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RESIDENT ANGIOGENIC MESENCHYMAL STEM CELLS FROM MULTIORGAN DONOR THORACIC AORTAS

Tesi di Dottorato

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INTRODUCTION

1. STEM CELLS

1.1 DEFINITION and CLASSIFICATION OF STEM CELLS

In the last years stem cells have became a new fascinating topic of research in biology due to their extensive regeneration potential and their functional differentiation capacity. Stem cells rapidly became attractive for many applications like tissue engineering, cellular therapies and drug screening. The biology of stem cells is very important to understand how an organism develops from a single cell and how healthy cells replace damaged cells in adult organisms. Stem cells are particular types of cells that differ from other kinds of cells in the body. All stem cells- regardless of their source- must fulfill three criteria (Fig.1) (*National Institute of Health, 2002;Ulloa-Montoya et al.,2005*)

1. *They must be capable of self-renewal,* i.e. dividing and renewing themselves for long periods: when cells replicate themselves many time over it is called proliferation. If the cell descendants continue to be unspecialized, like the stem cells parents, the cells are said to be capable of long term self renewal.
2. *They are unspecialized cells* : they don't have any tissue-specific structures that allow it to performs specialized functions, but unspecialized cells can give rise to specialized cells.
3. *They can give rise to specialized cell types:* when unspecialized cells give rise to specialized cells, the process is called differentiation that is trigger by internal signs (cells genes) and external signs (cytokines secreted by other cells, physical contacts with neighboring cell and different molecules present in the microenviroment).

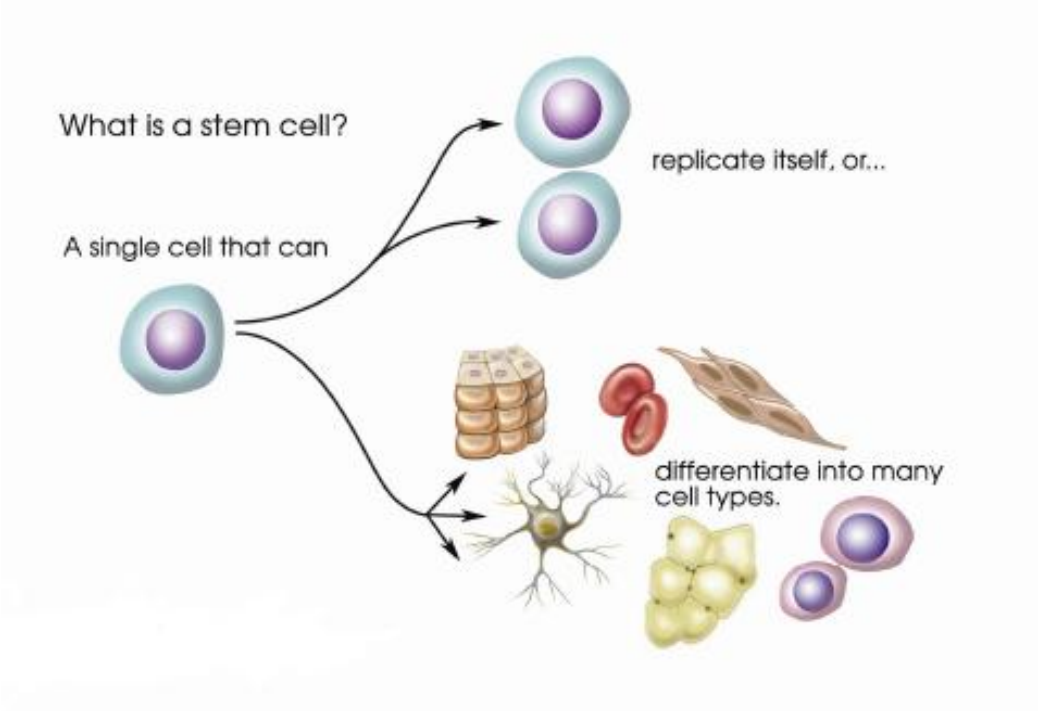

Fig. 1: Criteria for definition of "stem cells"

Stem cells are classified according to their differentiation capability. The *Zygote* is defined the totipotent stem, whereas the *Embryonic Stem Cells* are considered Pluripotent Stem Cells for their capacity to differentiate into the three somatic germ layers: mesoderm, ectoderm and endoderm. The *Adult Stem Cells* or *Somatic Stem Cells* can be divided into multipotent and paucipotent stem cells. The multipotent stem cells maintained the undifferentiated state and their multipotentiality through asymmetric divisions. The paucipotent stem cells or *committed progenitors* can differentiate only into cells of one tissue or germ layer in which they reside like bone marrow, brain, skin, liver, skeletal muscle, peripheral blood and blood vessels.(Fig.2)

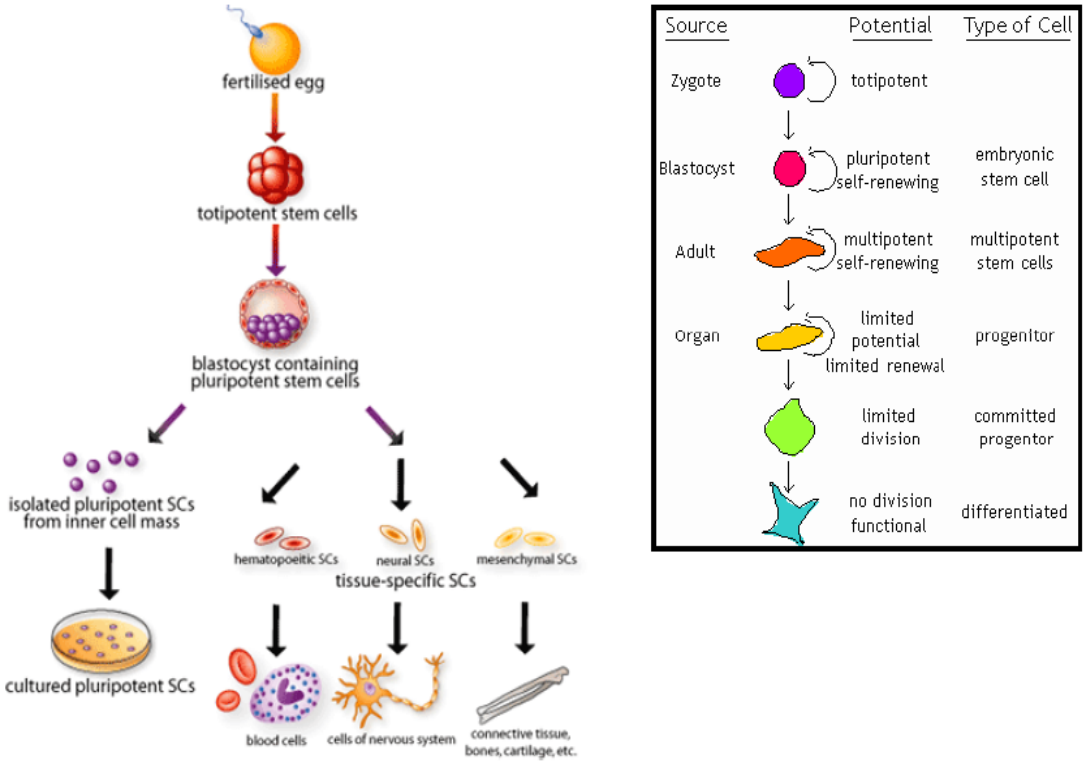

Fig.2: Classification and destiny of stem cells

1.2 THE EMBRYONIC STEM CELLS

The *Embryonic Stem Cells (*ESCs) are derived from the inner cell mass of the blastocyst from the 3 to 5 day old embryos. These cells can give origin to hundreds of highly specialized cells needed to make an adult organism. The ESCs have unlimited self renewal and differentiation potential. They are capable of giving rise to cells of the three somatic germ layers that constitute an organism: mesoderm (muscle, bone), ectoderm (neurons, skin, etc.) and endoderm (hepatocytes, pancreatic beta cells,)(Fig.3); for this property the ESCs are defined pluripotent stem cells.

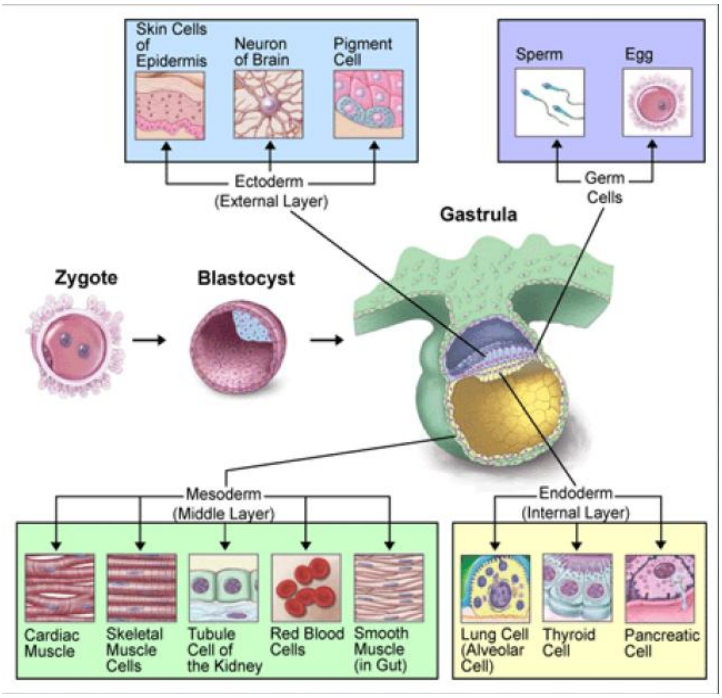

Fig.3: Characteristics and differentiation potential of ESCs

The ESCs were first isolated from mice in the 1981 (*Martin, 1981,Evans et al., 1981)*, then from non human primates and in the 1998 from humans (*Thomson et al., 1995,1998*). At the beginning, ESCs were created for infertility purposes through in vitro fertilization procedures and when they were not longer needed for that purpose, they were donated for research with the informed consent of the donor. Mouse ESCs are able to give rise embryogenesis when injected into a pre-implantation embryo, producing functional differentiated progeny in all tissue and organs even after numerous propagation and manipulation *in vitro* (*Smith, 2001*). In theory, also the human ESCs should be able to give the same results, but for ethical reasons this cannot demonstrated. The pluripotency of ESCs is tested by the colony-forming ability in culture, the expression of numerous surface markers like SSEA-1 in the mouse and SSEA3, SSEA-4 and TRA-1 in humans (*Henderson et al., 2002, Carpenter e al., 2003*). Further it is crucial the expression of transcription factor correlated with the stem ability and

the undifferentiated state like OCT4, NANOG and SOX2 (*Niwa et al., 2000, Chambers al., 2003, Avilion et al 2003*). Besides, the pluripotent differentiation of ESCs has been show when they were transplanted into post natal animals: they generated tumors called *teratomas* consisting of different type of tissues belong to the three germ layers (*Wobus et al, 1984, Reubinoff et al., 2000*). However, this feature represents a challenge on ESCs application for clinical therapies because of the need to control undifferentiated cells to avoid teratoma formation after transplantation.

Another aspect about the property of ESCs that must be considerate for the clinical application is their immunogenicity. In particular the ESCs expression of Human Leukocyte Antigen Class II (HLA-II) can trigger immune rejection in the case of heterologus transplantation. Considering this problem and for the ethical issue connected with the ESCs, the researchers have addressed their study on the Adult Stem Cells (*Watt et al., 2000*).

1.3 ADULT STEM CELLS

Also the *Adult Stem Cells* or *Somatic Stem Cells* answered to the criteria that defined Stem Cells. They had a self-renewal degree and differentiation potential lower than ESCs. The adult stem cells are undifferentiated cells able to renew itself and to differentiate into specialized cell types of the tissue or organ in which they reside (multipotent potential). Thus the fundamental role of Adult Stem Cells in living organism is to maintain the homeostasis and to repair the damaged tissues. Scientists have identified adult stem cells in different tissues and this finding had led to speculate if these cells are suitable for transplantation. The possibility to control their differentiation potential could propose these cells as the best candidate for cellular therapy in common diseases (*National Institute of Health, 2002).*

The researches on adult stem cells starts in the 1960 when were found in the bone marrow two kind of cells: the HEMATOPOIETIC STEM CELLS (*Islam, 1985*) and the MESENCHYMAL STEM CELLS (*Friedenstein et al., 1974*) (Fig.4).

