitochondria and free glycogen were found (*Fig.28)*. These last features are coherent with those described for mesenchymal stromal cells in bone marrow.

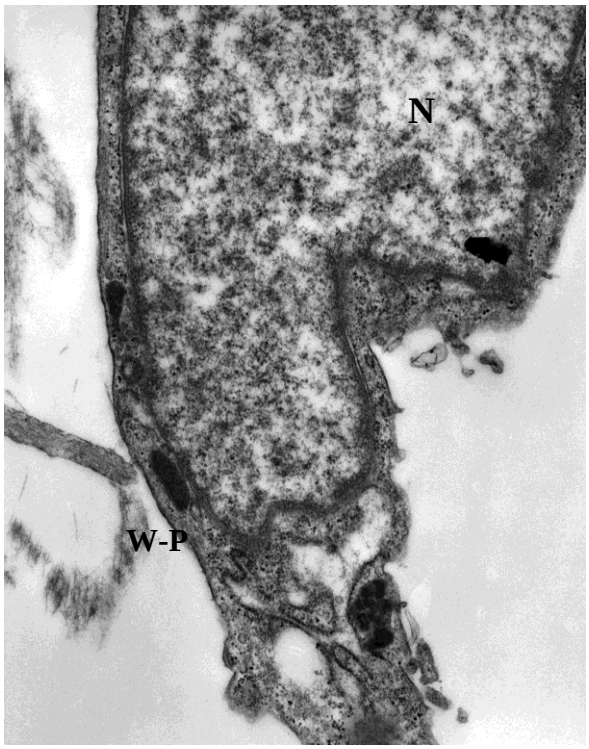


Fig.26: Ultrastuctural features of endothelial phenotype into adventitial tunica.Abbreviation: N: nuclei, W-E: Webel Palade boby

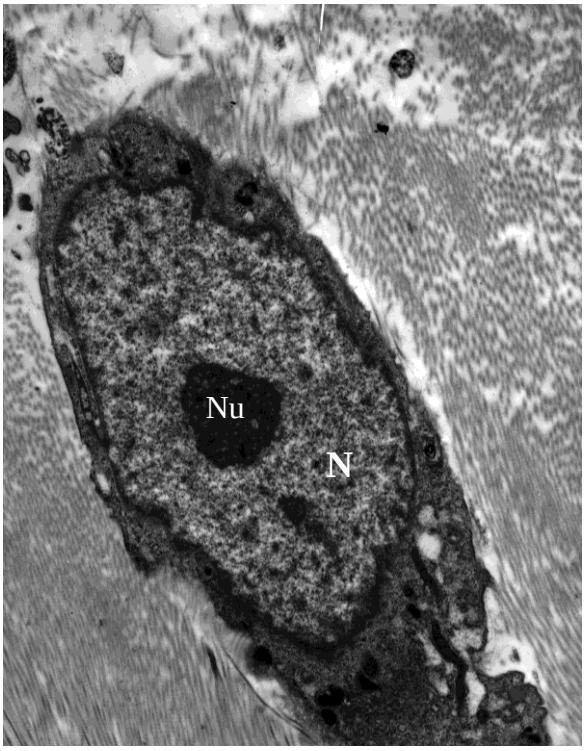


Fig.27: Ultrastuctural features of mesenchymal stem phenotype into adventitial tunica. Abbreviation: N: nuclei, Nu: nucleoli

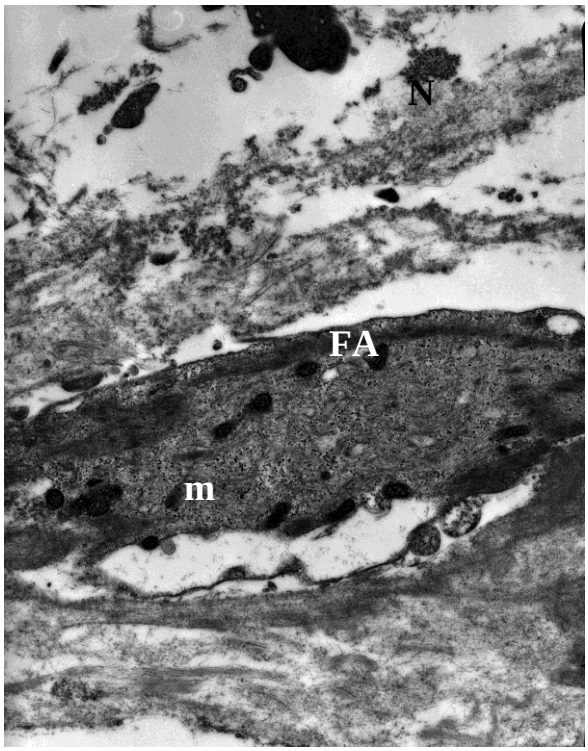


Fig.28: Ultrastuctural features of smooth muscle phenotype into adventitial tunica. Abbreviation: m: mitochondria, FA: actin filament

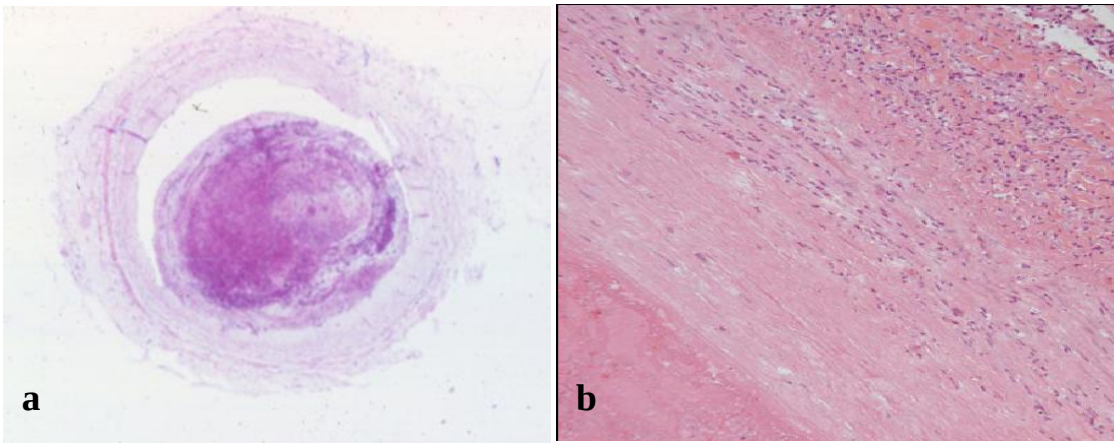
8.2 HISTOLOGICAL ANALYSIS OF HUMAN VASCULAR WALL HOMOGRAFT

The hematoxylin and eosin staining, performed on human vascular wall homografts, was used for histopathological assessment. With the optic microscope both posterior left femoral-tibial homograft rings appeared with a compromised case history. In both segments were found an aneurysmal dilation mainly in the medial tunica, causing its thinner and its fibrosis.

Moreover, the sample show a 2°grade arteriosclerosis with an associated lipid core(*Fig.29d*); the observation showed also focus of adventitial angiogenesis with an associated chronic inflammation(*Fig.29b*).

The lumen of the arterial segment appeared obstructed by an overlying parietal thrombosis (*Fig. 29a*) richly vascularized(*Fig.29c*).

Finally were observed signs of a preexisting parietal haemorrhage rich in monocytes macrophages containing haemosiderinic elements especially in the border line between the medial and adventitial tunicas.



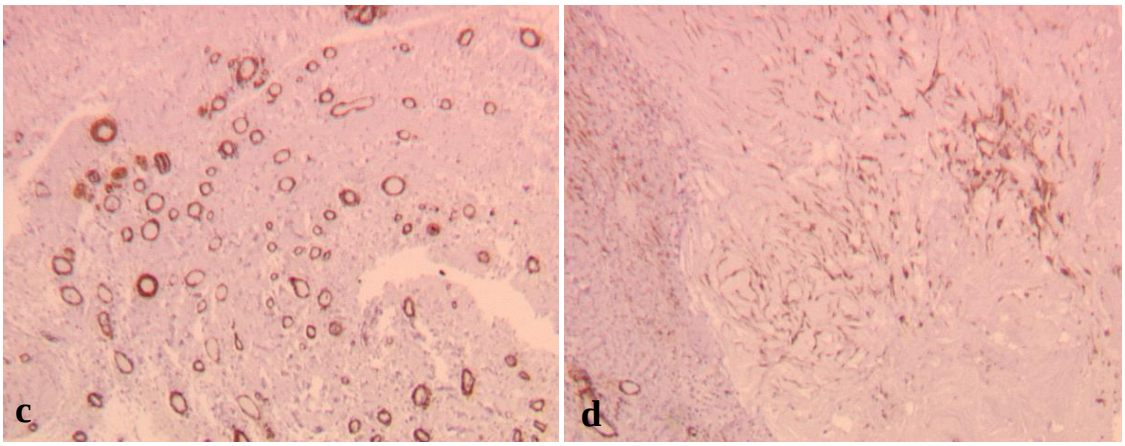


Fig.29: Histological analysis of vascular wall homograft. a) overlying parietal thrombosis. b) chronic inflammation. c) thrombo richly vascularized. d) lipidic core

8.3 VASCULOGENIC ZONE" IN SITU: IMMUNOHISTOCHEMICAL ANALYSIS

The immunohistochemical analysis was performed, on each vascular wall homograft, to identify and to localize the adult stem cell populations that could be reside in the vasculogenic zone using specific antibodies commonly expressed by mesenchymal stem cells like:

- **CD44**: is a receptor for hyaluronic acid and it is involved in adhesion between hyaluronate and other proteoglicans in the extracellular matrix including collagen and fibronectin.

- **CD105:** also known as endoglin. This antibody is present on endothelial cells, erythroblasts, monocytes, and connective tissue stromal cells and facilitates enrichment of stromal progenitors from bone marrow.

- **CD90 or Thy1**: cell surface protein that can be used as a marker for a variety of stem cells.

- **CD166 or ALCAM** (activated leukocyte adhesion molecule): a transmembrane glycoprotein involved in the osteogenic differentiation and also is expressed on lymphocyte B, T and macrophages.

The immunohistochemical analysis showed numerous cells highly positive for CD44 and some other cells weakly positive for CD90 localized mainly in the vasculogenic zone corresponding to the border line between medial and adventitial tunicas of the homograft wall. Besides, even pericytal and perivascular cells of the rich vascular plexus present in the adventitial tunica showed an high positivity for CD44 and a low positivity for CD90.
Near the vasculogenic zone were also observed sporadic cell weakly positive for $CD105^{low}$ and $CD166^{low}$ (*Fig.30*).
Those observation confirmed the existence of a vasculogenic zone in the border line between the medial and adventitial tunicas in the vascular wall, in which resides a population of stem cell potentially able to generate, in a specific hierarchic organization, stromal supporting elements (MSC).

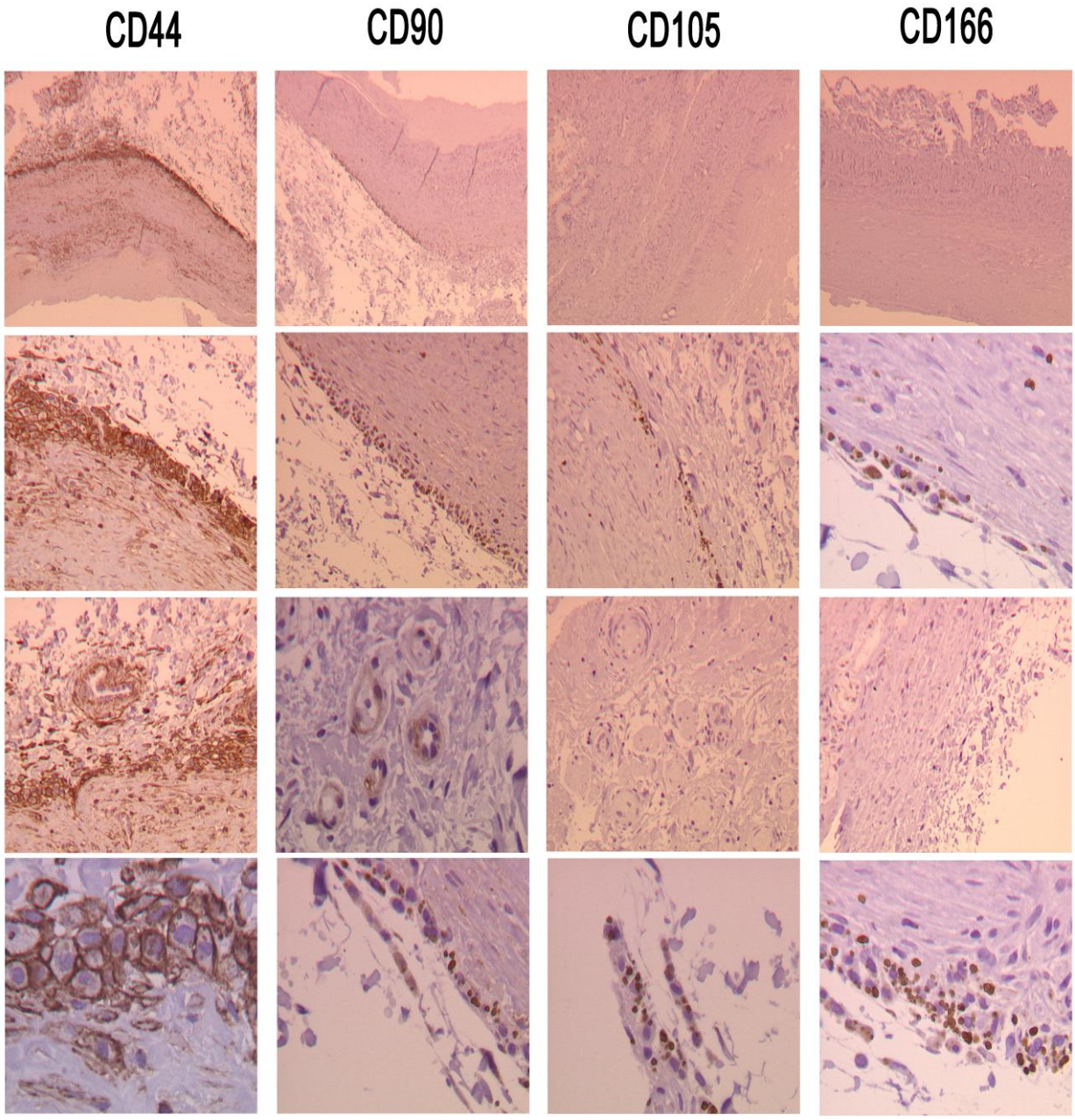


Fig.30: "Vasculogenic zone" in situ immunohistochemical analysis

8.4 ISOLATION AND CELL CULTURE

From 25-30cm^2 of each sample of vascular wall homograft we succeeded in isolating mononuclear cells using a combination of enzymatic and mechanical procedures, as previously described in materials and methods section. The initial number of primary cell cultures varied from 1,5x10^6 to 3,5x10^6. Cells isolation was carried out successfully in only one case. The remaining cases were discarded due of a fast growth of contaminant bacteria at passage 2 to avoid delivering erroneous experimental results. After approximately 2-3days from initial seeding, the observation to phase contrast microscopy from cells in P_0 demonstrated a strong adherence to plastic and fibroblast-like spindle-shape morphology and had a tendency to grow in colonies, estimating 36 hours as doubling time. During the cell culture, this spindle-shape cells were highly proliferating increasingly assuming the morphological properties of MSCs from bone marrow (*Fig.31*).

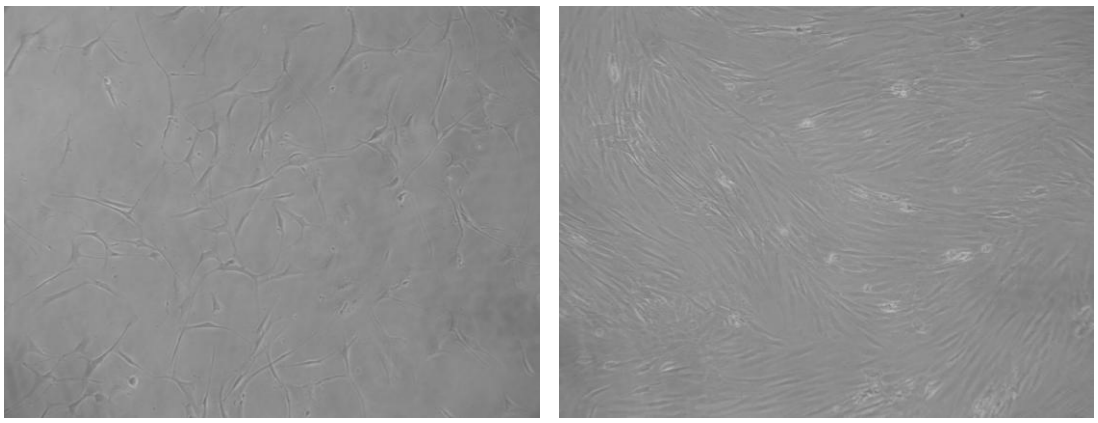


Fig.31: MSCs isolated from vascular wall homograft. a) The cell morphology after 3 days of culture, bar =20 μm; b) Typical phenotype of mesenchymal stromal cells after P_3 in culture (Magnification 10X)

The final number of resident mesenchymal stem cells isolated from 25 cm^2 of homograft segment was estimated around ≈ 0,5-2,0x10^4 cells, thus demonstrating that MSCs population is present in homograft. The cells were cultured in collagen type I flasks in DMEM supplemented 10%FBS for up to P_{10}-P_{12} for expansion without losing the proliferative capacity, like the culture of BM-MSCs.

8.5 IMMUNOPHENOTYPING: FLOW CYTOMETRY ANALYSIS

The cells isolated from vascular wall homograft were subsequently analyzed for the single expression of characteristic markers generally used to identify the MSCs and stem cells using a flow cytometric analysis.
The fibroblast-like cells culture taken at P_3 were evaluated at their surface with a panel of antigens found in MSCs (CD90, CD44, CD105, CD29, CD73); hematopoietic lineage (CD45), hematopoietic progenitor (CD117, CD34, CD133), endothelial cells (CD31, vWF, CD146, KDR) and stemness markers (c-Kit, Notch-1, Oct-4, Sca-1, Stro-1) and perycite cells (NG2). As other mesenchymal stem cells, the majority of H-MSCs expressed typical mesenchymal antigens such as CD90, CD44, CD105, CD29, CD73 and NG2. On the contrary, they were negative for the early and mature hematopoietic markers CD34 and CD133 and for mature hematopoietic markers CD45. H-MSCs were also negative for KDR, CD146, CD31 and vWF which are markers of differentiated endothelium whilst c-Kit was not detected (*Fig.32*).

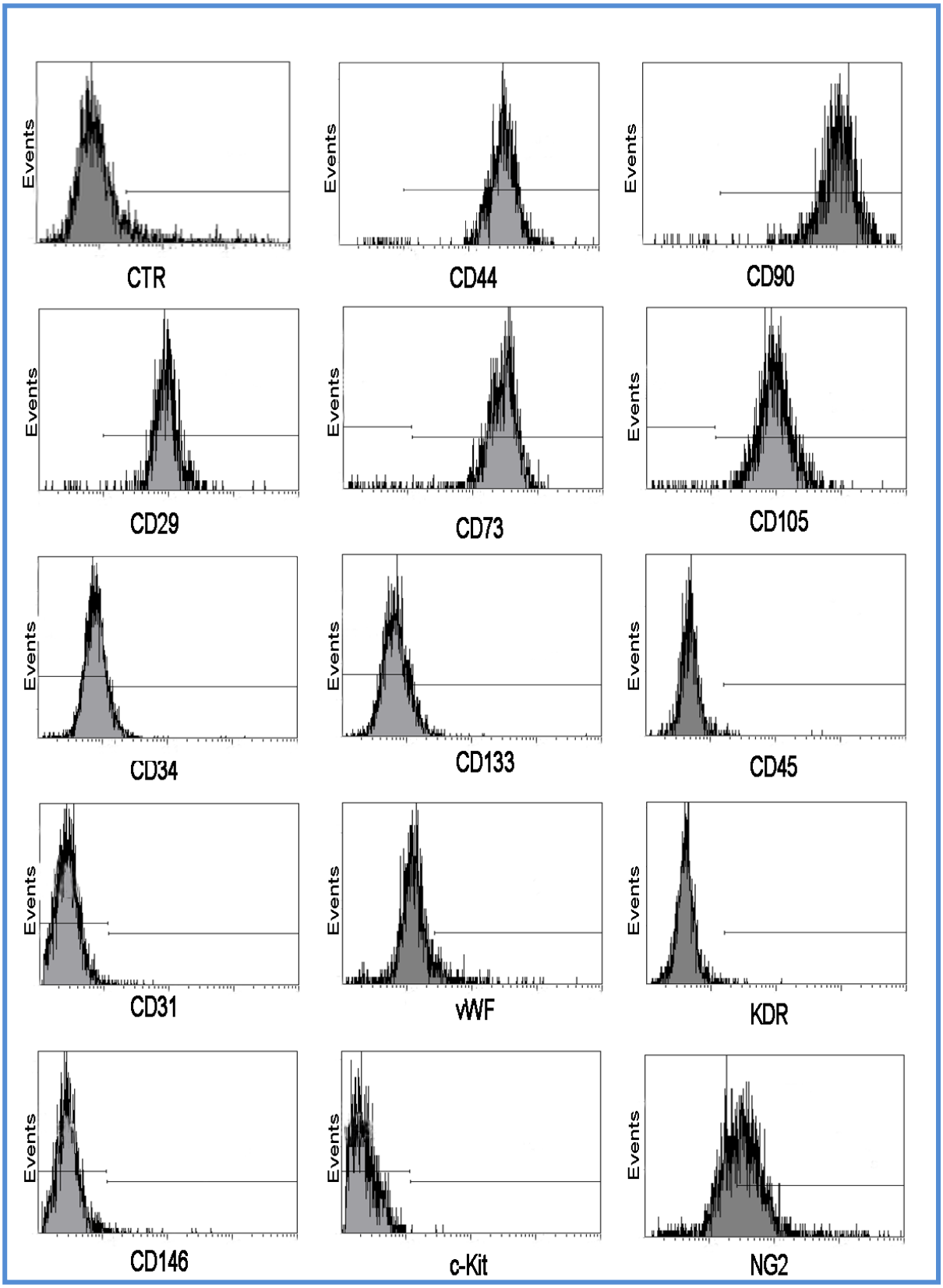


Fig.32: Flow cytometry analysis of typical CD markers present on MSCs and Pericytes and typical hematopoietic and endothelial markers which are not expressed by MSCs

The cells intensely expressed stemness markers such as Oct-4 and Notch-1; Sca-1and Stro-1 were also expressed although at lower level (*Fig. 33*).

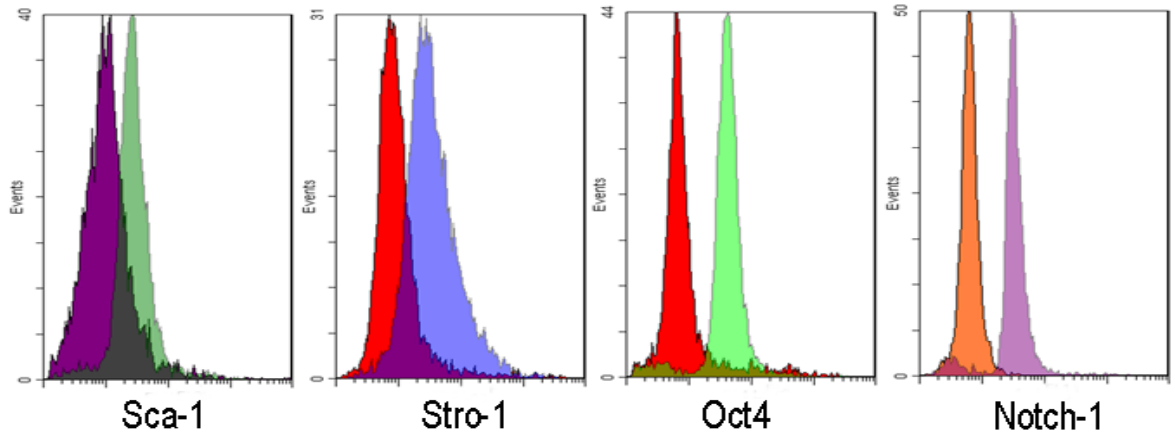


Fig.33: Immunophenotype of stemness markers expressed by H-MSCs in flow cytometry analysis.

In conclusion, the phenotype of H-MSCs isolated was $CD44^{+}$, $CD90^{+}$, $CD105^{+}$, $CD29^{+}$, $CD73^{+}$, $Sca\text{-}1^{low}$, $Stro\text{-}1^{+}$, $Oct4^{+}$, $Notch\text{-}1^{+}$, $CD45^{-}$, $CD34^{-}$, $CD133^{-}$, KDR^{-}, $CD31^{-}$, vWF^{-}, $CD146^{-}$, $c\text{-}Kit^{-}$ and $NG2^{+}$.

8.6 IMMUNOFLUORESCENCE ANALYSIS

In addition to flow cytometry analysis, a single immunofluorescence staining was performed to investigate the smooth muscle phenotype of H-MSCs isolated. The cells, taken at the same passage used in flow cytometry analysis, were stained with intracytoplasmatic antigens against to smooth muscle markers (α-SMA, Calponin, h-Caldesmon, Vimentin, Desmin, and PDGF-rβ) and Ki-67 a proliferation's marker. The fibroblast-like cells showed a mesenchymal phenotype resulted positive for Vimentin and Calponin while, surprisingly, we observed rare cells positive for the α-

SMA staining and the absence of other smooth muscle markers like h-Caldesmon, Desmin and PDGF-rβ (*Fig.34*).