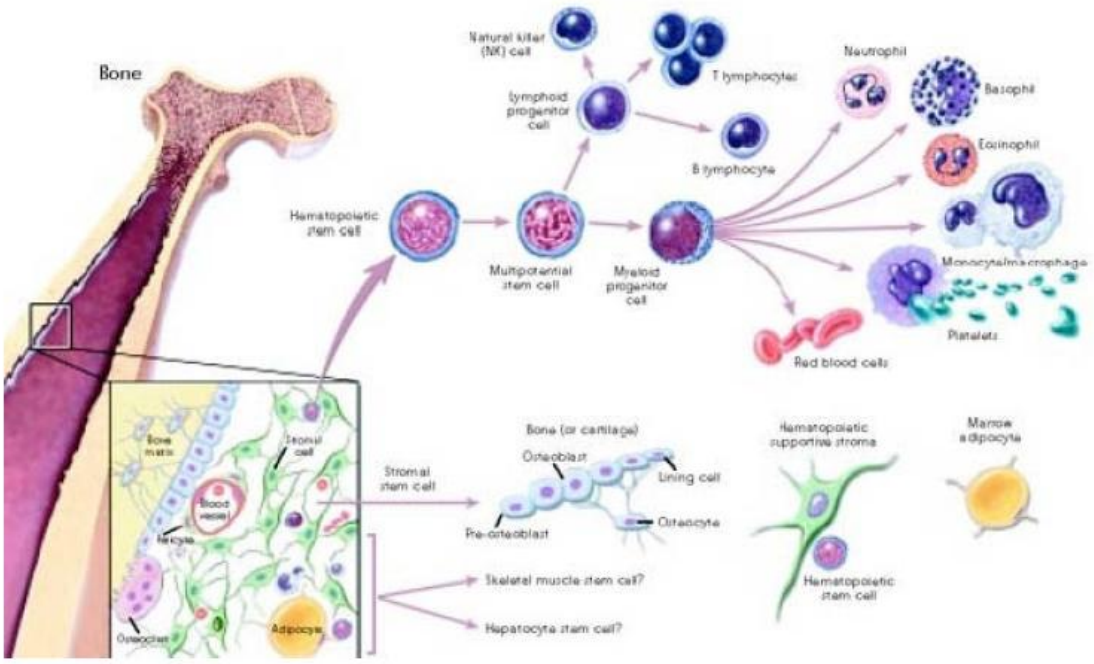

Fig.4: The ADULT STEM CELLS in the bone marrow

The most extensively studied adult stem cells are the *HEMATOPOIETIC STEM CELLS (HSCs)*: a type of the adult stem that is the source of all blood cell lineages. The HSCs were first isolated from bone marrow in the mouse (*Spangrude et al., 1988*) and now they can be obtained in humans from bone marrow, peripheral blood and umbilical cord blood (*Ulloa-Montoya, et al., 2005*); the human HSCs have been characterized for the expression of cell surface marker CD34+/CD38-. The principal role of

HSCs is to continuously differentiate into multiple lineages of different blood cell types (Fig. 5) , simultaneously replicating themselves through self-renewal to prevent depletion of the stem cells pool in the bone marrow (*Huang et al, 2007*).

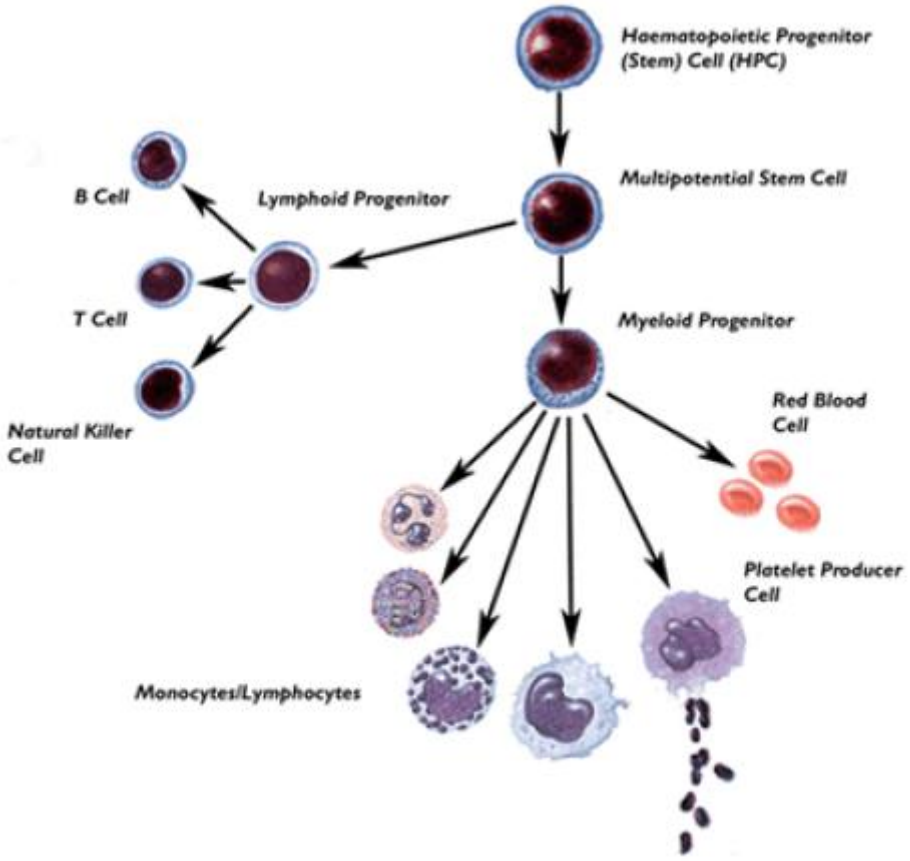

Fig.5: HSCs and their differentiation lineages

HSCs are used in the clinical practice (bone marrow transplantation) for reconstitution of patient's hematopoietic system after undergoing chemotherapy or radiotherapy to treat cancer and diseases such as aplastic anemia, thalassemia, etc. (*Ulloa-Montoya et al.,2005*). Bone Marrow-HSCs (BM-HSCs) are normally quiescent, the fate choice to either self-renewal or

differentiate is controlled by complex interplay mechanisms between intrinsic and extrinsic signals, e.g the microenvironment of the niche in which they reside. Furthermore, it is well demonstrated that the HSCs undergo asymmetrical divisions, in which individual HSCs give rise to non identical cell descendants, one keeping the features of HSCs and the other becoming a differentiated progenitor cell. External environmental signals must integrate with intrinsic molecular mechanisms to control the fate decisions of HSCs. In particular several transcription factors, playing as intrinsic molecular mechanism, are implicated in the regulation of self renewal like: Transcription factor translocation Ets leukemia (tel)(*Hock et al., 2004*), Hox4 (*Sauvageau et al., 2004*), Stat5 (*Kato et al., 2005),* Stat3 (*Chung et al., 2006*). In addition several proteins involved in the modulation of gene expression have been found to regulate HSCs self-renewal like BMI-1,which, together with proteins, 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 self renewal are numerous like the transduction pathways of Notch, WNT, BMP and Sonic hedgehog (Shh). Functional genomics and newly developing technologies will continue to extend the understandings of HSCs characteristics in order to obtain protocols for HSCs *in vitro* generation, expansion and of differentiation control (*Huang et al., 2007*).

Different kind of adult stem cells have been studied although they are not well characterized as HSCs. Neuronal stem cells give rise to neurons, astrocytes and oligodendrocytes (*Gage et al.,2000*), where as Mesenchymal stem cells can differentiate into fibroblast, osteoblastas, chondroblasts, adipocytes and skeletal muscle (*Friedestein, 1982, Prockopt 1997, Pittenger et al., 1999*). In the last years, other stem cells have been identified like gastrointestinal stem cells (*Potten, 1998*), epidermal stem cells (*Watt, 1998*) and hepatic stem cells (*Alison, 1998*). Recently a new

population of pluripotent stem cells defined Multipotent adult progenitors cells (MAPC) have been isolated from bone marrow of post natal human and rodents, that can differentiate in vitro in cells of the three germ lineages (*Reyes et al., 2001*).

The hypothesis that Somatic Stem Cells can differentiate only in the cell types of the tissue in which they reside has been revaluated. In the last years, several experiments showed the possibility that adult stem cells from one tissue may be able to give rise to cell types of a completely different tissue . This Property is called *Plasticity* (Fig.6). For example, bone marrow derived cells differentiate or trans–differentiate into myoblasts (*Ferrari et al,1998),* endothelium (*Rafii et al., 1994, Asahara et al., 1997, Lin et al., 200, Orlic et al., 2001*), liver, biliary duct (*Peterson et al., 1999, Theise et al 2000*), lung, gut (*Lagasse et al., 2000*) or neural tissue (*Mezey et al., 2000*); MAPC were shown to differentiate into neurons and hepatocytes (*Zhao et al 2002, Schwartz et al., 2002*); neuronal stem cells differentiate into hematopoietic stem cells (*Bjornson et al.,1999*), bone marrow cells regenerate infarcted myocardium (*Orlic et al, 2001*) and Mesenchymal Stem Cells (MSCs) from fetal membrane enhanced cardiac repair in infarcted rat hearts when induced in culture with a mixed ester of hyaluronan with butyric and retinoic acid (HBR) (*Ventura et al., 2007*).

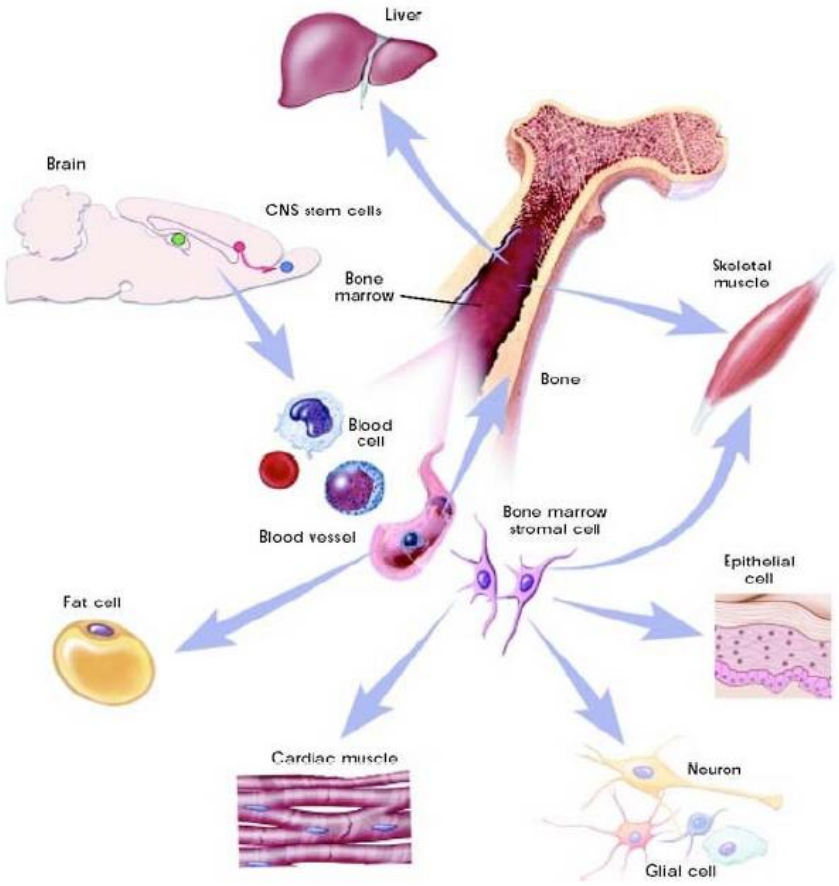

Fig.6: Plasticity of Adult stem cells

Even if cumulative experimental in vitro and in vivo evidences suggested the plasticity of the adult stem cells lines, their clinical use can be consider safety in replacing cells and human tissues. Finally, the ability of adult stem cells to differentiate into specific cell types offer the possibility of a renewable sources of replacement cells and tissues to treat diseases like Parkinson, Alzheimer, stroke, heart disease, diabetes and rheumatoid arthritis. However the principal problem for the therapeutically use of adult stem cells is the lack of a sufficient number of stem cells available. Unlike embryonic stem cells, adult stem cells like MSCs lacked telomerase activity (*Zimmermann et al., 2003*) and showed limited ex vivo proliferation capability, reaching senescence and losing multilineage differentiation potential after 34-50 population doublings in culture.

1.4 MESENCHYMAL STEM CELLS

For many years the researchers hypothesized the presence of cells with the property of stem cells among the non hematopoietic stem cells. The pioneering work of Friedestein et al, who first demonstrated that bone marrow derived cells were capable of osteogenesis (*Friedenstein et al., 1961*), described the existence of a subpopulations of stromal cells that support normal adult hematopoiesis (*Dexter et al., 1977, Calvi et al, 2003*)**.** The Term Mesenchymal Stem Cells (MSCs) was popularized by Caplan (*Gao et al 2001*) describing a cell with a fibroblast like morphology isolated by Percoll density centrifugation that grew adherent when cultured in a plastic support and that expressed SH2 and SH3 antigen. Today the general protocol for isolating MSCs from bone marrow involves the isolation of mononuclear cells using a gradient centrifugation and seeding these cells on tissue culture plates in medium with fetal bovine serum. After attachment of the adherent fraction, the medium is removed to eliminate non–adherent cells and remaining cells are expanded in vitro for a limited number of passages (*Ulloa-Montoya et al., 2005*). The adjective Mesenchymal can give some ambiguity. In fact, the mesenchyme is correlated with tissue of mesodermal origin, the middle embryological germ layer, giving rise to blood, vascular, musculosketal, urinogenital system and to connective tissue including dermis. So, the term mesenchymal should include both blood and connective tissue ; in fact, now there are some evidences of the existence of a common precursors for the HSCs and endothelial cells (EC), called haemangioblast (*Ema et al., 2003, Jaffredo et al., 2005),* a cell derive from embryonal mesoderm. Today, the more reliable hypothesis is that hemangioblast derived from mesenchymal stem cells. Accordingly, the nomenclature for this type of cells is not consistent, so the cells with non hematopoietic multipotency included " colony-forming units-fibrobalsts", "stromal cells stem", "mesodermal progenitors cells", "skeletal stem cells", "mesodermal progenitors cells", " non hematopoietic stem cells", and so on

(*Sethe et al., 2006*). In 2005, the International Society for cellular Therapy have defined the nomenclature for MSCs: In this position paper, the Society proposed that the plastic-adherent cells currently described as mesenchymal stem cells could be termed Multipotent mesenchymal stromal cells, while the term mesenchymal stem cells should be reserved for a subset of these (or other) cells that demonstrate stem cells ability by clearly stated criteria. For both the population, the acronym MSCs may be used (*Horwitz et al., 2005*).

Now is clearly described the presence of a niche within the bone marrow that support the survival and the growth of the HSCs. This niche is formed by stromal cells (endothelial cells, adipocytes, macrophages, reticular cells, fibroblasts, osteoprogenitors cells) and extracellular microenvironment where MSCs are presumed to exist (*Baksh et al., 2004*). Since the early work of Castro-Malaspina (*Castro-Malaspina et al., 1980*), many researchers have developed different way to isolate and purified a populations of mesenchymal stem cells. In the 1999 MSCs were isolated from human post natal bone marrow (*Pittenger et al 1999*) and even if bone marrow is considered a well-accepted source of MSCs, in the last years, MSCs were isolated from many other human sources like umbilical cord blood (*Erices et al., 2000*), adipose tissue, connective tissue , peripheral blood, skeletal muscle (*Baksh et al., 2004*) and more recently from deciduous tooth (*Miura et al., 2003*), umbilical cord mesenchime (*Romanov et al., 2003*) and fetal membrane (*Zhang et al, 2004, Alviano et al , 2007*). Term Amniotic membrane is a very attractive source of MSCs due to the fact that is a fetal tissue usually discarded without ethical conflicts leading to a high efficiency in MSCs 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 as an initial site to liver and then to bone marrow with a consequent migration of HSCs and MSCs

In the last years, considerable progress has been made towards characterizing the cells surface antigenic profile of Human bone marrow derived MSCs populations using FACS (*Fluorescence activated cell sorting)* and magnetic bead sorting techniques. To date, a single marker to definitely delineates this cells has yet to be identified, so now the principal criteria for the definition of MSCs are (*Prockopt et al, 1997*): the ability to adhere to tissue culture plastic, the typical fibroblast-like morphology and the formation of colonies (termed colony forming unit-fibroblasts [CFU-f]). However the primary important feature of MSCs is their ability to differentiate in vitro, in different culture conditions, into several cell types (Fig.7).

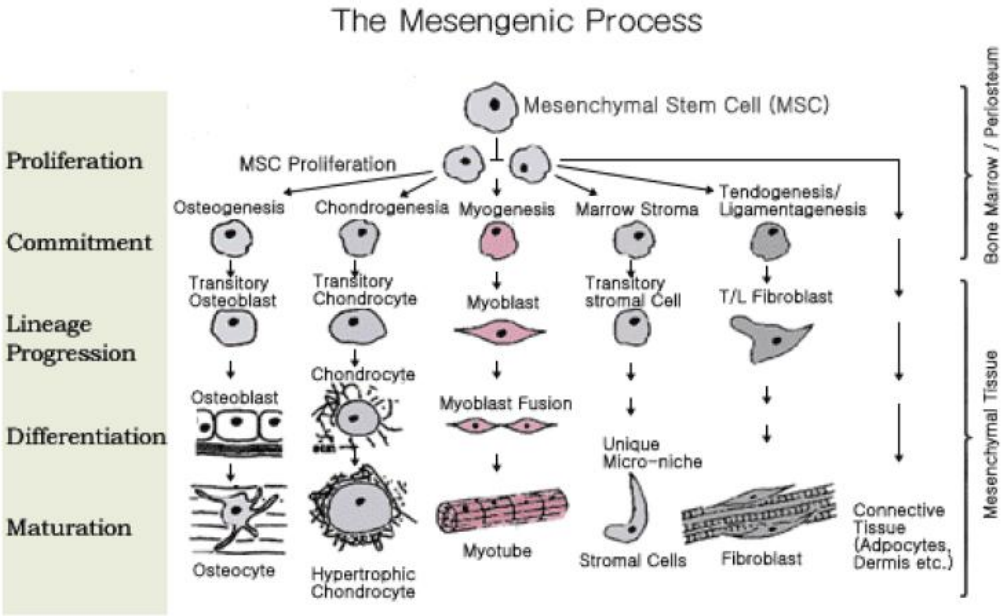

Fig.7: The Mesengenic Process of Mesenchymal Stems Cells

Now is well accepted that MSCs are capable of multipotent differentiation into connective tissue like bone, cartilage, tendon , muscle , adipose tissue, hematopoietic supporting stroma (*Baksh et al., 2004*) and also non-

mesoderm-type cells, for example, neuronal-like and endoderm-like cells (*D'Ippolito et al., 2004, Zhao et al, 2002,Sanchez-Ramos et al, 2000*).

The MSCs are a heterogeneous populations in terms of their multilineage differentiation potential. For instance, Pittenger et al demonstrated that only one-third of the initial adherent bone-marrow derived MSCs clones are pluripotent (osteo/adipo/chondro)(*Pittenger et al 1999*). Several studies both in vivo and in vitro confirmed this observations. (*Baksh et al, 2004*). This characteristic of MSCs, e.g. their heterogeneity, could be explained by the fact that the MSCs in the bone marrow are a pool of cells that comprises Mesenchymal stem cells and different subpopulations at different state of differentiation. Baksh et al in 2004 suggested a model (Fig.8) in which the MSCs in the bone marrow constituted a "primitive stem cells", like HSCs, with multipotent differentiation potential and self-renewal capacity, that with asymmetric divisions, give rise to MSCs with limited self renewal and different multilineage potential (*Baksh et al, 2004*).

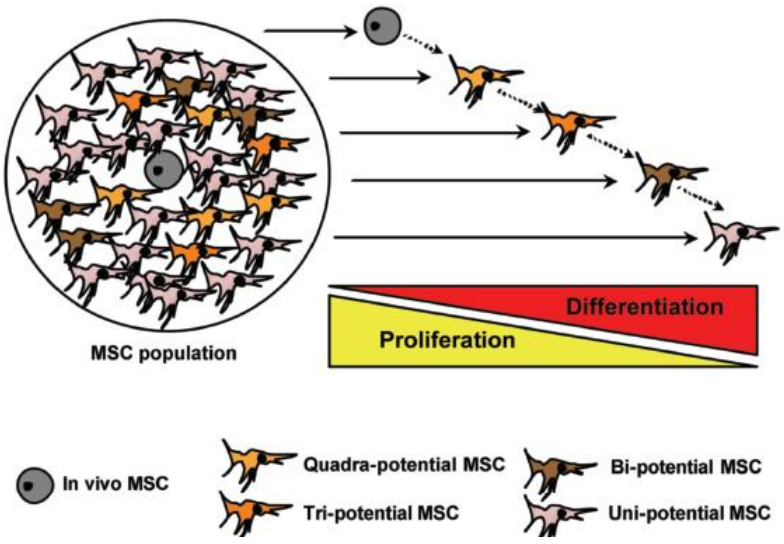

Fig.8: Models MSC differentiation (*Baksh et al., 2004*)

The multilineage differentiation potential of MSCs remain today an important feature to define their stemness by in vitro test. To date a specific universal antigen to define the immunophenotype of MSCs is still lacking.

For this reason MSCs are described negative for hematopoietic surface marker : CD34 (a transmembrane protein that defined ˜ 1% of normal bone marrow mononuclear cells including hematopoietic precursors/stem cells and normal endothelial cells), CD45(recognizes a family of proteins known as the leukocyte common antigen exclusively expressed on the surface of almost all haematolymphoid cells and their progenitors), CD14 (LPS-receptor) , CD31(glycoprotein also designed platelet endothelial cell adhesion molecule-1- PECAM-1-that is normally expressed on endothelial cells, circulating and tissue phase hematopoietic cells including platelets, monocytes/macrophages, granulocytes and B-cells) and positive for the coexpression of several antigen like (*D'Ippolito et al, 2004*, *Short et al, 2003*, *Reyes et al 2001*, *Pittenger et al*, *1999*):

CD105: this antibody SH2 identifies an epitope of endoglin (CD105), the 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*)

CD166 or ALCAM (activated leucocyte adhesion molecule) : is involved in the osteogenic differentiation and also is expressed on lymphocyte B, T and macrophages.

CD54 or ICAM-1: Intercellular adhesion molecule -1 is an inducible cell surface glycoprotein expressed at a low level on a subpopulations of hematopoietic cells, vascular endothelium, fibroblasts and certain epithelial cells.

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.

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.

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

CD29: is a beta 1 hyntegrin involved in the cell-cell interactions and in adhesion with extracellular marker.

In the last years , the research have tried to improved isolation and ex vivo expansion of MSCs by optimization of initial plating density, and by a immunoselection of a homogeneous MSCs population based on cell surface markers, cell size or expression of telomerase (*Ulloya-Montoya et al, 2005*). STRO-1, yet uncharacterized cell marker, is one of the first antibody used to enrich approximately 10 - 20 fold for CFU-F in fresh aspirates of human BM respect their incidence in unsepareted BM. Further, the STRO1 enriched subset of marrow cells is capable of differentiating into hematopoiesis-supportive stromal cells with a vascular smooth-like phenotype, adipocytes, osteoblasts and chondrocytes(*Short et al., 2003*). The use of antibody anti-STRO1 combined with anti-vascular cell adhesion molecule-1 (V-CAM-1/CD106) has allowed to obtained a population of cells that could be expanded for over of 40 population doublings and that showed a 50% cloning efficiency to form CFU-F (*Short et al., 2003, Ulloya-Montoya et al., 2005).*

For the Immunological profile, Mesenchymal Stem Cells express intermediate levels of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I molecules and negligibly low

levels of HLA class II and Fas ligand; they do not express the costimulatory molecules B7-1, B7-2, CD40, or CD40L. MSCs in vitro were able to suppress T-cell proliferation, and intravenous infusion of MSCs didn't elicited toxicity. 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 , 2003 a,b*). For the properties above described (multipotential potential, extensive self renewal and immunological property), adult mesenchymal stem cells shown a great promise in cell therapy, gene therapy and in the new emerging area of tissue engineering. In several animal transplantation studies , MSCs expanded ex vivo were able to differentiate in the cells of the tissue in which they were implanted, to repair the tissue and, in some cases, partially to restore its normal function without any immunoreactivity in the host. (*Baksh et al, 2004*). Recently, the use, with local implantation, of MSCs or subset of the cells with vascular endothelial phenotype or a mixture of hematopoietic stem cells have been tried in a small number of patients with vascular ischemia (*Tateishi-Yuyama et al., 2002*), coronary artery disease (*Assmus et al., 2002*), but this results must to be confirmed in a randomized clinical trials. In particular the researchers have studied the possibility to use MSCs as a carrier to deliver genes into the tissue for gene therapy application to either promote repair and regeneration of diseased or damaged tissue or rescue defective genes. For this purpose, the researchers have used different methods for transfecting MSCs (*Kassem et al.,2004*). Another important area where the use of MSCs seems to be very useful, is the tissue engineering. Ex vivo engineering procedures, by the means of three-dimensional bio-scaffoldf seeded with mature cell or stem cells and cultivated in bioreactors, could lead to the formation of tissues or organs (*Stock, 2001*) . Now several kind of scaffolds are available (collagen type 1, fibronectin, alginate, polylactide, plyglicolide and different combination) and different pre-clinical animal model showed the success of this

approach, particularly for treatment of large bone defects (*Kon et al., 2000*). Now there are many researches based on the study of tissue engineering with applications in different field of Regenerative Medicine (Fig.9). The hope is to achieve an established human transplantation of these engineered tissues in the near future.