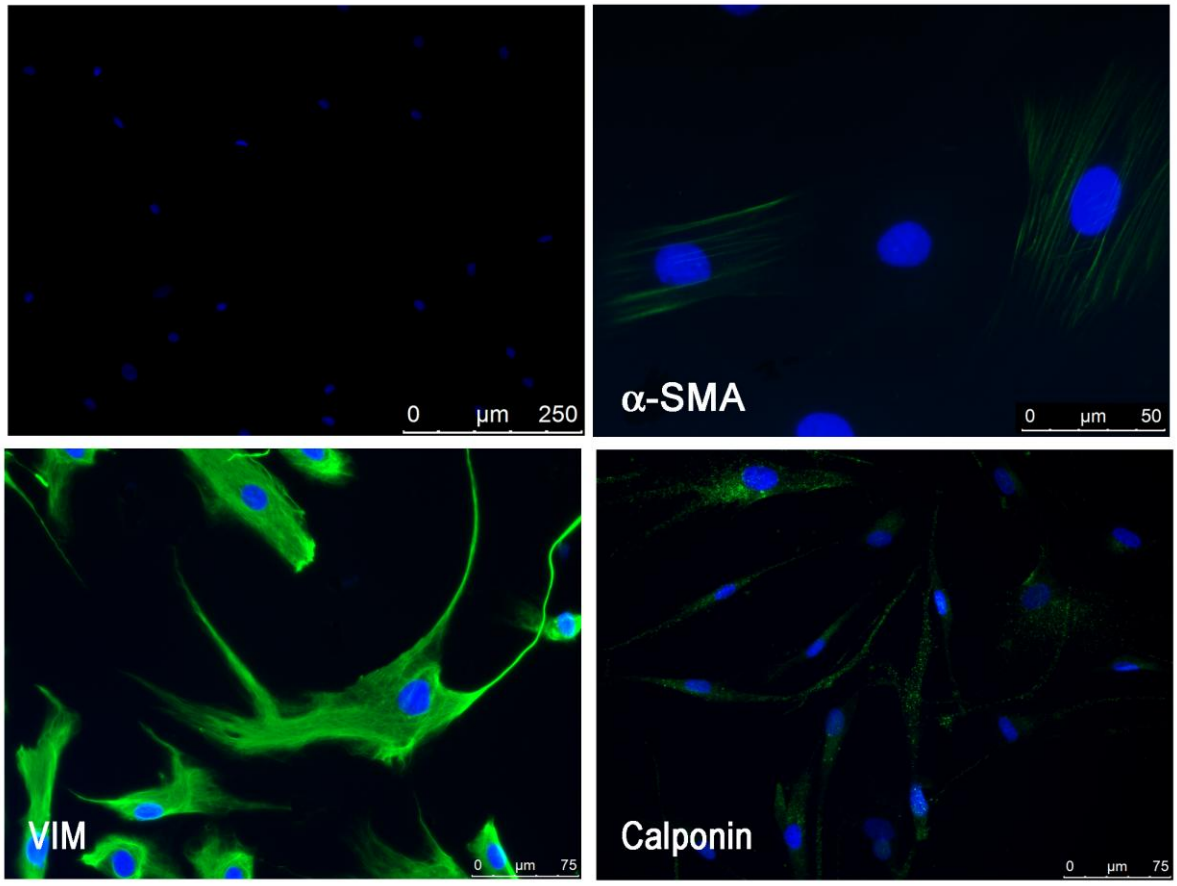


Fig.34: Immunofluorescence characterization of H-MSCs cultured on slide chambers. Cells were stained with moAbs directed against to smooth muscle markers. Negative control (A), α-SMA (B), Vimentin (C) and Calponin (D). Nuclei in blue and cell positive in green

8.7 ULTRATRSTUCTURAL ANALYSIS: TRANSMISSION ELECTRON MICROSCOPY (TEM)

At P_4, the subcellular characteristics of these cells derived from vascular wall homograft were investigated by transmission electron microscopy (TEM). The observation revealed cells with a high ratio nucleus/cytoplasm with a large euchromatic nuclei and prominent nucleoli. The abundant

cytoplasm containing numerous organelles, e.g., some mitochondria, a few lipid droplets, the rough endoplasmic reticulum (rER) with cisternae dilated, free glycogen. More careful observation of peripheral cytoplasm shows the presence of collections of clear vesicles, vacuoles and blisters giving a labyrinthine appearance (*Fig.35*).

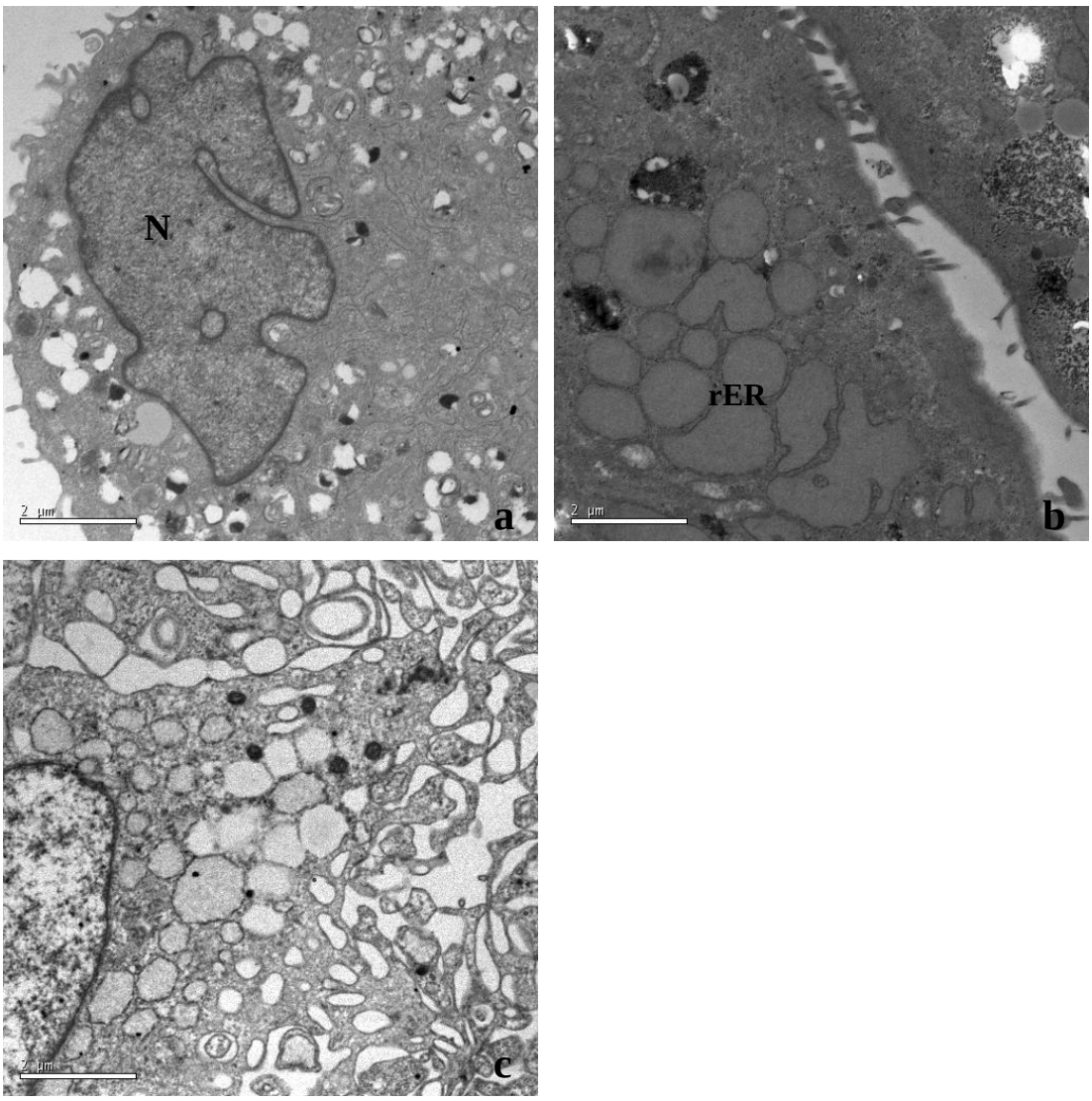


Fig.35: Ultrastructural features of H-MSCs. a) High ratio nucleus (N)/cytoplasm b) Abundant cytoplasm containing the rough endoplasmic reticulum (rER) with cisternae dilated and free glycogen c) peripheral cytoplasmic collections of clear vesicles, vacuoles and blisters Scale bar=2 µm.

All these ultrastructural features are coherent/accord with those described for mesenchymal stromal cells isolated from bone marrow as already reported in our previous study (*Pasquinelli et al. 2007, b*).

8.8 STEM CELL GENES EXPRESSION ANALYSIS: RT-PCR

The gene expression performed on H-MSCs was evaluated by RT-PCR. This semiquantitative analysis confirmed stem cell gene expression showing that these cells constitutively expressed transcripts associated with embryonic stem cell marker, Oct4, and some molecules involved in stem cell critical regulatory pathways i.e., Notch-1, BCRP1, as well as, to a lower extent, hematopoietic stem cell transcripts such as KDR and CD133. The RT-PCR analysis demonstrated that these cells at P_4 expressed BCRP1, that maintained the undifferentiated state of embryonic stem cells; Notch-1, that regulated the stemness and the undifferentiated status of H-MSCS; and lower expression level of Oct4 governing pluripotency and cell fate determination of embryonic stem cells (*Pan et al., 2002*). Moreover, the H-MSCs showed very low expression of KDR, receptor type 2 of VEGF, as well as CD133, hematopoietic stem cell transcripts. (*Fig.36*).
The positive control, BM-MSCs, expressed all the molecules involved in stem cells conditions, like CD133, Oct-4, BCRP-1, BMI-1 while they didn’t expressed the hematopoietic stem cells molecules c-Kit and KDR (Pasquinelli et al., 2007 b).

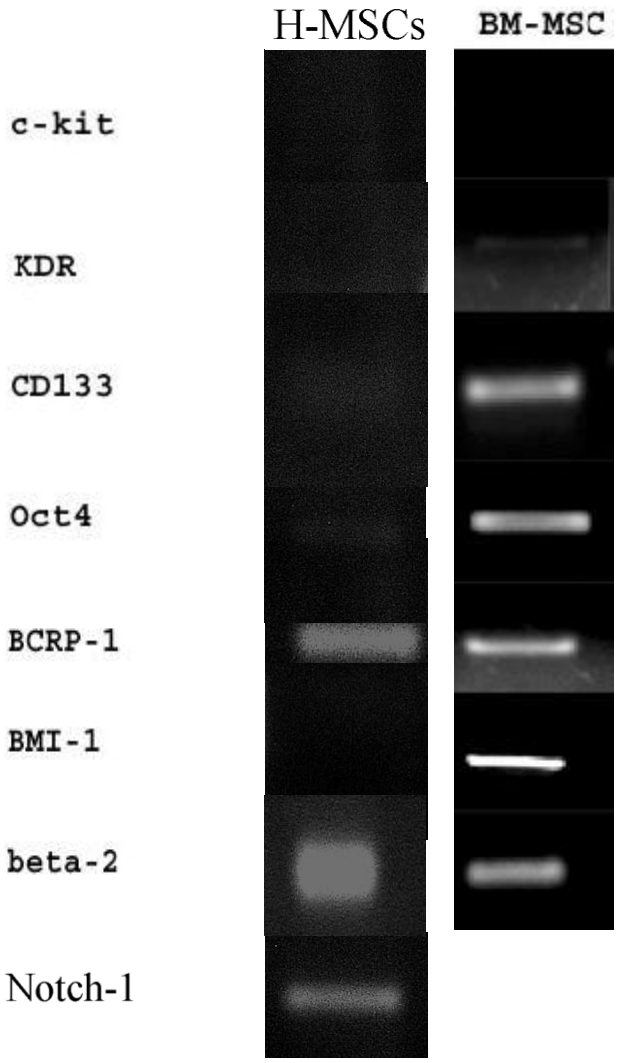


Fig.36: Stem cell genes expression analysis by RT-PCR performed on cells isolated from arterial homograft. On the left, the lane shows transcript expression found in H-MSCs. On the right, the lane indicates the genes expression by BM-MSCs. β2μ was used as a house-keeping gene.

8.9 PROLIFERATION ASSAY: ALAMAR BLUE

The kinetics of cell proliferation was evaluated directly "in vivo" for up 21 days using Alamar Blue™ assay a non-toxic aqueous dye which is reduced

by all components of the mitochondrial respiratory chain and highlights the cells metabolically active (intact and functional mitochondria). The results of assay highlight that proliferative capacity of these cells increases with time, reaching a plateau on day 10 and then remain almost constant until day 21 (end of the experiment) (*Fig.37a*).

The marked cell proliferation of cells under study was confirmed also by positive immunofluorescence for Ki-67. Ki-67 is a cell proliferation marker able to recognize a nuclear and nucleolar protein expressed in the G1, S, M and G2 phases of the cell cycle. This data suggested that H-MSCs have high-growth potential (*Fig.37b*).