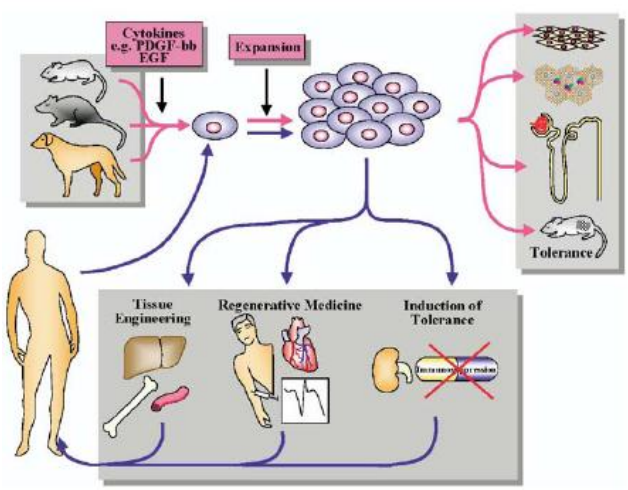

Fig.9: Potential use of adult stem cells in regenerative medicine and recustructive surgery. (*Conrad et al., 2004*)

2. STEM CELL NICHE

After birth adult stem cells are located in a special microenviromental termed “Niche” , that varies in relationship with the tissue in which they reside. The principal role of adult stem cells is to maintained the tissue homeostasis , replacing cells lost for natural cell death (apoptosis) or after injury. For this particular function, a delicate balance between self-renewal and differentiation must be maintained. In the 1978, Schofield, for first, hypothesized the presence of the “Niche”, a special microenviromental that support the stem cells’s maintaining (*Schofield., 1978*). This particular structure has the important function to sequester stem cells from stimuli of differentiation, apoptosis and others that would challenge stem cells reserves. Further, the niche prevents the excessive stem cells production that could lead to a cancer development. The stem cell niche is composed of cellular components and molecular signals produced by the support cells. The studies to identified the niche started in different genetic models like Drosophila and C. Elegans. In the last years, different type of Niche in the mammals systems have been identified and described (*Li et al., 2005*).

2.1 THE HEMATOPOIETIC STEM CELLS IN THE BONE MARROW

The HSCs represent the best characterized stem cell population, but little is known about the in situ information that define the anatomical and structural relationships of stem cells with their progeny and the microenviromental cells (*Moore et al., 2006*). The anatomical sites in which this cells reside are located in the bone marrow proximal to the endosteal surface of trabecular bone. (Fig .10 A)

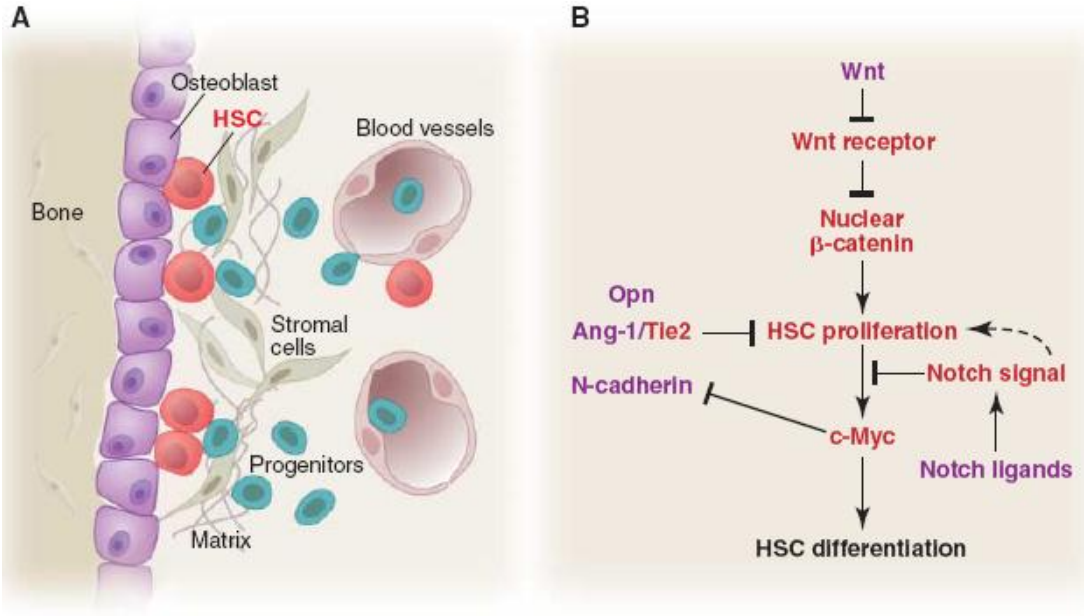

Fig 10: Stem cells and their niche in the bone marrow . A) HSCs and their niche cellular component ; B) possible molecular signals that HSCs send and receive for regulating their proliferation and differentiation (*Moore et al., 2006*)

Recently two studies indicated that: 1) osteoblastic cells are required for the physically attach of the HSCs in the bone marrow , 2) N-Cadherin/ β Catenin complex between HSCs and Osteoblast has been identified 3) Jagged1,generated from osteoblasts, influences HSCs by signaling through the Notch receptor, 4) the number of osteoblasts controls the number of HSC (*Calvi et al., 2003,Zhang et al., 2003*)

In vitro studies of HSCs coculture with osteoblasts demonstrated the expansion of HSCs (*Taiehman et al.,1998*), while a depletion of osteoblasts leads to the loss of hematopoietic tissue (*Visnjic et al., 2004*). The Osteoblast presence is relevant and play a fundamental role in the BM-HSCs niche even if others type of cells may supply the same function. The contribution of stromal cells or perivascular cells is yet to be defined.

N- Cadherin is fundamental for anchored stem cells to the niche and other type of adhesion molecules, like Integrins, are involved (*Simmons et al., 1997*) Further, several studies provide direct evidence for the involvement of matrix components in HSCs regulation (*Moore et al., 2006*).

About the analysis of the molecular signals, different gene expression studies of HSCs have revealed some signals involved in the regulation of self renewal / differentiation balance (Fig.10B). These components include: WNT/β catenin (*Reya et al., 2003*), important for the maintenance of self renewal , Notch that maintained HSCs in undifferentiated state (*Calvi et al., 2003, Ducan et al., 2005*), Transforming growth factor βeta –BMP (TGF/β-BMP), for the control in the number of HSCs (*Zhang et al., 2003*). In addition, a comprehensive genomic analysis of an HSC-supportive microenviromental cells has been performed (*Hackney et al., 2002*).

2.2. THE EPHITELIAL STEM CELL NICHE IN SKIN

The hair follicle present in the skin is composed by a basal layer or permanent portion that normally give rise to stratified skins layers and a specially zone called the Bulge, in which reside the hair follicle stem cells that are responsible for the regeneration of hair and sebaceous gland (Fig.11A)(*Alonso et al., 2003*). So, the bulge area is a sort of Niche where epithelial stem cells are located and maintained (*Niemann et al.,2002, Cotsarelis et al., 1990, Sun et al., 1991*). This stem cells give rise to daughter cells that either migrate upward to serve as epidermal progenitors for generating epidermal cells during wound repair or migrate downward to convert to hair-matrix progenitors which give rise to the hair shaft (*Niemmann et al., 2002, Oshima et al., 2001, Taylor et al., 2000*). In the last years different studies confirmed that the hair follicle stem cells (HFSCs) are quiescent. Multipotentiality of single hair follicle stem cells has been

shown by using cells expanded in vitro; it is possible that in situ single cells in the bulge are destined to produce distinct lineages. Freshly isolated cells from bulge have been used as a populations and not as a single cell in transplantation (*Morris et al., 2004*). Such techniques, or an ability to track the progeny of single HFSCs in situ, will be required to accurately assess the multipotential activity of these cells in normal homeostasis (*Moore et al., 2006*). The molecular analysis performed on the hair follicle stem cells reveled the following features (*Li et al., 2005*):

1 The expression of adhesion molecules is essential for the interactions between stem cell and niche
2 The presence of growth inhibition factors such as TGF*β*/BMP molecules and cell cycle
3 The components of Wnt signaling pathways, including receptors and inhibitors

The expression of several signaling pathways like WNT and BMP have important role in the regulation of HFSCs development and regeneration. The WNT/β catenin systems is important for controls the stem cells activation, fate determination and differentiation while BMP signaling , as opposed to WNT, restricts the activation of stem cells and favors epidermal cell fate (Fig.11 B) (*Moore et al., 2006*).

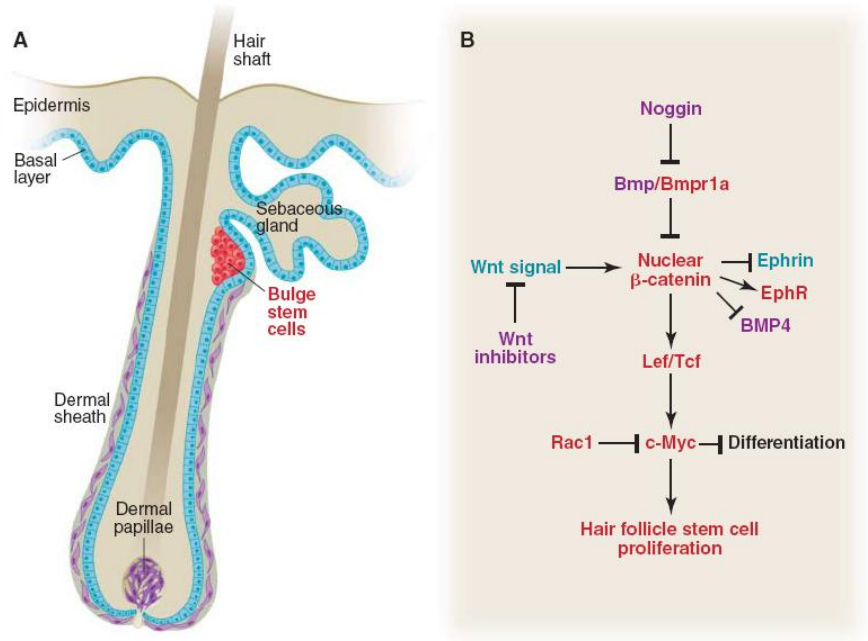

Fig 11: Stem cells within their niche in the hair follicle. A) cells present in the hair follicle and B) molecular mechanisms that mediate the hair follicle stem cells proliferation (*Moore et al., 2006*)

2.3. THE INTESTINAL STEM CELL NICHE

The presence of a stem cell niche, in the intestine reside in the architecture of the epithelial villus-crypt structure based on surrounding perycryptal fibroblasts and mesenchymal cells. (Fig.12 A).The intestinal regeneration begins with intestinal stem cells that give rise to four different type of cells: columnar enterocytes, mucin producing goblet cells, paneth cells and eteroendocrine cells (*Bjerknes et al., 1999, Hermiston et al., 1995, Winton et al, 2000*). In every crypt, there are four or six intestinal stem cells that are located in a ring from the bottom crypt; the activated intestinal stem cells migrate upward to became progenitor or transit amplifying cells and at the top of the crypt, they stop proliferating and differentiate (*Moore et al., 2006*). The intestinal stem cells (ISCs) are normally quiescent and their asymmetric cell division must to be yet demonstrated. The mesenchymal

stem cells surround the crypt and during postnatal intestine regeneration, play a important role in directing epithelial cell proliferation, differentiation and apoptosis; for example BMP4 expressed by mesenchymal stem cells is one of the molecular mechanisms purposed for the niche signals (*He et al., 2004*). The endothelial cells that composed the vascular vessels provided the intestinal stem cells survival with different signals like FGF (*Paris et al., 2001*), while myofibroblasts, that surrounding epithelial cells, are proposed to be the candidate "niche" supporting ISCs and influencing other epithelial cells (*Millis et al., 2001*). Also in the intestinal stem cells niche, some molecular signals have been proposed for regulate the balance of intestinal stem cells between self-renewal and differentiation. (Fig. 12 B).This molecule and gene expression included WNT, BMP, FGF, Notch, Myc, PI3K/Akt (*Li et al., 2005*). In this complex mechanisms, WNT play a positive role in promoting intestinal stem cells activation/self renewal and the crypt cell fate (*Van de Wetering et al.,2002*), while BMP restricts this process. (*Haramis et al., 2004*, *He et al., 2004*)

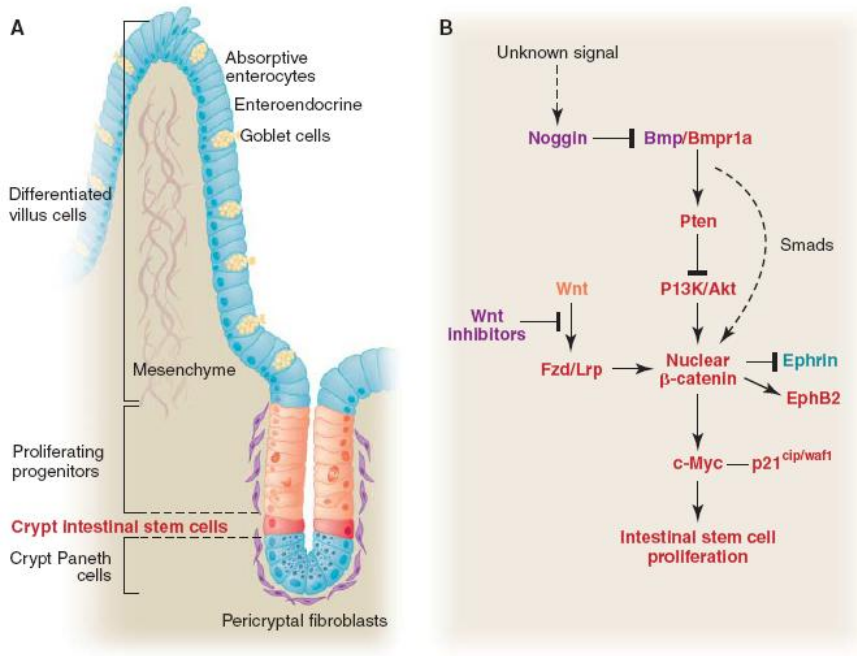

Fig 12: The intestinal stem cells niche. A) Principal type of cells and their spatial disposition in the crypt niche. B) Molecular signals pathways that mediate proliferation of Intestinal Stem Cells (*Moore et al., 2006*).

2.4. THE NEURONAL STEM CELL NICHE

Neuronal stem cells have been identified in the 1990 (*Alvarez-Buylla et al., 1990*).The well characterized germinal regions in which the neuronal stem cells reside and support the neurogenesis in the adult brain are two important regions in the hippocampus: the SUBVENTRICULAR ZONE (SVZ) and the SUBGRANULAR ZONE (SGV)(*Doetsch et al., 1999, Lois et al., 1993,Palmer et al.,1997, Temple et el., 2001*). In the SVZ there are different kinds of cells. Ependymal cell layers the lateral ventricle; SVZ astocytes are located adjacent to the ependymal cells. The immature cells derived from SVZ astrocytes are precursors for neuroblasts; this cells have stem cell features: self-renewal and give rise to transient amplifying precursors C cells that give rise to neuroblasts, which than differentiate into neurons. Also the astocytes in the SVZ can generate oligodendrocytes

(*Doetsch et al., 2003, Mirescu et al., 2003, Temple et al., 2001*). In this zone a specialized basal lamina extending from the blood vessels wrapped all types of cells (Fig. 13).

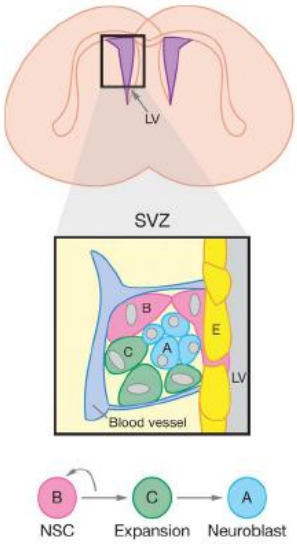

Fig.13: Neuronal stem cells niche in the SVZ zone: B are astocytes, E are ependymal cells, C are amplyfing cells and A are neuroblast. All types of cell are wraped by the basal lamina of the blood vessel (*Li et al., 2005*).

The SUBGRANULAR ZONE (SGZ) is a zone in the hippocampus between the limbus and the dentate gyrus; in the SGZ the neurogenesis occurs directly with the contribution of blood vessels. The astrocytes act as stem cells able to produce granule neurons (Fig.14) (*Doetsch et al., 2003, Temple et al., 2001*).

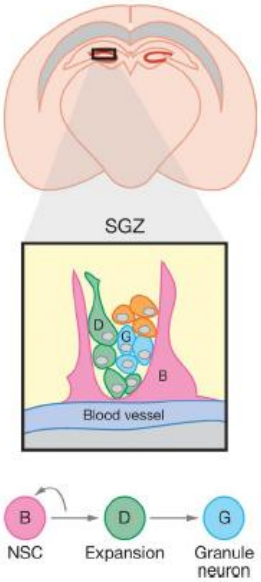

Fig.14 : The SGZ neuronal stem cell niche: B are astrocytes directly attached to the blood vessels, D are proliferenting cells and G are differentiated cells into granule neurons.(*Li et al., 2005*)

In both zone (SVZ and SGZ), the blood vessel (endothelial cells and basal lamina) are principal components of the niche; these endothelial cells secreted signals and factors essential for control self-renewal and lineage commitment like BMP and their antagonists, WNT and β catenin, Noggin, FGF, IGF, VEGF, TGF α and BDNF (*Shen et al., 2004*, *Doetsch et al., 2003*, *Temple et al., 2001*).

2.5 THE HEART STEM CELL NICHE

Recently, a new concept of niche was postulated for the heart. Urbanek et al in 2006 demonstrated the presence of a niche in the adult heart of the mice. The author demonstrated that this niche is present particularly in the atria and in the apex of the heart. The Cardiac Niche is constituted by Cardiac stem cells (CSC) and early lineage committed cells that are supported by similar cells found in the bone marrow and in the brain. Homeostasis of cardiac niche is mediated by asymmetric and symmetric division of CSCs. Different internal and external signals like Integrin, Numb, histone H3 , Connexins and Cadherin seem to be involved in the regulation of the niche in the heart (*Urbanek et al., 2006*) The presence of a stem cells niche in the human heart must be demonstrated yet.

Taken together, the concept of stem cells niche presents in different adult tissue, could give important insights to identification of the stem cells niche in other systems, like in the vascular system, where isn't yet identified a complete understood niche. Further, in the concept of the niche, the blood vessels plays a fundamental role, therefore in the adult vessels would be reside stem cells and the other components that created a niche, in which the stem cells could be maintaining in a undifferentiated state and could be involved in the vessels homeostasis and turnover.

3. THE BLOOD VESSELS SYSTEM

Blood vessels constitute the first organ in the embryo and form the largest network in the adult. There are three types of vessels - arteries, veins, and capillaries; they are not anatomically the same and they are not just tubes through which the blood flows. Arteries have to expand to accept the blood being forced into them from the heart, and then squeeze this blood on to the veins when the heart relaxes. Arteries have the property of elasticity, meaning that they can expand to accept a volume of blood, then contract and squeeze back to their original size after the pressure is released. It is the elasticity of the arteries that maintains the pressure on the blood when the heart relaxes, and keeps it flowing forward. If the arteries did not have this property, the blood pressure would be more like 120/0, instead of the 120/80 that is more normal. Arteries branch into arterioles as they get smaller. Arterioles eventually become capillaries, which are very thin and branching. It is in the capillaries that the exchange between the blood and the cells of the body takes place. Here the blood releases its oxygen and takes on carbon dioxide, except in the lungs, where the blood picks up oxygen and releases carbon dioxide. As the capillaries begin to thicken and merge, they become venules. Venules eventually become veins and head back to the heart. Veins do not have as many elastic fibers as arteries. Veins do have valves, which keep the blood from pooling and flowing back to the legs under the influence of gravity. When these valves break down, as often happens in older or inactive people, the blood flows back and pool in the legs. The result is varicose veins, which often appear as large purplish tubes in the lower legs. Capillaries are the smallest diameter vessels and the site of exchange of metabolites between blood and tissues. Capillaries consist of a single layer of endothelial cells and their basement membrane. The endothelial cells are joined together by tight junctions. At intervals, these tight junctions are interrupted, leaving small spaces allowing the passage of fluid (*Pasqualino,1996*).

3.1. HYSTOLOGY OF THE BLOOD VESSELS

The blood vessels are made of three layers, called from the luminal side outward, the tunica intima, the tunica media and the tunica adventitia and the thickness of these three layers varies greatly depending upon the size and type of vessel (large, medium & small arteries and veins; capillaries).(Fig. 15)(*Gallegher, 1992*).

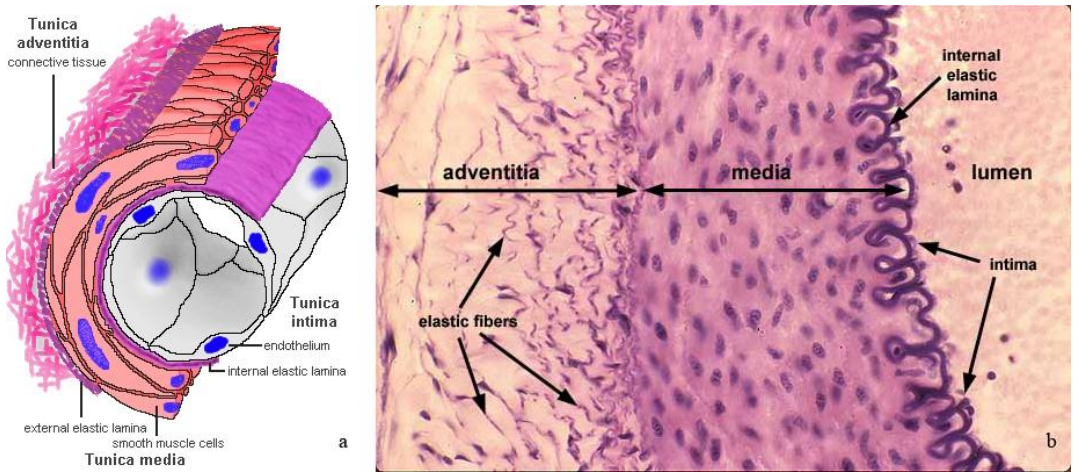

Fig. 15: The layers in the artery (intima, media, adventitial layer). a) Schematic representation; b) Hystology (H&E).

The tunica Intima:

The intima is the inner layer of a vessel. It consists of endothelium (present in all vessels) and subendothelial connective tissue may be present. The subendothelial layer is highly variable, depending on the type of vessel and also grows with age or disease conditions like arteriosclerosis. The endothelium of vessels entering or leaving the heart is continuous with that of the heart. Both connective tissue and smooth muscle are present in the intima. The border of the intima, in the arteries, is delineated by the internal elastic lamina (elastic tissue) that forms the boundary between the intima and the media. Veins do not contain the elastic membrane lining that is found in arteries. In some veins the tunica intima layer also contains valves.

The tunica intima increases in thickness with age, and may also become expanded by lipid deposits, so the integrity is critical since damage can lead to atherosclerosis or clotting.

The tunica Media

The tunica media is the middle layer of arteries and veins wall. It is composed of smooth muscle and elastic fibers. This layer is thicker in arteries than in veins. The tunica media is the layer of concentrically-arranged smooth muscle, the autonomic control of which can alter the diameter of the vessel and affect the blood pressure. Smooth muscle cells (in contrast to cardiac and skeletal) have secretory capabilities, and (depending on the vessel), the tunica media contains varying amounts of collagen fibres, elastic fibres, elastic lamellae, and proteoglycans secreted by the smooth muscle cells. The elastic tissue that forms the boundary between the media and the adventitial is called external elastic membrane.

The tunica Adventitia

The tunica adventitia is the strong outer covering of arteries and veins. It is composed of connective tissue as well as collagen and elastic fibres. These fibres allow the arteries and veins to stretch to prevent overexpansion due to the pressure that is exerted on the walls by blood flow. This adventitial connective tissue is usually more or less continuous with the stromal connective tissue of the organ in which the vessel is found. The presence of adventitial connective tissue tightly adhering to vessels facilitates the surgical isolation and repair of vessels. It tends to be much larger in veins than arteries. Adventitia may also contain numerous elastic fibres. In the arteries with a diameter > 1 mm, blood vessels supplying the adventitia and outer media are also present, these are called vasa vasorum ("vessels of the vessels").