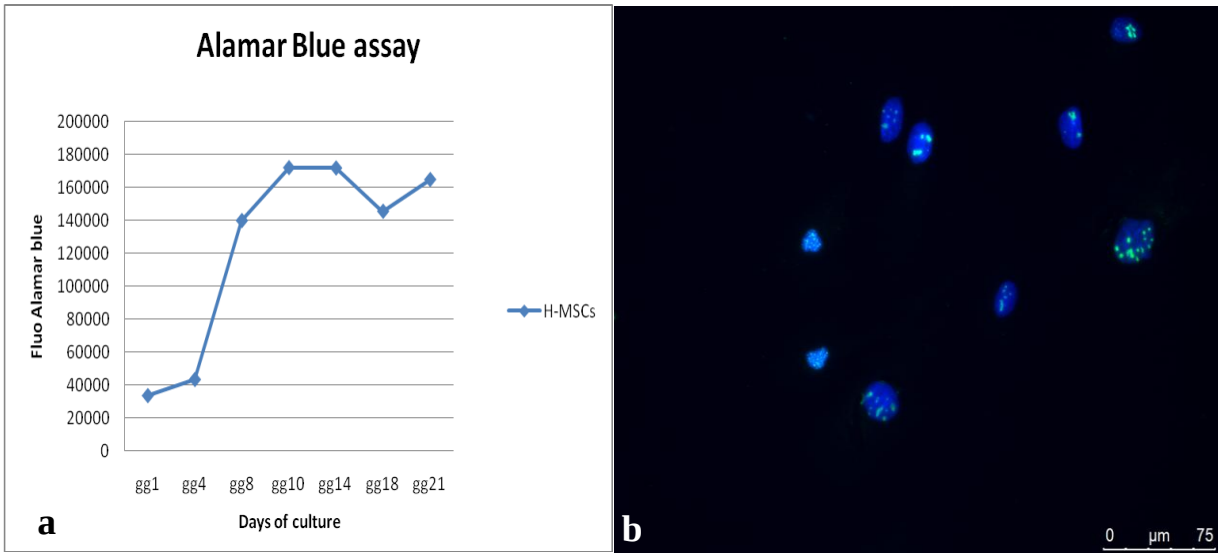


Fig.37: Evaluation of cell proliferation of H-MSCs. a) By Alamar Blue fluorescence assay. Values are given as means of fluorescence of a single experiment in triplicate. b) Ki-67 immunofluorescence staining confirmed the high proliferation of H-MSCs. Nuclei in blue and nuclear/nucleolar positivity in green.

8.10 ANGIOGENIC DIFFERENTIATION

The angiogenic capacity of MSCs isolated from vascular wall homograft, induced for 7 days with 50 ng/ml VEGF to form capillary-like structures, was performed according to Oswald (*Oswald et al., 2004*). The features of angiogenic differentiation were evaluated using the *in vitro* angiogenesis assay and confirmed by various technique.

8.10.1 In vitro angiogenesis assay: Matrigel

The ability to form capillary-like tube formation was tested in a semisolid matrix with an in vitro angiogenesis kit. Cells, taken at P_4, were cultured until near confluence for 7 days in absence or presence of VEGF and observed at regular intervals during the following 24 hours. Undifferentiated and differentiated H-MSCs were trypsinized and seeded on the top of the ECMatrix or Matrigel gel solution. After 2 hours, the undifferentiated H-MSCs organized themselves in a few capillary structures and most of the cells remained scattered in the medium. When cultivated in the presence of VEGF, the cells rapidly aligned themselves and formed hollow tube-like structures with thin cytoplasmic projections sprouting from the cell periphery. After 4 hours, the same cells appeared connected by thicker projections forming an evident capillary-like network but after only 6 hours this capacity was lost.

On the contrary, HUVEC, used as positive control, spontaneously aggregated in a capillary-like network when seeded on Matrigel (*Fig.38*).

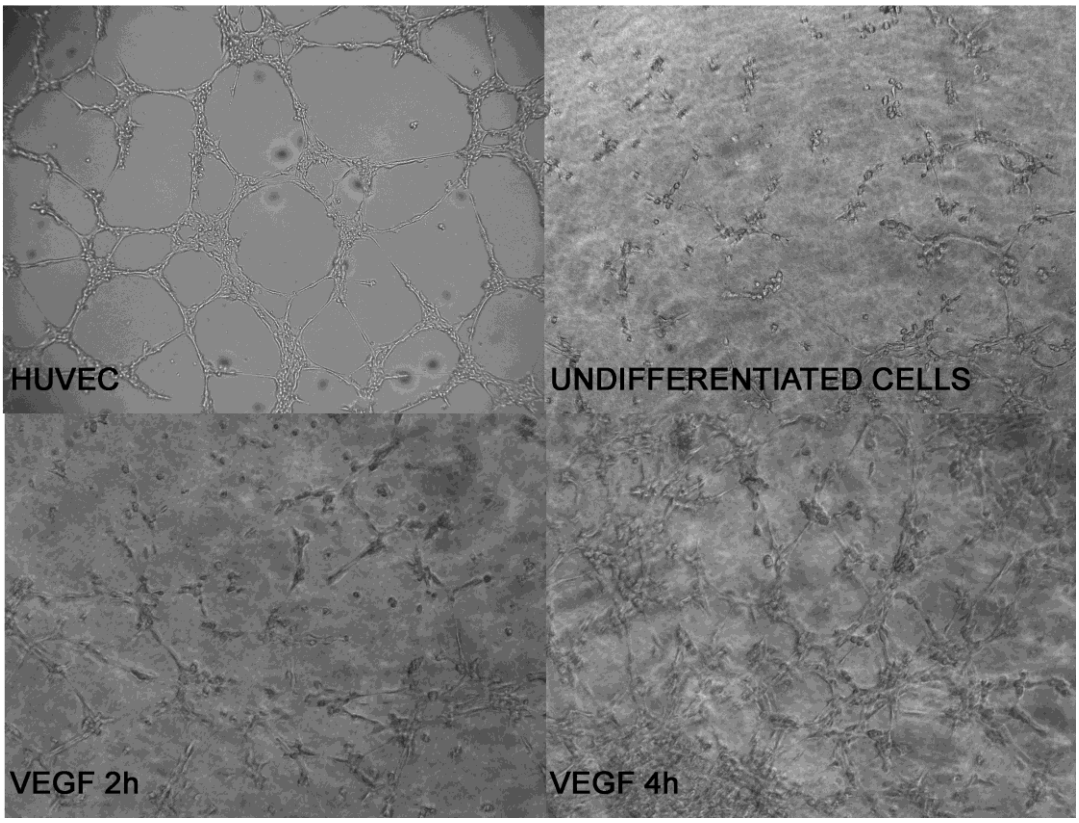


Fig.38: Light microscopy analysis of differentiated and undifferentiated H-MSCs on semisolid matrix in presence and absence of VEGF. A) HUVEC as positive control; B) Undifferentiated H-MSCs; C) After to 2 hours from Matrigel seeding; D) After 4 hours from Matrigel seeding

8.10.2 Flow cytometry assay

After 7 days, the cells cultured in a conditioned medium with or without VEGF were evaluated with an indirect immunofluorescence flow cytometry to quantify the endothelial-specific marker expression. Differentiated H-MSCs showed a completely absence of expression of the VEGF receptor-II or KDR and a lower expression of the VEGF receptor-I or FLT-I. Moreover, a single labeling experiment demonstrated the VEGF promoted the vWF cytoplasmatic expression (marker of mature endothelium) and the synthesis of VEGF (*Fig.39*).

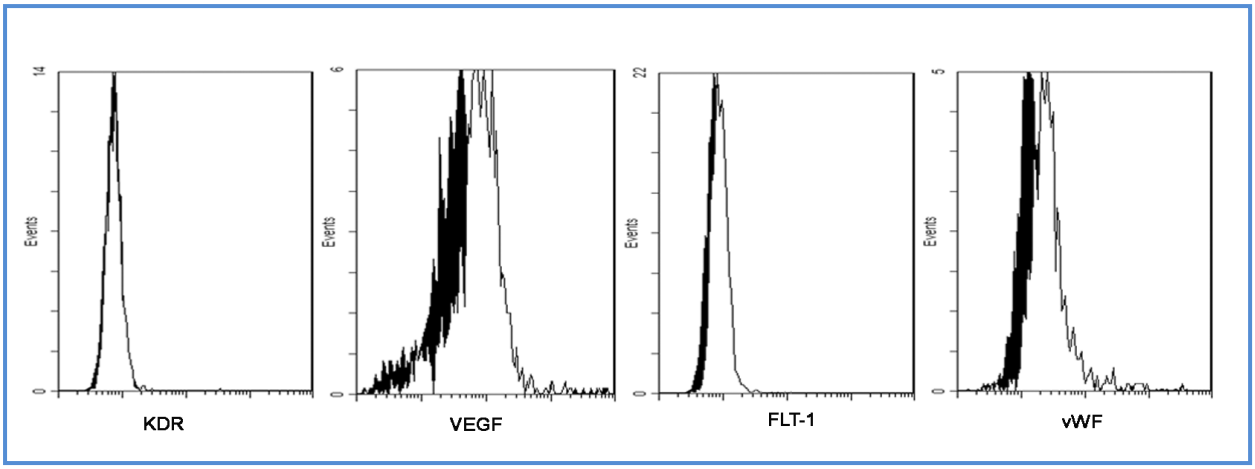


Fig.39*:* Flow cytometry analysis for the expression of endothelial-specific markers after angiogenic differentiation of H-MSCs

8.10.3 Immunofluorescence staining

The immunofluorescence staining, performed on undifferentiated and differentiated cells to quantify the endothelial-specific molecules, confirmed the data obtained in flow cytometry (data not show).

8.10.4 Ultrastructural analysis: TEM

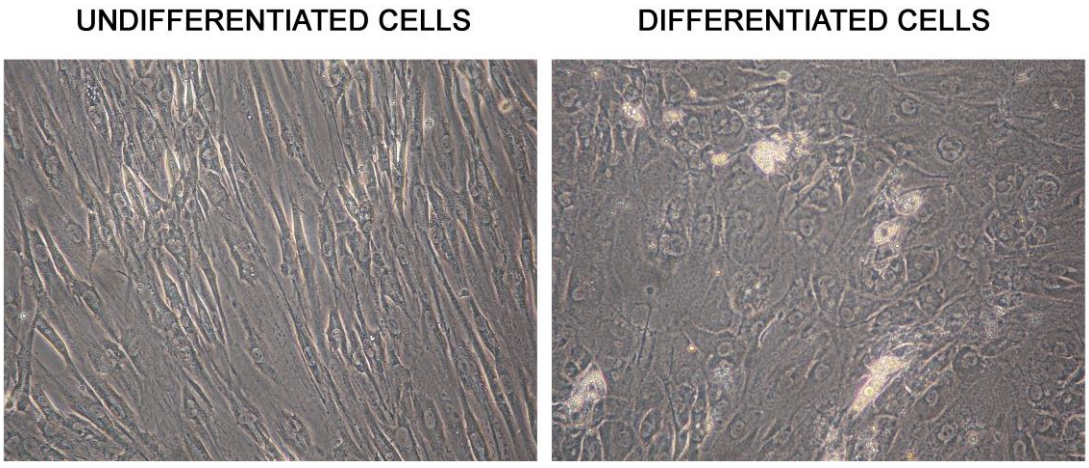
The subcellular characteristics of undifferentiated and differentiated H-MSCs, after 7 days of angiogenic differentiation, were evaluated with transmission electron microscopy. The observation on H-MSCs pellet, when cells had been treated with VEGF, not showed features consistent with an early endothelial phenotype such as electron-dense oval-shaped granules possibly representing immature Weibel-Palade bodies (data not show).

8.11 ADIPOGENIC DIFFERENTIATION

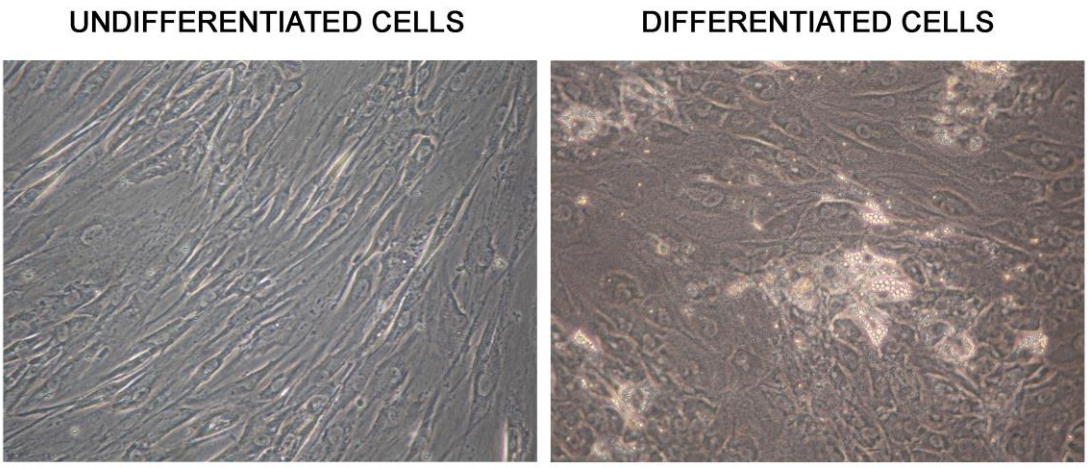
After the three complete rotations of cell culture with adipogenic induction medium and adipogenic maintenance medium, oil Red O and ultrastructural analysis was performed.

8.11.1 ***oil Red O staining***

H-MSCs treated with adipogenic media showed the presence of multiple lipid-rich vacuoles into cytoplasm that increased in size and number with the time of induction (*Fig.40*).



NON INDUCTION 10DAYS ***INDUCTION 10 DAYS***



NON INDUCTION 20DAYS ***INDUCTION 20 DAYS***

Fig.40: During the adipogenic differentiation, fresh specimens, observed at light microscopy after 10 days and 20 days of induction, showed the presence of multiple lipid-rich vacuoles (Magnification 20X)

Controls, by contrast, retained their morphology and did not display significant cytoplasm vacuoles.

Vacuoles within cells were stained with the lipid dye Oil Red O (Fig.41).