Arteries carry blood away from the heart. They are classified into three types according to their size: large or elastic arteries; medium (or muscular or distributive) arteries; and small arteries or arterioles, which are less than 0.5 mm in diameter. The aorta is the largest artery in the body and principal artery of the body that carries oxygenated blood to most other arteries in the body. The aorta in humans rises from the left ventricle (lower chamber) of the heart, arches back and downward through the thorax, passes through the diaphragm into the abdomen, and divides into the right and left iliac arteries at about the level of the fourth lumbar vertebra. The aorta gives rise to the coronary arteries, which supply the heart muscle with blood, and to the innominate, subclavian, and carotid arteries, which supply the head and arms. The descending part of the aorta gives rise, in the thorax, to the intercostal arteries that branch in the body wall. In the abdomen it gives off the coeliac artery, which divides into the gastric, hepatic, and splenic arteries, which supply the stomach, liver, and spleen, respectively; the mesenteric arteries to the intestines; the renal arteries to the kidneys; and small branches to the body wall and to reproductive organs. The aorta is subject to a condition known as atherosclerosis, in which fat deposits attach to the aortic walls. If left untreated, this condition may lead to hypertension or to an *aneurysm* (a swelling of the vessel wall), which can be fatal (*Pasqualino, 1996*)(Fig.16).

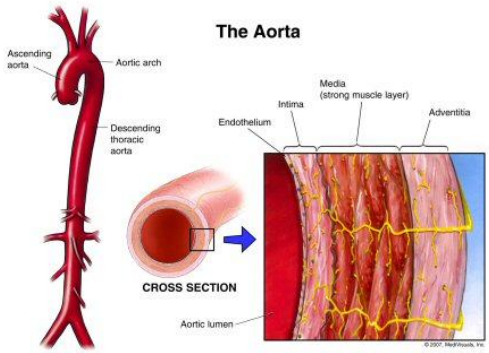

Fig.16: The Aorta system

3.2. THE VASCULAR WALL AS A SOURCE OF STEM CELLS

During the embryogenesis, the cardiovascular system is the first functioning developed apparatus, which requires coordinated development of the heart, the vessels and the blood and its function is critical for early embryo survival (*Copp et al., 1995*). First in 1920 Sambin proposed, in the vascular wall of the chick dorsal aorta, the existence of a common progenitors cells , for endothelial cells and hematopoietic cells, called Hemangioblast (*Sabin et al., 1920*). When the blood islands are developed in the yolk sac, haematopoietic and endothelial cells (ECs) are intimately related, so this association has prompted embryologists to assume the existence of a putative common ancestral progenitor. ECs and HSCs shared several markers and several genes like flk1, flt1, tie1, tie2, tal1 and Runx1 (*Ema et al., 2003*) .The clearest evidence for this common progenitors came from Choi and colleagues who found that differentiating embryonic stem cells contain blast-colony forming cells, which are able to generate both primitive hematopoietic and endothelial cells in appropriate conditions in vitro (*Choi et al., 1998*). In the intraembryonic aorta-gonad-mesonephros region (AGM), hematopoietic precursors can be seen as clumps of cells apparently budding off from the endothelial cells of the ventral wall of the dorsal aorta and the umbilical and vitelline arteries (*Ema et al., 2003*). Thus, during definitive as well as primitive yolk sac haematopoiesis, there is a close association between endothelial and hematopoietic cells. In the postnatal life, the adult haematopoiesis take place in the bone marrow, where Hematopoietic stem cells reside in a special microenviromental or niche that promotes stem cells renewal and differentiation. In has been showed that CD34+/KDR+ bone marrow cells, in mice and human, are enriched for endothelial precursors (*Asahara et al., 1997*, *Peichev et al., 2000*) and single CD34+/KDR+ cells from adult bone marrow can generate both endothelial and hematopoietic cells in vitro. This studies raised the possibility of an adult hemangioblasts that might play a key role in normal

haematopoiesis and vessels turnover. In the 2002, Minasi et al identified in the quail embryonic dorsal aortas the existence of a self-renewal, multipotent stem cell that expressed emo-angioblasts markers (CD34, C-kit, flk1) and that was able to differentiate in most mesodermal tissue (chondrogenic, osteogenic, adipogenic, skeletal muscle). The authors identified these vessels associated stem cells with the name of the meso-angioblasts, on the assumption that it represents common progenitors for endothelial and other mesodermal cells, that participate in postembryonic development of the mesoderm (*Minasi et al., 2002*). Yet, the existence of mesangioblasts in adult tissue has not been reported. The process for the blood vessels formation during the early embryogenesis is called Vasculogenesis, i.e. represents the primary differentiation of endothelial cells from undifferentiated precursors cells called angioblasts. New vessels in the adult arise mainly through angiogenesis, a process defined as outgrowth of new vessels from preexisting blood vessels (*Carmeliet, 2003*). Recent studies indicate that postnatal vasculogenesis may occur indicating a role for circulating (C-EPCs) and/or bone marrow-derived endothelial precursor cells (BM-EPCs), cells with property of embryonal angioblasts, involved in the new blood vessel formation in response to various stimuli (*Asahara et al., 1999a, Kalka et al., 2000, Pelosi et al.,2002*). Once mobilized in the blood, EPCs are supposed to participate in physiological and pathological arterial wall remodeling during their lifetime (*Carmeliet, 2000, 2003*), even if the homing and the contribute of EPCs for the formation of new vessels at the sites of ischemia have been demonstrated (*Hristov et al.,2003***)**, up to now the exact role of this cells in this site has been matter of debate (*Asahara et al., 1999b, Bagley, et al.,2003, Carmeliet et al., 2001, Rajantie et al., 2004***).**

Immature mesenchymal cells with vascular progenitor features have to been shown to reside in pheripheral tissue such as skeletal muscle, where they participate in angiogenesis after injury (*Majka et al., 2003*). In the last years

, some experimental evidences suggest that also the vascular wall is much more dynamic than ever before believed. In fact, apart from the unquestionable link with the hematopoietic system, some progenitors seem to be located within the adult vessel wall. Otherwise, the presence of ectopic tissue like cartilage , fat, muscle, bone and vascular tissue in the wall of diseases vessels is a common finding during routine histopathological observations. Several studies also indicated that putative smooth muscle progenitor cells are present within the adult vasculature, and more precisely in the vascular adventitia (*Liu et al 2004*). In the last years, some experimental artherosclerosis studies demonstrated that the vascular adventitia could be implicated in neointimal proliferation. In 2004, Hu et al identified the presence of stem cell population in the vessel wall. In this study, the author isolated from the adventitia of mouse vessel wall, especially in the aortic root, a population of cells positive for Sca1+, cKit+, CD34+,FLK1+ and negative for the stage-specific embryonic antigen 1 (SSEA-1). In primary culture this cells were heterogeneous, including fibroblast-like, rounded-shaped and adipocyte-like cells. In order to characterize only the adventitial progenitor cells, the cells from primary culture, were sorted for Sca1.The Sca1 sorted cells resulted also C-kit+ and Lin-; they were able to differentiate into smooth muscle cells (SMCs) in vivo and ECs in vitro; they contributed to experimental atherosclerosis in vivo and also they did not originate from the bone marrow. This study also suggested the abundant presence of resident progenitor cells in the aortic root respect others vascular areas demonstrating the embryological role of the aorta.(*Hu et al., 2004*). In according with this experimental evidence, afterwards it has been demonstrated that postnatal aorta contained an immature subpopulation of vascular progenitor cells, CD34+/ASMA-. These cells expressed markers of early pericyte lineage, had an undifferentiated ultrastructural morphology and differentiated into mural cells in vitro but not into ECs (*Howson et al., 2005*). Ingram et al in 2005

showed that a complete hierarchy of EPCs can be identified also in mature endothelial cells such as human umbilical vein endothelial cells (HUVEC) and human aortic endothelial cells (HAEC) (*Ingram et al,2005a*). Otherwise, the human fetal aortas contained immature vascular progenitor cells (CD133+,CD34+, KDR+) coexpressing endothelial and myogenic markers in undifferentiated state, where as they can differentiate into ECs and muscular cells in vitro and secrete a complex combination of angiogenic factor that ameliorate ischemic tissue outcome in vivo (*Invernici et al, 2007*) This Vascular Progenitor Cells (VPCs) did not express CD45, indicating that may not arise from hematopoietic system but may derive from resident MSCs located in the niche of the fetal paraortic membrane or at the periphery of aorta wall parenchyma (*Alessandri et al., 2001*). In 2006, Zengin et al. suggested the existence of a vasculogenic zone in the adult human vascular wall located between the smooth muscle and the adventitial layer that contains vascular wall resident stem cells that could be serve as a reservoir of cells for postnatal vasculogenesis. This vasculogenic zone hypothetically contains cells of different subpopulations: vascular wall resident EPCs, that represents the first evidence of EPCs outside the bone marrow (*Zengin et al., 2006*), even if Ingram showed that vessel wall derived endothelial cells proliferate because they contain a complete hierarchy of EPCs (*Ingram et al., 2005a*) This zone may contain multipotent stem cells that are capable of differentiating into macrophage but probably also into hematopoietic progenitor cells (HPCs); theses cells could be a reservoir for inflammatory cells important for local immune response. In this study the author also hyphotized in the vasculogenic zone the presence of multipotent mesodermal stem cells that may serve as a precursors of SMCs, fibroblasts and perycyte (*Zengin et al., 2006*). According with this, several experimental observations shown that postnatal artery walls contain MSCs with multilineage differentiation. In 2003, Tintut et al described a subpopulation of vascular cells, isolated from bovine aortic

medial cells, called "Calcifying vascular cells"(CVC) that are able to differentiate in vitro along different mesenchymal lineages, e.g osteogenic, chondrogenic, leiomyogenic and stromogenic lineages, where as the adipogenic potential of this cells was limited. CVCs, at flow cytometry analysis, were positive for surface markers commonly expressed by marrow-derived mesenchymal stem cells (positive for CD44 and CD29 , and negative for hematopoietic markers like CD14 and CD45). Otherwise, CVCs replicated for 20-25 passages with retention of multipotentiality. Since these cells did not differentiate toward adipogenyc lineage, they could represent a stage of commitment generation below the mesenchymal stem cells in the mesengenic hierarchy (*Tintut et al., 2003*). In another elegant study, Sainz et al in 2006 defined the possibility to isolate a "Side Population" (SP) of constitutively resident progenitor cells from healthy arteries of adult mice. In this study, living SP was found only in the tunica media layer of thoracic and abdominal aortas and their immunophenotype was Sca1+/Ckit $^{-/low}$ Lin^{-} with low CD34 and flk1 expression. Arterial SP cells did not express SMCs or ECs markers at the basal state but, in the presence of vascular endothelial growth factor (VEGF), differentiated into endothelial cells phenotype and when cultured on Matrigel showed vasculogenic potential, while, in the presence of TGF beta 1 or PDGF-BB, became more similar to SMCs phenotype. Arterial SP cells were unable to generate hematopoietic colonies on Methylcellulose, indicating no capacity of this cells to differentiate toward neither the myeloid lineage not the lymphoid lineage. This cells could participate in arterial homeostasis and remodeling (*Sainz et al., 2006*). Furthermore MSCs isolated from the human saphena vein are able to differentiate in vitro into osteoblasts, chondrocytes and adipocytes (*Covas et al., 2005*). Recent observations demonstrated that human vascular adventitia (pulmonary arteries) contains mesenchymal stem/ progenitors cells (Vimentina+, Cd29+, Cd44+, Cd105+ ,CD34-,Cd133-,CD14-,CD45-) with the capacity to differentiate in

vitro into adypocyes, osteoblsts, and myofibroblasts. (*Hoshino et al., 2008*). Taken together these experimental evidences suggest the presence of resident stem cells (multipotent or a mixture of committed progenitors cells) within the postnatal arterial wall, which are involved in the postnatal vasculogenis and in the formation of ectopic tissue. Adult stem cells in the vessels seem to be located in the vasculogenic zone between the media and the adventitial layer. Even so, the definition of a functional microenvironment (niche) in the human postnatal vessels, where stem cell related to mouse and chicken mesoangioblast can be maintained in a quiescent state, must be determinate.