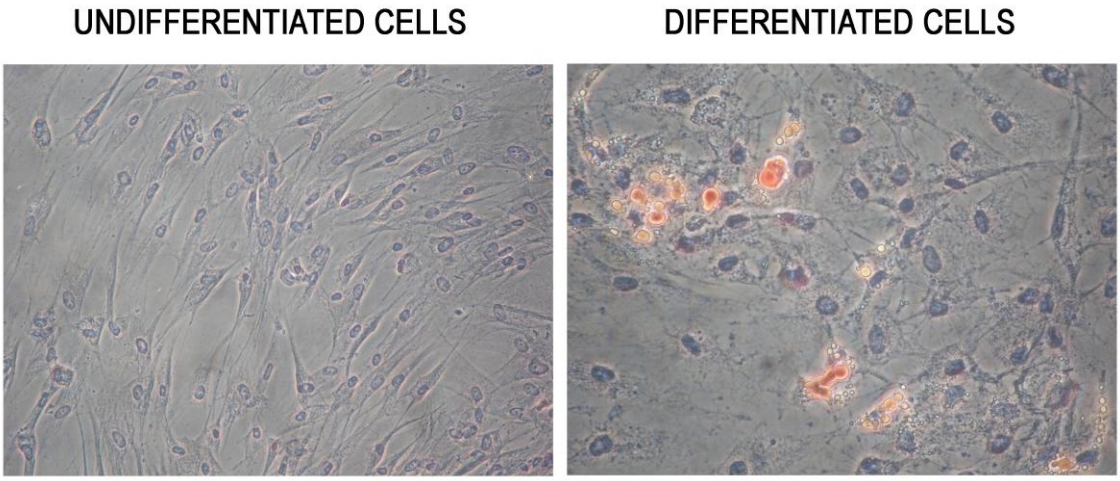


Fig.41: Adipogenic differentiation of H-MSCs shown by positive staining for neutral lipid vacuoles (red) demonstrated by the Oil Red O reaction (Magnification 10X)

8.11.2 Ultrastructural analysis: TEM

Ultrastructural observation showed that H-MSCs contained multiple, confluent lipid droplets in the cytoplasm and small dense mitochondria, besides an elevated endocytic activity (*Fig.42*).

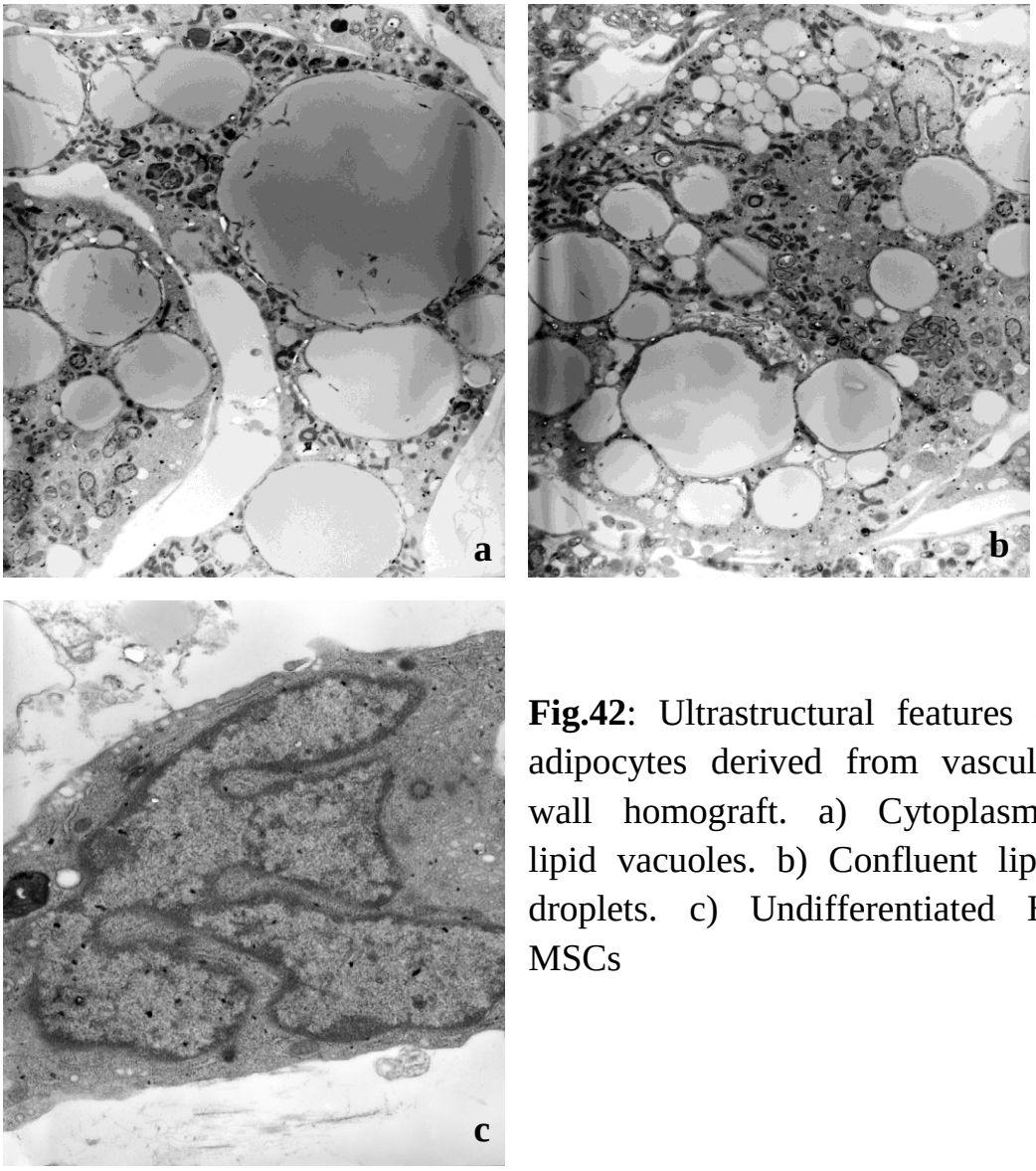


Fig.42: Ultrastructural features of adipocytes derived from vascular wall homograft. a) Cytoplasmic lipid vacuoles. b) Confluent lipid droplets. c) Undifferentiated H-MSCs

8.12 OSTEOGENIC DIFFERENTIATION

The osteogenic differentiation potential, of mesenchymal stem cells isolated by vascular wall homograft and cultured for up to 21 days in an osteogenic induction medium, was evaluated in light microscopy and transmission electron microscopy to identify the calcium salt deposits in extracellular matrix.

8.12.1 von Kossa staining

At light microscopy, the morphological changes in H-MSCs during osteogenic induction were first noted around 10 days after plating, when the differentiated cells have lost their cytoplasmic extension (*Fig.42*). The quantity of calcium deposition increased with the time of induction (*Fig.43*).

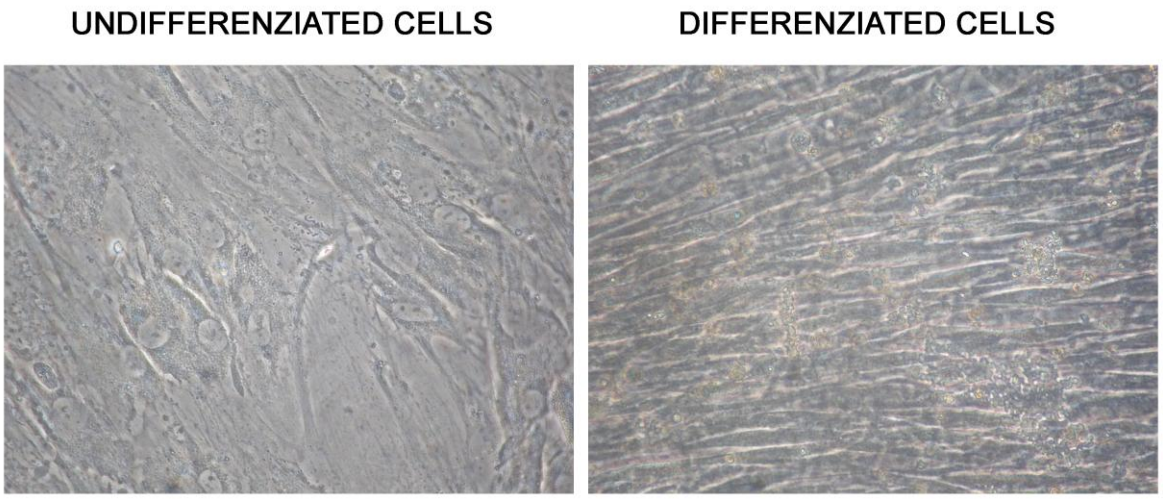


Fig.42: Fresh specimens were observed during the experiment. The morphological changes of H-MSCs were noted after osteogenic induction around 10 days after plating (Magnification 20X)

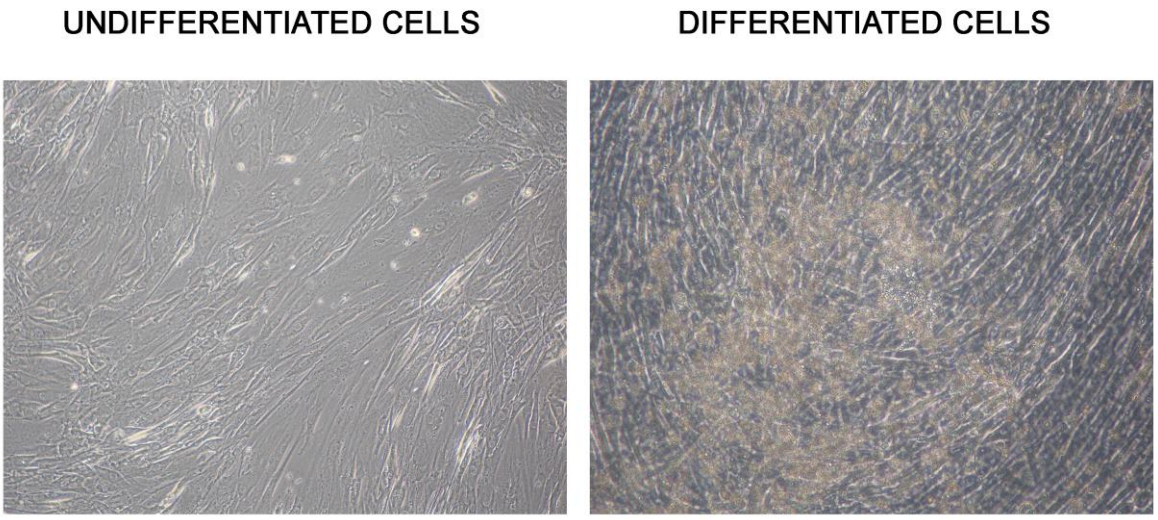


Fig.43: Fresh specimens were analyzed during the experiment. At light microscopy, the calcium deposits increased with the time of induction (Magnification 10X)

After the morphological change, marked calcium deposition was observed under phase contrast microscope, increased over time and it was confirmed by von Kossa staining. Brownish calcium deposits were spread evenly across the plate of induced H-MSCs and completely absent in non induced cells. Controls, by contrast, retained their morphology and did not display calcium deposition in extracellular matrix (*Fig. 44*).

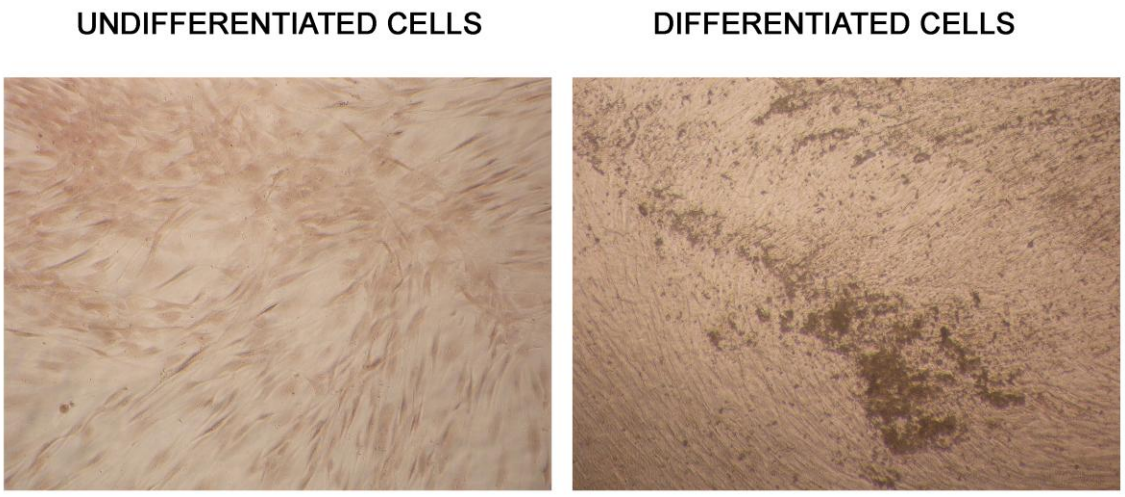


Fig.44: Osteogenic differentiation of H-MSCs shown by positive staining for calcium deposits (brownish) demonstrated by the von Kossa reaction. (Magnification 10X)

8.12.2 Ultrastructural analysis: TEM

The ultrastructural analysis of H-MSCs, after 3 weeks of osteogenic induction, revealed multiple foci of electron dense fibrillary deposits which were decorated with needle-shaped hydroxyapatite crystals (*Fig.45*).

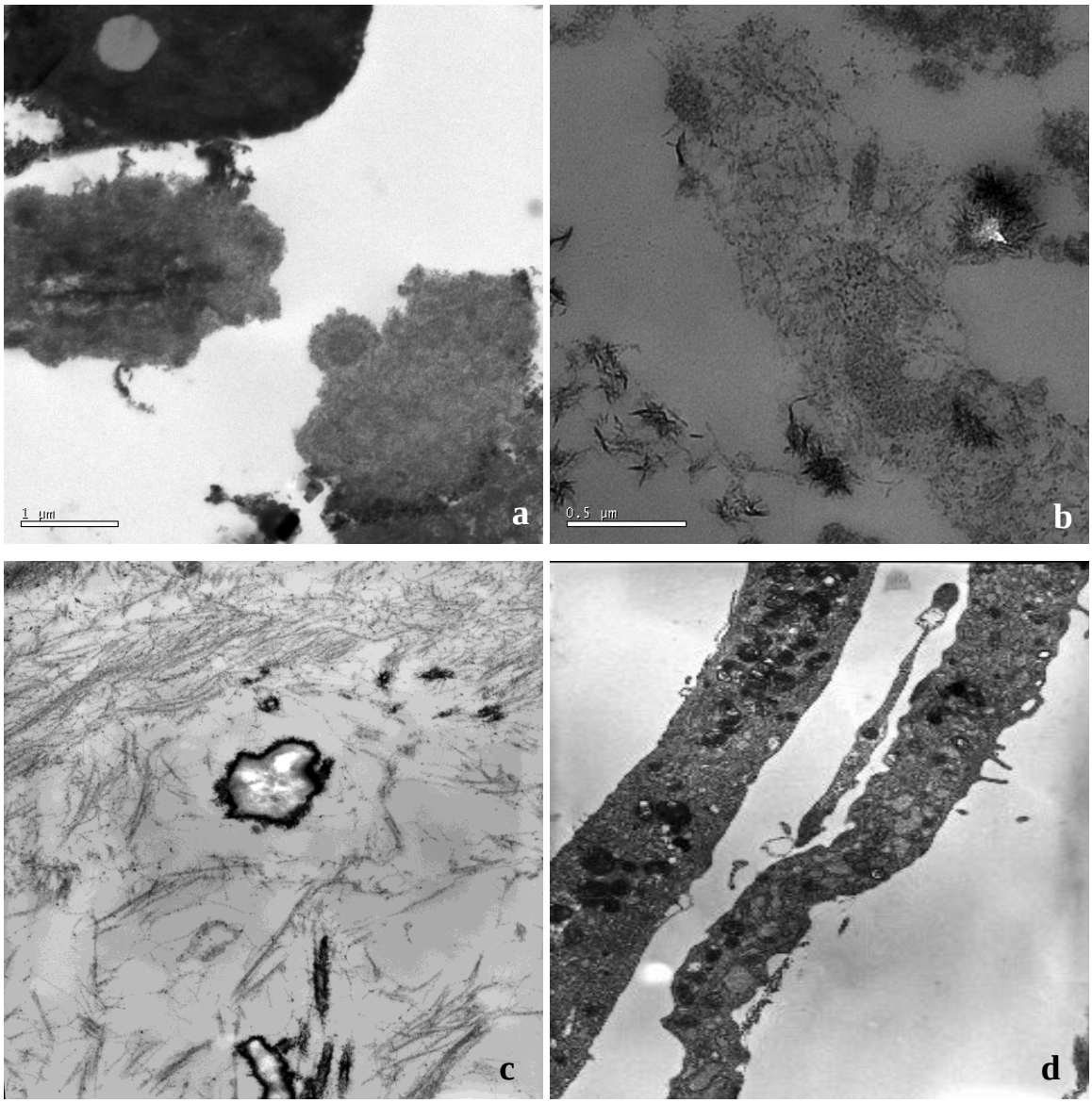


Fig.45: TEM analysis of H-MSCs after osteogenic committed. a) Osteoid matrix. b) Needle-shaped hydroxyapatite crystals. c) Terminal osteogenesis. d) undifferentiated H-MSCs

8.13 CHONDROGENIC DIFFERENTIATION

The chondrogenic potential of undifferentiated and differentiated H-MSCs pelleted and cultured in a medium without serum and containing

transforming growth factor-β3, under hypoxic condition, was assessed with alcian blue staining, immunohistochemical and ultrastructural analysis.

8.13.1 Alcian blue staining

Light microscopy on high-density H-MSCs pellets revealed that the cells were embedded in an abundant extracellular matrix during culture. The presence of a proteoglycan-rich extracellular matrix was shown by alcian blue staining in induced cells but not in controls (*Fig.46*).

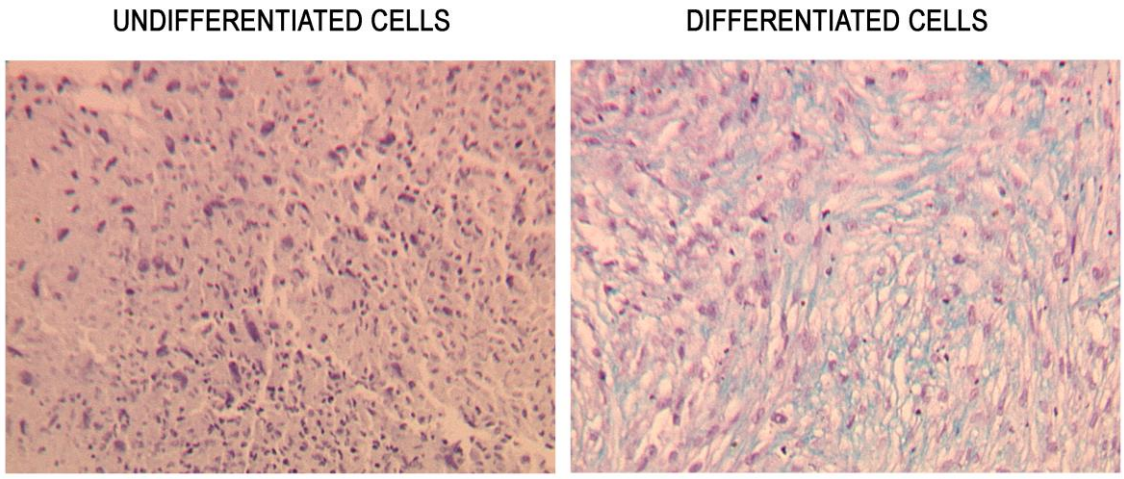


Fig.46*:* Alcian Blue staining of H-MSCs cultured as a pellet. There are abundant collagen bundles in the extracellular matrix of differentiated cell.

8.13.2 Type II collagen: immunohistochemical analysis

Chondrocyte-like lacunae were evident in histological sections, and immunohistochemistry analysis focally revealed, in extracellular matrix, the presence of human type II collagen, specific marker for chondroblasts, which is typically found in joint cartilage (*Fig.47*).

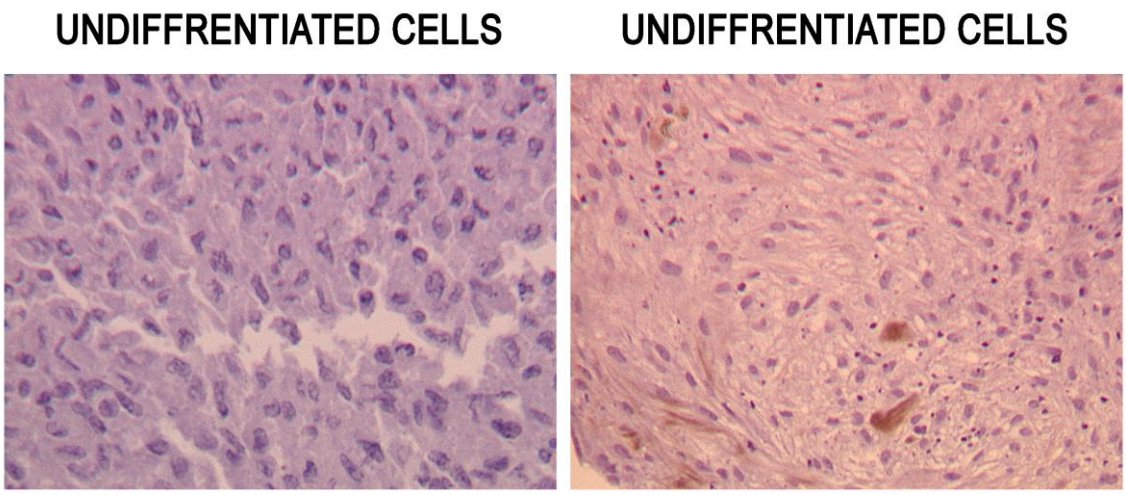
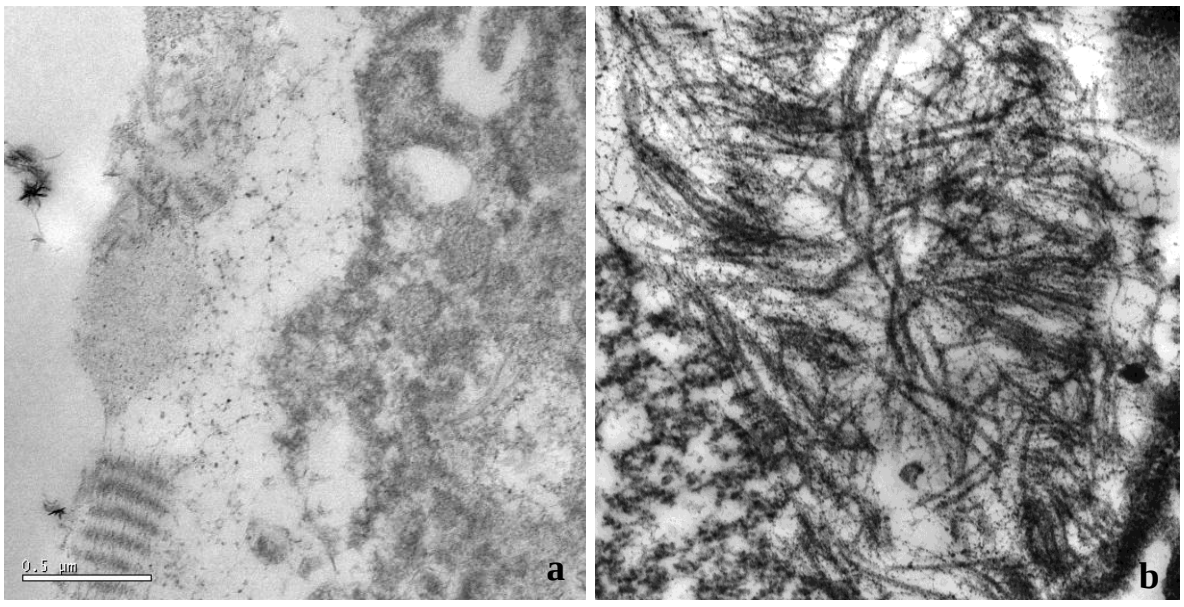


Fig.47: Immunohistochemical staining was focally positive for human type II collagen in extracellular matrix

8.13.3 <u>Ultrastructural analysis: TEM</u>

More careful ultrastructural observation confirmed the presence of abundant extracellular matrix containing mature collagen fibers and proteoglycan particles in induced cells exclusively (*Fig.48*).



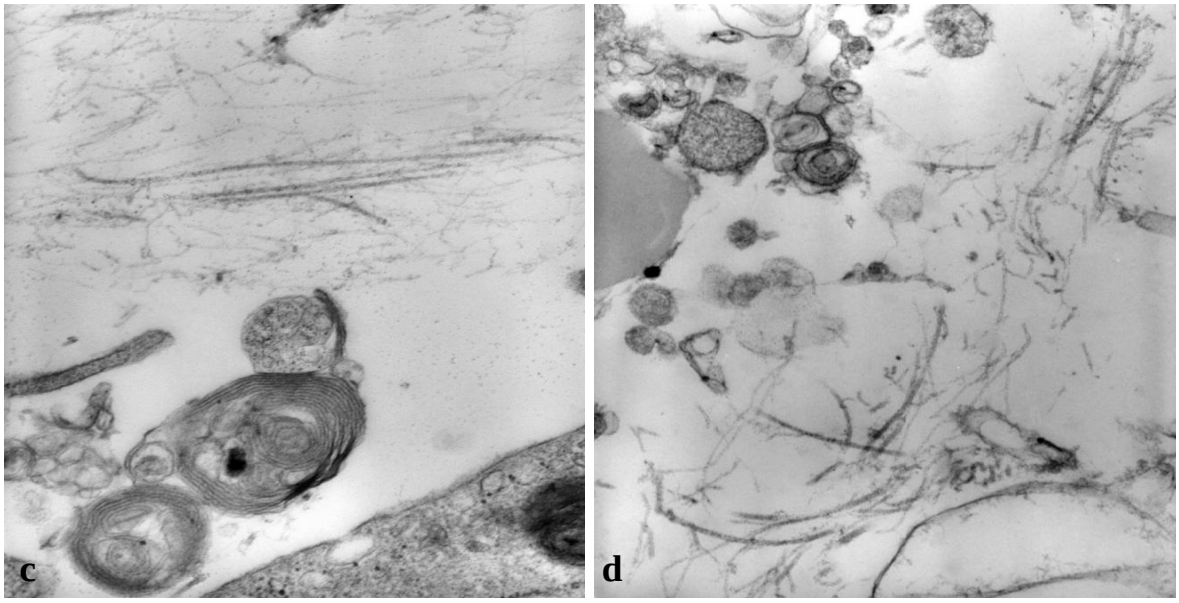


Fig.48: TEM analysis of H-MSCS after chondrogenic differentiation. a)Collagen fibers and proteoglycan particles. b) Increased mature collagen fibers. c) and d) Undifferentiated H-MSCs.