3.3 ANGIOGENESIS

Vessels formation occurs mainly through two mechanisms: Vasculogenesis and Angiogenesis. Vasculogenesis represents de novo formation of blood vessels during embryonic development. Mesoderm-derived stem cells (hemangioblasts) form aggregates (blood islands), and they develop into primitive hematopoietic and endothelial cells (angioblasts). The angioblasts, undifferentiated precursor cells, proliferated and differentiated in situ into endothelial cells to form a vascular labyrinth (*Carmeliet, 2000*). In postnatal life adult hemangioblasts seemed to be present even if their location and characterization must be determinate. On the other hand, the formation of new vessels from preexisting vessels is called angiogenesis. New vessels in the adult arise mainly through angiogenesis, although vasculogenesis also may occur. Angiogenesis is a tightly regulated process required for a number of physiological process like wound healing, ovulation and menstruation as well as embryonic development. Excessive angiogenesis is seen in a wide range of diseases including tumors, inflammatory diseases and diabetic retinopathy (*Kiumura et al., 2003*). Angiogenesis is a very complex mechanism that include different steps. Angiogenesis initiates with vasodilation, a process involving nitric oxide. This increase in permeability

lead to a preexisting vessel destabilization and loss of the endothelial cell interactions. Different angiogenic growth factors are released. These molecules lead to matrix metallo proteinase activation, crucial for extracellular matrix degradation. After this, endothelial cells proliferate and migrate to assemble in a solid cords that subsequently acquire a lumen. Subsequently, pericytes and SMCs migrate in order to reorganize the cellular interactions and surround the new formed vessels. Angiogenic sprouting is controlled by a balance of activators and inhibitors. Important activator factors are VEGF (vascular endothelial growth factor), FGF (fibroblast growth factor), TGF-β (transforming growth factor β), IL-8 (interleukin 8) and Angiopoietin-I. Inhibitors factors are Interferon α, β, γ, IL-12 (interleukin 12), Angiostatin and Endostatin (*Carmeliet, 2000*).

3.3.1 VASCULAR ENDOTHELIAL GROWTH FACTOR

The family of VEGF includes VEGF-A, B, C, D and placenta growth factor. VEGF (denoted as VEGF-A) was initially named vascular permeability factor for its ability to induce vascular permeability. Later this vascular endothelial specific mitogen was named VEGF for its ability to promote proliferation of endothelial cells. VEGF play a key role during angiogenesis process as activator. VEGF induces endothelial cells proliferation, migration differentiation and survival. Knock-out experiments have demonstrated that heterozygous VEGF-A knout-out mice were embryologically lethal and that blood vessels formation was dramatically impaired (*Ferrara, 1996*). The main receptors which seem to initiate signal transduction cascades in response to VEGF binding consist of three kinds of tyrosine kinases : VEGF-R1 or Flt1 (fms-like tyrosine-kinase 1), VEGF-R2 or KDR (kinase-insert domain receptor) and VEGF-R3 or Flt3(fms-like tyrosine-kinase 3). Among them, KDR may mediate the major action on cell growth and permeability. (*Gale et al., 1999*). Different factors have

been found to induce VEGF expression like hypoxia, cytokines, growth factors.

3.3.2 MSC and ANGIOGENIC DIFFERENTIATION

As above described, endothelial and hematopoietic cells share a common progenitor : the hemangioblast, a cell derive from embryonal mesoderm. Today, the more reliable hypothesis is that hemangioblast derived from mesenchymal stem cells. However, it must be determinate if adult mesenchymal stem cells maintained this differentiation capacity. Reyes et al. in 2002 isolated from adult bone marrow a multipotent population of adult stem cells, called MAPC (multipotent adult progenitors cells), able to differentiate into endothelial cells and other mesodermal tissues. These cells were negative for endothelial markers like CD34, VE-Cadherin (vascular endothelial cadherin), AC133 and Flk1(fetal liver kinase) and can differentiate in vitro into mature endothelial cells (*Reyes et al., 2002*). Recent studies demonstrated that also adult mesenchymal stem cells are able to differentiate in vitro into endothelial cells. In 2003, Annabi et al suggested that hypossic culture conditions (1% O_2, 5% CO_2, 94%N_2) induced mouse BM-MSC migration and tree-dimensional capillary like structures formations on Matrigel (*Annabi et al., 2003*). Further, Oswald et al. showed that human BM-MSC are able to differentiate into cells with phenotypic (vWF, KDR,FLT) and functional (capillary like structures on Matrigel) features of endothelial cells when cultivated under confluence, presence of 2% of fetal calf serum (FCS) and 50 ng/ml VEGF for 7 days at 37°C-5% CO_2.(*Oswald et al., 2004*). Moreover, Alviano et al. demonstrated that human MSCs from amniotic membrane (AM-hMSCs) spontaneously form capillary-like structures when cultured in Matrigel. This behavior has been enhanced by the presence of VEGF containing culture medium, and the endothelial committement of the cells was demonstrated by the expression of von Willebrand factor (*Alviano et al., 2007*).

3.4 THE VASCULAR TISSUE BANKING

In the 1990, due for the increasing demand of vascular homografts used in the vascular surgery like for prothesic infections, peripheral arterial occlusive disease or abdominal aortic aneurysm , raised in Europe the establishment of Cardiovascular tissue Banking, where the vascular homografts from multiorgan donors are procured, prepared, cryopreserved and distributed. Emilia Romagna Cardiovascular Tissue Banking is located in Bologna, S.Orsola-Malpighi Hospital, and it is certified ISO 9000 from 2003; so all the procedures, included the setting standards for quality and safety for the donation, procurement, testing, processing, storage and distribution, followed the " Directive of the European Parliament and of the Council" (28-05-2003). The vascular homografts were harvested by a vascular surgery team in brain-dead multiorgan donors. The principal criteria for the baking of the vessels are: age 15-55 years, absence of cardiovascular disease, negativity for HIV, Hepatitis B and C, Treponema Pallidum, CMV and Toxoplasma. Harvested vessels were kept in a sterile boxes with RPMI or Celsior Media and transferred in a isothermal boxes filled with ice to the Cardiovascular Tissue Bank. Under a laminar flow, a specialized vascular surgery team prepared and classified the samples in according to the quality of the tissue. For arterial vessels:

Class III: Good gross morphology, only small lipid streaks, pressure test positive.

Class II: Focal intimal thickennings; fibrocalcific plaques without evidence of hemorraghe and / or intimal ulcer (<15%),

Class I : Enlarged wall (aneurysm), presence of mural calcification (>30%), intimal ulcer, pressure test negative

After preparation , the samples were transferred in an antibiotic solution for

72 h at 4°C, and successively transferred in a sterile bags containing the cryosolution (RPMI plus human albumin and DMSO at the concentrations of 10% as a cryoprotectant), cryopreserved in a programmable freezer (cooling rate of -1°C/min to -120°C) and stored, with a specific barcode for the identification, in the vapor phase of liquid nitrogen at -140°C (not more of 5 years). Microbiology tests were performed before and after decontamination (aerobic and anaerobic bacteria, fungi); positive serology and persistent positive microbiology after decontamination were criteria for rejection. A small pieces of tissue were fixed in formalin-buffered for the histology examination. When the vessels were requested, the cryopreserved homograft were sent in dry ice to the hospital with the thawing protocol included. The bags , in which the sample were stored, were quickly submerged in a water bath at 39°C and let thaw for 10 min. When thawing was achieved, the bags were cut under sterile conditions and the samples were washed in cooled saline solution (NaCl 0.9%) for 3 times to remove the cryoprotectant that may be toxic at temperature above 10°C. (*Buzzi et al, 2005*).

EXPERIMENTAL DESIGN

4. AIM OF THE WORK

Stem cells are one of the most fascinating areas of biology today, and in particular, in the last years, Adult Stem Cells have generated much interest especially for their application potential as source for cell based regenerative medicine and tissue engineering. Adult stem cells have been found in different tissues like bone marrow, skin, intestine, nervous system, where they reside in a special microenviromental termed "Niche". For this reason, scientists have tried (attempted) to identify and discover Adult stem cells in different organ and tissue, where they may play a primary role to maintaining and repairing the tissue. The arterial wall of the blood vessels is much more plastic than ever before believed. Several studies demonstrated that some progenitor cells seem to be located within the adult vessels. Resident vascular progenitor cells have been isolated from different vessel territories: thoracic and abdominal aortas of healthy adult mice (*Sainz et al., 2006*); human internal thoracic arteries (*Zenging et al., 2006*); adventitia of apoE-deficient mouse aortic root (*Hu et al., 2004*); human and quail dorsal aortas(*Invernici et al., 2007, Minasi et al.,2002*) . Recently, Zenging et al have postulated the presence of a *"vasculogenic zone"* in human adult arteries in which a complete hierarchy of resident stem cells were nicked in the arterial walls. Moreover, the authors hypothesized the presence of adult vascular wall- resident *Mesenchymal stem cells*, that have the ability to differentiate in smooth muscle cells, in surrounding pericytes and fibroblasts. (Fig. 17) (*Zengin et al., 2006*).

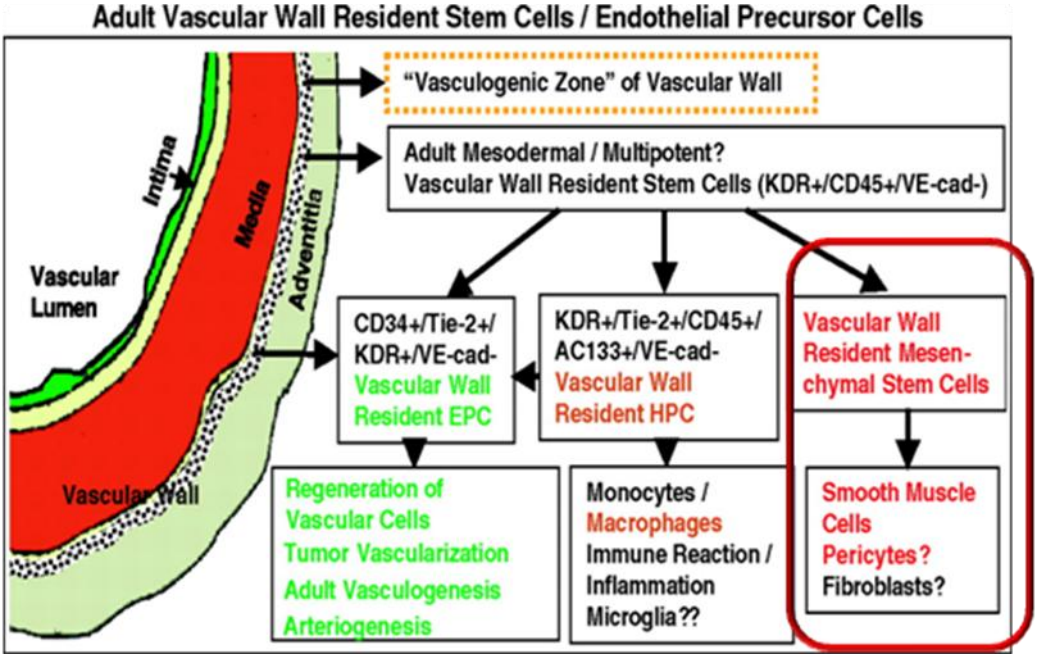

Fig. 17: Hypothetical scheme of the " Vasculogenic zone" in the arterial wall (*Zengin et al., 2006*)

Thanks to a collaboration with Cardiovascular Tissue Bank, that cryopreserved human vessels for arterial reconstructive surgery, the present work aimed to identify in situ and isolate adult stem cells (MSCs) from thoracic aortas of young and healthy heart-beating multiorgan donors. Further, because the clinical therapeutic approaches based on EPCs is hampered by difficulties in obtaining an adequate number of functional progenitors, this study aimed to establish whether MSCs from human thoracic aortas can be differentiate *in vitro* in endothelial cells.

5. MATERIALS AND METHODS

5.1 HUMAN ARTERIAL SAMPLE

Working in collaboration with the Cardiovascular Tissue Bank, Service of Transfusion Medicine, Policlinico S. Orsola- Malpighi, Bologna, Italy, we included in the present study human vessels (human thoracic aortas and human femoral arteries) harvested from multi-organs donors , which, if were considered suitable for arterial reconstructive surgery by a vascular medical team, were cryopreserved and then used in the common vascular surgery like a homograft. The requirement to define this are: age between 15-55 years, absence of cardiovascular disease, negativity for virological tests (HIV, Hepatitis B and C, Treponema Pallidum, CMV,Toxoplasma), negativity for microbiological tests for bacteria and fungi, quality of the tissue (Class III: perfect, Class II: lesser quality but still acceptable, class I: discarded) (*Buzzi et al., 2005*).