8.14 LEIOMYOGENIC DIFFERENTIATION

TGF-β1 and PDGF-BB were added to the culture medium for 2 weeks to induce leiomyogenic differentiation of mesenchymal stem cells isolated from human vascular wall homograft and the cellular response was assessed by immunofluorescence and RT-PCR for smooth muscle-specific markers expression and verified throughout ultrastructural analysis.

8.14.1 ***Immunofluorescence analysis***

Immunofluorescence labeling of undifferentiated and differentiated H-MSCs illustrated that, after culturing of 2 weeks in a medium containing TGF-β1 and PDGF-BB, they acquired the ability to express *de novo* α-SMA and Caldesmon while caused an increase of Calponin protein in the cytoplasm (*Fig.49*).

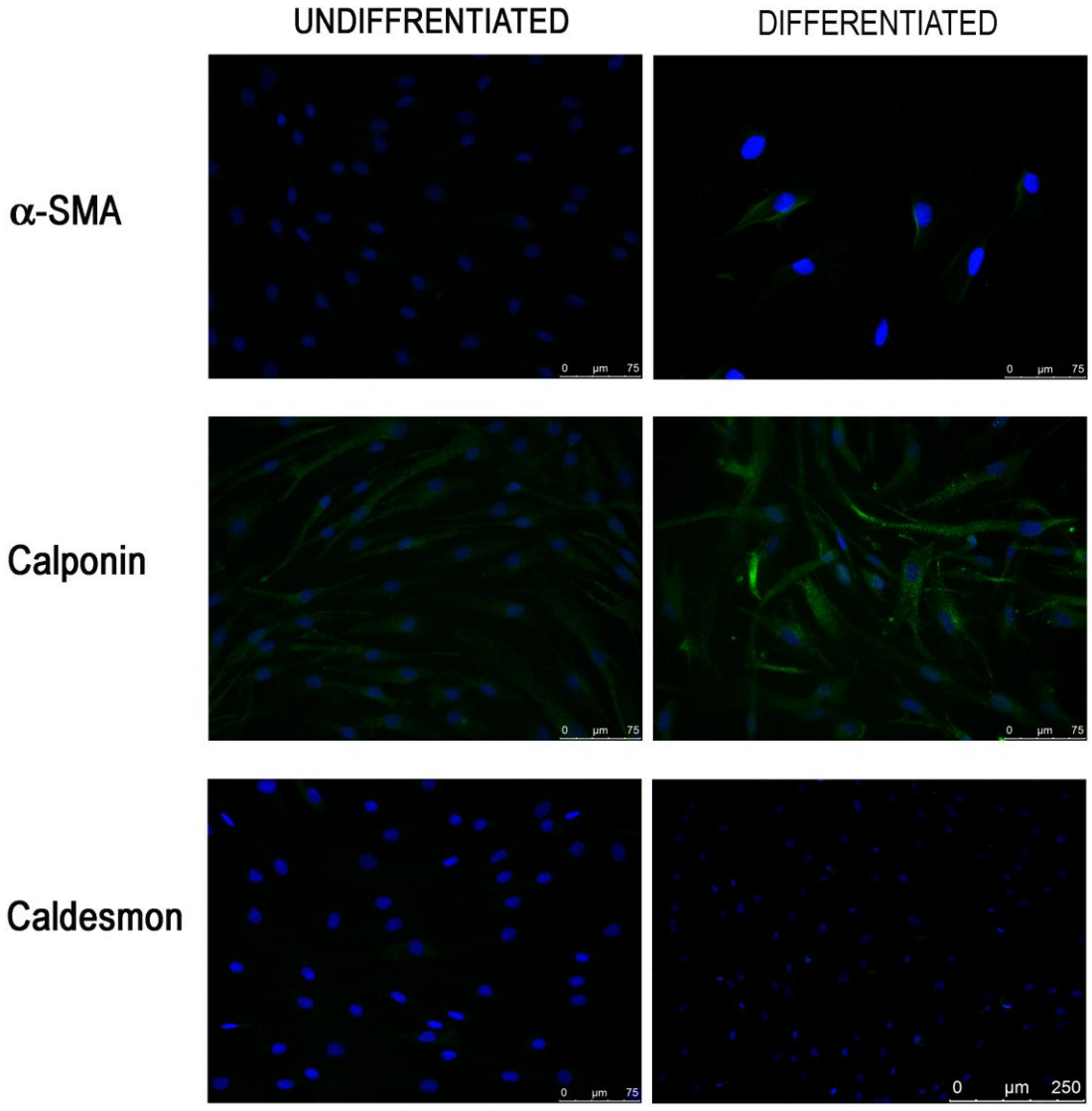


Fig.49: Immunofluorescent analysis of leiomyogenesis. Protein expression modulation of smooth muscle markers such as α-SMA, calponin, caldesmon and in differentiated and undifferentiated cells. Nuclei in blue and cell positive in green.

8.14.2 Semiquantitative analysis: RT-PCR

The expression of smooth muscle specific genes, such as α-SMA, calponin 2 and caldesmon, was evaluated by RT-PCR. Surprisingly we observed an upregulation of all genes and did not notice any differences between induced and uninduced samples (*Fig.50*).

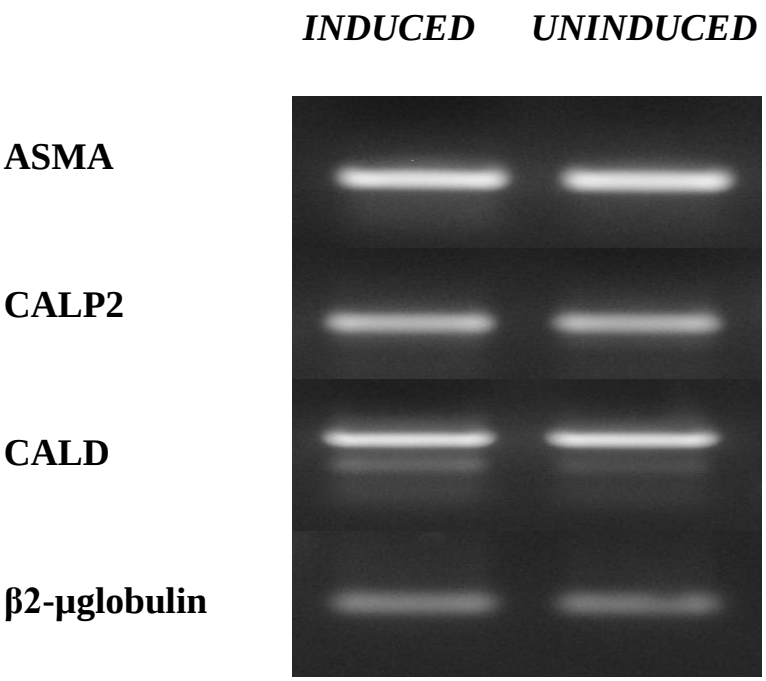


Fig.50: RT-PCR of smooth muscle markers. Beta-2globulin was used as control gene.

8.14.3 Ultrastructural analysis: TEM

The subcellular characteristics of undifferentiated and differentiated H-MSCs, after 3 weeks of leiomyogenic differentiation, were evaluated with transmission electron microscopy. The observation on H-MSCs treated with two well noted leiomyogenic inducible factors such as TGF-β1 and

PDGF-BB not showed features consistent with myofibroblast phenotype The induced H-MSCs showed some peripherally arranged contractile filaments along with profiles of rough endoplasmic reticulum; similar ultrastructural features was seen in the control cells (*Fig.51*).

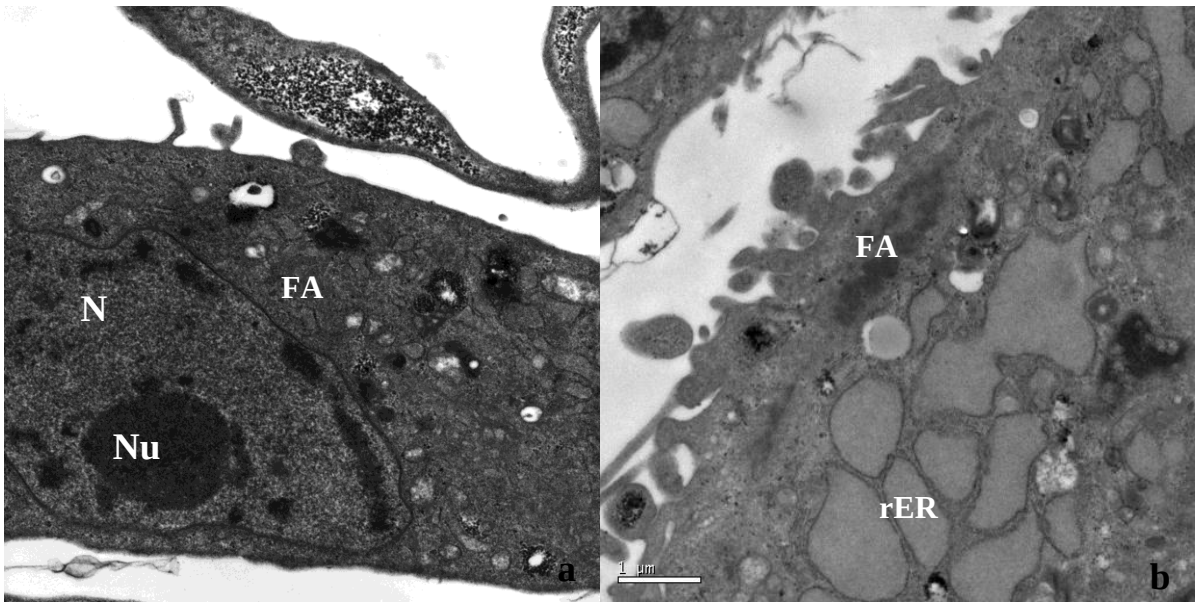


Fig.51: TEM analysis of leiomyogenesis. a) Smooth muscle cells with filaments contractile used as positive control b) Induced H-MSCs with arranged filaments contractile . Abbreviations: N: nuclei, Nu: nucleoli, rER: rough endoplasmic reticulum, FA: filaments contractile of actin

9. DISCUSSION

Cell proliferation and vascular wall remodeling are the first responses by arteries to pathological environmental stress (*Libby 2005, Ross 1999, Newby 2000*). In adult life, bone marrow derived stem cells and progenitors are continuously mobilized into the circulating blood to target areas of tissue injury. These cells may pertecipate not only in physiological renewal, providing rapid targeted rescue for regeneration after injury by giving rise to fully component resident vascular cells, but also in phatological conditions.

Most of the knowledge about resident vascular progenitors comes from animal models. Tintut et al. first demonstrated that MSCs are also present in the adult animal vessels. They described and isolated a subpopulation of vascular cells with self-renewal capacity and lineage plasticity (*Tintut et al. 2003*). Vascular progenitor cells, having stem cell markers, were found in the adventitia of ApoE-deficient mice and they contributed to experimental atherosclerosis and did not originate from the bone marrow (*Hu et al., 2004*). In 2006, it has been demonstrated the presence of resident precursors of endothelial and smooth muscle cells in healthy arteries of adult mices as defined "side population" by authors, using flow cytometry method (*Sainz 2006*). More recent studies are focused on their presence and role in the human vascular wall. Consistent with this view, the existence of a capillary-rich vasculogenic zone has been identified in adult human inner thoracic artery wall; in this area, situated at the border between the media and adventitial layer and possibly correspond to a vascular wall stem cell niche, might be found not only the VW-EPC but also resident stem cells including MSCs and hematopoietic progenitor cells (HPCs) (*Zengin et al., 2006).* This hypothesis is supported by several studies claiming to have found the in vivo reservoir of MSCs in post-natal tissue (*da Silva Meirelles*

et al., 2006, 2008). Several experimental observations shown that the same area in postnatal artery walls may contain resident MSCs with multilineage differentiation into different connective tissues. The presence of multipotent progenitors is not restrict to microvasculature; in fact, these resident MSCs have been isolated from different human large blood vessels such as the adult thoracic aorta (*Pasquinelli et al., 2007*) and pulmonary artery (*Hoshino et al., 2008*), and middle size vessels such as femoral artery *(Pasquinelli et al, 2010)*, saphena vein (*Covas et al., 2005*) and umbilical veins (*Covas et al., 2003*).

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 a previous study, our group identified, in normal human adult thoracic aortas and femoral arteries from young and healthy heart-beating multi-organ donors, the vasculogenic zone previously described by Zengin using immunohistochemical staining which make it unique in the arterial wall and congenial with a niche-like function (*Pacilli et al., 2009, Pasquinelli et al., 2006, 2007c*); we also isolated and described the presence of resident MSCs (VW-MSCs) with angiogenic ability (*Pasquinelli et al., 2007 a*) and adipogenic, osteogenic, chondrogenic and leyomiogenic potential (*Pasquinelli et al. 2010*) that present features similar to the mesenchymal stromal cells in bone marrow. All these evidences justify the research for adequate alternative sources of these cells for autologous and allogenic use.

To furnish a direct prove of VW-MSCs involvement in vascular remodeling, repair and in stress conditions, we optimize a new useful approach consisting in a long-term organ culture system, up to 70 days,

which gives the opportunity to follow the spontaneous vascular wall remodeling in a three-dimensional system without the influence of circulating blood components. The experiment was performed on healthy adult human femoral artery segments. The morphological analysis of vascular tissue revealed that, while the structural organization of intimal layers was unchanged over time, the media and adventitial tunic had the different behaviors. Until day 42 of culture, while muscular component gradually decreased, the adventitial layer shows an increase of cellularity due to the appearance of spindle cells. From day 42 onwards, the smooth muscle cells underwent a progressive cell death, while adventitia became repopulated. The most interesting finding of this study was that a new pseudo-intimal monolayer was formed during culture in the outer layers of vascular wall. The immunophenotype analysis of resident cellular components involved in parietal remodeling process confirmed their undifferentiated status. We also observed a well developed plexus of CD34+/CD31- small vessels at the border between the media and adventitia layers which topographically corresponds to the CD34+/CD31- cell layers already observed in human inner thoracic arteries and human thoracic aortas; recent studies reported that this zone is highly vasculogenic (*Zengin et al., 2006, Pasquinelli et al., 2007a*). Extending our observation, we found that the resident vascular wall cells involved in the process of remodeling in the adventitial tunic had a fibroblast-like appearance with long cytoplasmic projections and expressed molecules commonly found in MSCs such as CD44, CD90 and CD105. Again, ultrastructural investigation revealed a strict resemblance with MSCs. At the same time, the intima was lined with a similar layer composed by spindle cells, which showed a mature endothelial phenotype $CD31^+$ while focally expressing $CD34^+$. We could not exclude that this spontaneous phenomenon involve two distinct progenitors located in different functional areas of the wall; an

alternative hypothesis could invoke the role of ECM in driving functional differentiation close. Noteworthy was the observations of elongated elements expressing the stem cell surface marker c-kit. This subpopulation of c-kit+ cells was found in the adventitia stroma close to neoformed adventitial layer and in association with the vasculogenic area.

On the basis of these observations we retain that H-MSCs could have an important role in the onset of vascular pathologies, i.e. atherosclerosis and aneurysms.

We focused our attention on pathological cases of homografs of femoral arteries, furnished by operating room of Vascular Surgery of the S.Orsola-Malpighi General Hospital of Bologna, to identify and to localize in situ, with immunoistochemical analysis, the adult stem cell populations that could be reside in the vasculogenic zone and to understand their role in the vascular wall remodeling. Subsequently, we isolated this cell population and tested *in vitro* their multilineage differentiation potential through immunohistochemical, immunofluorescence, RT-PCR and ultrastructural analysis. Immunohistochemical tissue studies, using specific antibodies commonly expressed by mesenchymal stem cells, showed a highly CD44 positive cell layer and some other elongated cells weakly positive for CD90; these cells weremainly localized in the vasculogenic zone corresponding to the border line between medial and adventitial tunicas of the homograft wall. Besides, even pericyte and perivascular cells of the rich vascular plexus present in the adventitial tunica showed an high positivity for CD44 and a low positivity for CD90. Near the vasculogenic zone were also observed few CD105 and CD166 weakly positive cell.

These observations confirmed the existence of a vasculogenic zone also in pathological arteries which localize at the border line between the medial and adventitial tunicas, containing a resident population of stem cell potentially able to generate, in a specific hierarchic organization, stromal

supporting elements (MSC). The different expression of some mesenchymal markers from the healthy counterpart suggest a probably dissimilar grade of stemness, commitment and multipotential of the homograft derived MSCs which could explain the irreversible status of vascular damage. Hence, we isolated a cell population from vascular wall homografts which shared many properties with mesenchymal stem cells and we named these cells Homograft derived Mesenchymal Stem Cells (H-MSCs). From 25-30cm^2 of each homograft sample, we obtained 1,5x10^6 to 3,5x10^6 mononuclear cells using a combination of enzymatic and mechanical procedures and, subsequently, the cells were seeded in culture plate with D-MEM supplemented with 10% FBS and then cultured at 37°C in 5% CO_2 humified atmosphere. Cell isolation was carried out successfully in two cases. The remaining case was discarded due to fast growth of contaminant bacteria at passage 2. After 2 days from initial seeding, the non-adherent cells were discarded; the adherent cells shared many morphological properties of MSCs isolatedfrom bone marrow, such as: a strong adherence to plastic, a fibroblast-like spindle-shape morphology, a tendency to grow in colonies, and a highl proliferative ability (*Dominici et al., 2006*). An additional finding comes from results with alamar blue assay. The assay highlight that proliferative capacity of these cells increases with time, reaching a plateau on day 10 and then remaining almost constant until day 21 (end of the experiment). High cell proliferation was also confirmed by Ki-67 positive immunofluorescence, a cell proliferation marker able to recognize a nuclear protein expressed in the G1, S, M and G2 phases of the cell cycle. The final number of resident mesenchymal stem cells isolated from 25 cm^2 of each homograft segment was estimated around ≈ 0,5-2,0x10^4 cells. All these data demonstrate that MSC are present in failed homografts.

The fibroblast-like cells had the typical antigen expression pattern of culture-expanded MSCs; the majority was positive for a mixture of molecules commonly found in MSCs such as CD90, CD44, CD105, CD29, CD73; moreover stemness molecules such as Notch-1, Oct-4, Sca-1, Stro-1 were also expressed. These cells also expressed NG2, a pericyte marker; this finding support the recent evidence that pericytes may represent the MSC in situ counterpart (*Covas et al.,2008, Crisan et al., 2008, da Silva Meirelles et al., 2008, Doherty et al., 1998, Farrington-Rock et al., 2004*). On the contrary, they were negative for the early and late hematopoietic markers CD34, CD133 and CD45. H-MSCs were also negative for KDR, CD146, CD31 and vWF which are markers of differentiated endothelium whilst c-Kit was not detected. The immunophenotype of isolated H-MSCs was: $CD44^{+}$, $CD90^{+}$, $CD105^{+}$, $CD29^{+}$, $CD73^{+}$, $Sca\text{-}1^{low}$, $Stro\text{-}1^{+}$, $Oct4^{+}$, $Notch\text{-}1^{+}$, $CD45^{-}$, $CD34^{-}$, $CD133^{-}$, KDR^{-}, $CD31^{-}$, vWF^{-}, $CD146^{-}$, $c\text{-}Kit^{-}$ and $NG2^{+}$. Again, ultrastructural investigation revealed a strict resemblance with MSCs from bone marrow. After culturing, these cells showed high ratio nucleus/cytoplasm with a large euchromatic nuclei and prominent nucleoli; a multivacuolar appearance and a high synthetic competence. i.e. prominence of rough endoplasmic reticulum with dilated cisternae. More careful observation of peripheral cytoplasm showed the presence of collections of clear vesicles, vacuoles and blisters giving a labyrinthine appearance (*Pasquinelli et al., 2007 b*).

Besides flow cytometry analysis, a immunofluorescence staining confirms the mesenchymal status of H-MSCs as showed by intense vimentin positivity; the smooth muscle cell phenotype was excluded because of the presence of only rare cells positive for the α-SMA and the negativity for other smooth muscle markers like h-Caldesmon, desmin and PDGF-rβ. The loss of contractile filaments by vascular cells may explain the development of aneurysmatic pathology.

Stem cells gene expression analysis by RT-PCR revelead that H-MSCs express transcripts associated with stem cells; some of these molecules are indeed involved in stem cell specific functions such as BCRP1,maintaining the undifferentiated state of embryonic stem cells; Notch-1, suggesting a major role in regulating the stemness and the undifferentiated status of H-MSCS. To explain the low expression level of Oct4, we hypothesized that a subset of VW-MSCs had more ancestral characteristic Moreover, the H-MSCs showed very low expression of KDR, VEGF receptor type 2, as well as CD133 an hematopoietic stem cell transcript. The expression of the BCRP-1 molecule is also linked to the specific ability of stem cells to exclude dyes such as rhodamine and Hoechst. This property that identifies a small subset of stem cells termed the "side population" (SP), is related to the expression of specific transporter proteins(*Pasquinelli et al., 2010*).

We also studied the multipotency of H-MSCs; in particular we examined their angiogenic, adipogenic, osteogenic, chondrogenic and leiomyogenic abilities. VEGF preconditioned H-MSCs showed a poor ability in forming neovessels in a Matrigel assay. After 2 hours of culture, the H-MSCs rapidly aligned and formed hollow tube-like structures with thin cytoplasmic projections sprouting from the cell periphery. After 4 hours, the same cells appeared connected by thicker projections forming an evident capillary-like network but after only 6 hours this capacity was lost. This angiogenic ability was strictly depended on VEGF induction; controls never formed capillary structures. VEGF induction was accompanied by a moderate increase in vWF cell expression; VEGF expression was upregolated as documented by flow cytometry and RT-PCR assay and confirmed by immunofluorescence staining. This low angiogenic commitment could be explained with the complete absence of expression of KDR and a lower expression of the VEGF type-I receptor, FLT-I. The VEGF-A through the interaction with its receptors (VEGF-R1/Flt-1,

VEGF-R2/KDR/Flk-1) plays a major role in driving angiogenesis in vivo; it promotes cell proliferation as well as migratory and sprouting activity, and contribute to the formation of capillary-like structures. These effects seem to be maintained largely by the interaction of VEGF-R2 or KDR. Also, the ultrastructural features confirmed the H-MSCs angiogenic failure; we didn't observe characteristics consistent with endothelial phenotype such as Weibel-Palade bodies. On the contrary, the VW-MSCs isolated from healty vascular segments are more prone to angiogenesis and presumably able to repair vascular defects and to supply dead cells (*Pasquinelli et al., 2007a*). H-MSCs exhibit a good propensity for osteogenic commitment. After 10 day of osteogenic conditioning, we noted morphological changes, such as the loss of cytoplasmic processes; and the quantity of calcium deposition increased with the time of induction. The von Kossa staining, performed on H-MSCs after three weeks of osteogenic induction, confirmed the presence of marked brownish calcium deposits that were spread evenly across the plate. The TEM analysis revealed that ossification corresponded to a stage preceding the formation of the trabecular bone; in fact, at the end of osteogenic assay, we observed an osteoid matrix enriched with multiple foci of electron dense fibrillary deposits which were decorated with needle-shaped hydroxyapatite crystals. By contrast, undifferentiated H-MSCs retained their morphology and did not display calcium deposition in extracellular matrix. As for adipogenesis, after three complete rotations of cell culture with adipogenic induction medium and adipogenic maintenance medium, we observed the presence of multiple lipid-rich vacuoles into cytoplasm which were intensely stained with the lipid dye Oil Red O; the lipid dropletsincreased in size and number with the time of induction. Ultrastructural observation of multiple and confluent lipid droplets in the cytoplasm, small dense mitochondria and

numerous endocytic vesicles, confirmed the propensity of H-MSCs for adipogenesis. Unconditioned cells did not display cytoplasm lipid vacuoles. Regarding chondrogenesis, we noted that H-MSCs cultured for three weeks in a medium without serum and containing transforming growth factor-β3 under hypoxic condition, were embedded in an abundant proteoglycan-rich extracellular matrix intensly stained by alcian blue. Chondrocyte-like lacunae were observed and markers of cartilaginous differentation such as human type II collagen was detected focally in extracellular matrix by immunohistochemistry analysis. More careful ultrastructural observation confirmed the presence of abundant extracellular matrix containing mature and immature collagen fibers and proteoglycan particles in induced cells exclusively. Probably the intense mesengenic differentiation ability i.e. adipogenic, osteogenic and chondrogenic could be related to the pathology of the arterial wall. Moreover, it should be noted that "ectopic" tissues, e.g. cartilage, bone and fat can be seen during histopathological observation especially in atherosclerosis and other vascular diseases (*Abedin et al., 2004*). Unlike the healthy counterpart that has a low ability to form calcification, H-MSCs performed this task efficiently. This could explain a high propensity to ossify unlike his a low predisposition (*Pasquinelli et al., 2007a*).

Also interesting were the observations from leiomyiogenic commitment. We had already noted that basal cells did not express contractile proteins thus we performed a smooth muscle induction to evaluate if the expression of tipical smooth muscle proteins such as ASMA, calp2 and H-caldesmon could be induced. After differentiation we observed that no smooth muscle proteins are present in both uninduced and induced samples, although RT-PCR analysis showed an high expression of their transcripts. These results are very important because even if the H-MSCs did not acquire the smooth muscle phenotype, their genetic controls spountaneosly cause an

upregulation of contractile genes to compensate the loss of protein and to recover their native function. In aneurysmatic cells probably exist a post-transcriptional regulation of smooth muscle and contractile genes which determine the absence of the respective proteins and the loss of vascular function.

In conclusion, this study confirms the recent acquisition that the MSC compartment is more widely distributed than previously thought; it further highlights that MSCs are resident in a vasculogenic niche of healthy and pathological vascular walls.

The present work demonstrates the existence of resident H-MSCs with morphological, phenotypic and functional mesenchymal stem cells properties also in a human failed homograft. H-MSCs are long-lived in culture, highly proliferating and multipotent for their strong ability to differentiate into adipocytes, osteocytes and chondrocytes. Their vascular plasticity suggests that these cells could participate in arterial homeostasis and remodeling in a normal artery but in a pathological condition these results suggest that H-MSCs have an important role in the pathological vascular remodeling wall of the atherosclerosis and aneurysms.

The study of H-MSCs could elucidate the pathological mechanisms of vascular aneurysms and may be useful for the development of new therapeutic strategies.

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