5.2 PROCEDURES OF ARTERIAL SAMPLE CRYOPRESERVATION

After collection, the human vessels were kept in a sterile box with Celsior Media (IMTIX SANGSTAT, Lyon France), a flushing and cold storage solution for solid organ preservation, and transferred to the Cardiovascular tissue Banking in isothermal boxes filled with ice within 1 hour since procurement. Under a sterile conditions, e.g. a laminar flow in a sterile hood, the arteries were prepared, classified and transferred to a solution composed with RPMI 1640 (Cambrex Bioscience Vierviers, Belgium) plus antibiotics according to Strickett et al (*Strickett et al, 1983*). The medium was completed with a mixture of Mefoxin 240 mg/ml, Lincomycin 120mg/ml, Colimycin 100 mg/ml, and Vancomycin 50 mg/ml while

Amphotericin B was not included because her potential tissue tossicity. After decontamination in this medium for 72 h at 4°C , the samples were transferred into a sterile bags containing a fresh cryoprotectant solution RPMI 1640 with human albumin (Kendrion, Lucca, Italy) and Me_2SO4 at a final concentration of 10%, that was before cooled at 4°C for 30 min. The bags were kept at 4°C for 30 min to allow the Me_2SO4 to penetrate into the tissue completely. The bags were labeled and cryopreserved in liquid nitrogen vapor in a controlled rate freezing system (IceCube 1860, Sy-Lab, Wien, Austria) using an electronically monitored programs that allow to decrease the temperature at 1°C/min to – 45°C and at the faster rate thereafter until – 120°C has been achieved. The cryopreserved arterial homografts were stored in the vapor phase of liquid nitrogen (- 196°C). Each freezing curve was checked and validated in the laboratory (*Pasquinelli et al., 2006*).

5.3 PROCEDURES OF ARTERIAL SAMPLES THAWING

Human arterial samples were thawed as followed (based on the methods routinely used for reconstructive vascular surgery): the bags, in which the sample were stored, were quickly submerged in a water bath at 39°C and let thaw for 10 min. When thawing was achieved, the bags were cut under sterile conditions and the sample were washed in cooled saline solution (NaCl 0.9%) for 3 times to remove the cryoprotectant that may be toxic at temperature above 10°C (*Pasquinelli et al.,2006*).

5.4 FRESH AND CRYOPRESERVED ARTERIAL TISSUE: HISTOLOGY ANALISYS

A single pieces of a 1 cm long ring of each arterial sample before and after cryopreservation procedures was fixed in 10% buffered formalin, the gold

standard of fixatives for routine histology and immunohistochemistry. Fixation of tissues is necessary to 1) adequately preserve cellular components, including soluble and structural proteins; 2) prevent autolysis and displacement of cell constituents, including Ags and enzymes; 3) stabilize cellular materials against deleterious effects of subsequent procedures; and 4) facilitate conventional staining and immunostaining. Formaldehyde preserves mainly peptides and the general structure of cellular organelles. It also interacts with nucleic acids but has little effect on carbohydrates. It is a good preservative of lipids if the fixative contains calcium. In solution, formaldehyde is capable of binding the following amino acids: lysine, tyrosine, asparagines, histidine, arginine, cysteine, and glutamine. The basic mechanism of fixation with formaldehyde is the formation of addition products between the formalin and uncharged reactive amino groups (–NH or NH2), forming cross-links (*Ramos et al., 2005*). After fixation, the sample was washed , dehydrated in a gradual series of alcohol(3x5min each), waxed in xilene(2x 15 min each) and then embedded in paraffin (2x30 min at 4°C); then 5 µm thick sections, obtained at the microtome, were stained with haematoxylin and eosin according to the histology procedures. The slide were then observed under a light microscopy (Olympus CX42).connected with a CCD Camera to obtain images.

5.5 FRESH AND CRYOPRESERVED ARTERIAL TISSUE : TEM ANALYSIS

For Trasmission electron microscopy (TEM) analysis, human arterial specimens before and after cryopreservation were fixed in 2.5% glutaraldehyde in Phosphate Buffer 0.2 M over night at 4°C. Following washing in Phosphate Buffer 0.15 M, the samples were post-fixed in 1% Osmium Tetroxide in Phosphate Buffer 0.1 M for 1 hour at RT. After dehydration through increased concentration of ethanol (3x10 min each at

RT), the sample were immersed in polypropylene oxide (2x15 min at RT) and then embedded in a epoxy resin (50% Araldite CY212 + 50% Araldite HY + 1.5 % of accelerator, Benzyldimetylammina (BDMA) (all purchased from TAAB, England). Semi thin section obtained with ultramicrotome were stained with toluidine blue, while ultrathin sections were stained with uranyl acetate and lead citrate and then examined in a transmission electron microscopy Philips 400 T (*Pasquinelli et al., 2006*).

5.6IN SITU CELL PRESERVATION: IMMUNOHISTOCHEMISTRY PROTOCOL

For the evaluation of in situ cell preservation, formalin fixed paraffin-embedded 5 µm thick sections of the arterial sample before and after cryopreservation were stained for the immunohistochemistry (IHC) protocol. IHC was performed by using monoclonal antibodies against CD34 (clone QBend-10, dilution 1:80, Dako, Copenhagen, Denmark) to detect endothelial cells and monoclonal antibodies against smooth muscle actin (ASMA, clone 1A4, dilution 1:8000, Dako, Copenhagen, Denmark) to detect smooth muscle cells. In brief, the sections were dewaxed with xilene and rehydrated through decreasing concentrations of ethanol. Endogenous peroxidase was blocked by 10 min incubation at room temperature (RT) with absolute methanol containing 1.5% H_2O_2. Because fixation modifies the tertiary structure of proteins (Ags), many times making them undetected by specific Abs so antigen retrieval is necessary. To recover tissue antigenicity, the slides were put in Citrate Buffer ph 6 and then used a heat-based retrieval (120°C, 20 min., 1 atm :autoclave cycle). After cooling the slides at RT for 20 min and following several washes, section were incubated with primary antibody, above mentioned, diluted with PBS-1% BSA in a humidified chamber at 4°C over night (o.n.). Following washing, sections were incubated with a byotinylated antibody, affinity purified horse anti-mouse secondary antibody (Vector Laboratories, dilution 1:500

in PBS-1% BSA, Burlingame, CA, USA) for 30 min at RT and then with streptavidin:biotinylated peroxidase complexes (Biospa-Division, dilution 1:250 in PBS-1% BSA, Milano, Italy) for 30 min at RT and finally treated with diaminobenzidine (Sigma- Aldrich) in solution with H_2O_2 for 5 min at the dark. The reactions was stopped in distillated water and then the slides were counterstained with hematoxylin, dehydrated through increased concentration of ethanol, waxed in xilene, permanent mounted (Canada Balsam, Sigma, Milano, Italy) and then observed by light microscopy (LM)(Olympus CX42) (*Pasquinelli et al., 2006*).

5.7 IN SITU CELL DEATH: TUNEL ASSAY

Apoptotic cells on arterial samples before and after cryopreservation procedures were end –labeled in situ by TUNEL(Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining using *" In situ Cell Death Detection Kit- POD"* (Roche Applied Biosciences, Germany) according to the manufacturer's instructions. In brief, the slide were dewaxed in xilene and then rehydrated through a decreasing concentration of ethanol and distillated water. Endogenous peroxidase was blocked incubating the slide in a solution of 3% H_2O_2 in Methanol, for 5 min at the dark following washing in distillated water. To recover the tissue antigenicity, the sample were digested with a solution of Proteinase K (20 µg/ml PK in 10 ml Tris/HCl, pH 7.4, Roche Applied Biosciences Germany) for 30 min at 37°C. After several washing in PBS, 50 µl TUNEL REACTION MIX diluted 1:5 with Tunel dilution Buffer (Roche Applied Biosciences, Germany) for slides were used and then the samples were incubated in a humidified chamber for 60 min at 37°C at the dark. The TUNEL REACTION MIX diluted 1:5 was done, in ice, as following:

5 µl of Enzyme Solution + 20 µl of Tunel Buffer to make dilution 1:5

5 µl of the Enzyme Solution 1:5 + 45 µl of Label Solution

Two controls, positive and negative, were included in each experimental set up. Negative control was done incubating the sample only with 50 µl of Label Solution without the enzyme terminal transferase, while the positive control was done incubating with DNase I, grade I for 10 min at RT to induce DNA strand breaks, prior to labeling procedures with Tunel reaction Mix. After three washing in PBS, the slides were incubated with 50 µl of CONVERTED POD for 30 min at 37°C in a humidified chamber and finally treated with diaminobenzidine (Sigma- Aldrich) in a solution with H_2O_2 for 5 min at the dark. After washing in PBS, the slides were counterstained with hematoxylin, dehydrated, waxed in xilene and then mounted for the analysis by light microscope (Olympus CX42) (*Pasquinelli et al., 2006*).

5.8 ISOLATION CELLS FROM CRYOPRESERVED ALLOGRAFTS

Cells were obtained from cryopreserved allografts by scraping and by enzymatic digestion. Briefly, the samples were treated with type VII collagenase (0.1% Sigma-Aldrich) and scraped until reaching the adventitial layer. Minced tissue was incubated with the same concentrations of collagenase at 37°C in 5% CO_2 humidified atmosphere for 1 h. Then , cells isolated were filtered, centrifuged in Hepes Buffer, pH 7.4 , plus FBS 10 % (Seromed, Biochrom KG, Berlin, Germany) for 5 min 2x at 1800 rpm and then the pellets were suspended in 2 ml of PBS for Flow cytometry analysis (*Pasquinelli et al., 2006*).

5.9 FLOW CYTOMETRY ANALYSIS

Flow cytometry was used to assess in vivo cell viability and to establish the phenotype of cells from cryopreserved allografts. Cell viability was

performed as follows: to 5 $x10^5$ cells in 2 ml volume of PBS, 20 ml 7 – amminoactinomycin D (7 ADD) solution (Beckman-Coulter, Miami FL, USA) were added; after 20 min of incubation the cells were analyzed with a FC 500 Flow Cytometer (Beckman-Coulter). To analyze CD34 expression, $5x10^5$ cells in a 100 ml volume of PBS were incubated for 20 min with 20 ml of CD34-PE (Beckman-Coulter). ASMA analysis was performed by intracytoplasmic stain. Briefly, cells were fixed with reagent 1 of the Intraprep kit (Beckman-Coulter) following manufacturer's instruction. After two washes with PBS, the samples were permeabilized with reagent 2 and, after two additional washes, cells were first incubated with 5ml of ASMA (Sigma–Aldrich) and, subsequently, with FITC anti-Mouse IgG (Beckman-Coulter). Control samples were run with an irrelevant monoclonal antibody and FITC anti-mouse IgG. (*Pasquinelli et al., 2006*)

5.10 ORGAN CULTURE: LM AND TEM

Organ culture was used to establish the ability of cells to recover freezing/thawing injury. The samples were carefully placed in contact with the 100mm culture plates. RPMI 1640 with L-Glutamine (Cambrex, BioScience, Vierviers, Belgium), 10% FCS (Seromed, Biochrom KG, Berlin, Germany) and 1% Penicillin/Streptomycin 100X solution (EuroClone, Life Science Division, Milano, Italy) was added. The culture plates were placed in a 37 °C incubator with 5% CO_2. After culturing for 4, 8, and 15 days tissues were processed for LM and TEM as described above (for LM see paragraph 5.4, for TEM analysis see paragraph 5.5)(*Pasquinelli et al., 2006*).

5.11 ANTIGENICITY PRESERVATION : IMMUNOHISTOCHEMICAL STAINING FOR HC-10 E NAMB-1:

To detect if the cryopreservation procedures can modify arterial homograft antigenicity, we performed a immunohistochemical study of fresh and cryopreserved human arteries to visualize the expression of HLA class I heavy and light chains " in situ" by using the HC-10 and Namb-1 monoclonal antibodies. The HC-10 and Namb-1 MoAbs were generated, purified and characterized as described elsewhere (*Perosa et al. 2003; Pellegrino et al. 1982; Stam et al. 1986*). The HC-10 antibody recognizes an epitope expressed on virtually all b2-microglobulin- free HLA-B heavy chains and on b2-microglobulin-free HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32 and HLA-A33 heavy chains. The Namb-1 antibody recognizes both free and HLA class I heavy chain-associated human b2-microglobulin. About 5-µm sections from fresh and cryopreserved arterial segments were dewaxed with xylene and rehydrated through decreasing concentrations of ethanol. Endogenous peroxidase activity was blocked by a 20-min incubation at RT with absolute methanol containing 1.5% H_2O_2. To recover tissue antigenicity, tissue sections were immersed in a jar containing a Tris/HCl EDTA buffer (pH 9) and treated at 98 °C in a water bath for 20 min. After rinsing in PBS (pH 7.4) tissue sections were incubated with HC-10 (1:600) and Namb-1 (1:50) MoAbs in a humidified chamber overnight at room temperature. Monoclonal antibody tissue labeling was revealed by using a non-biotin polymeric system (BioGenex Super Sensitive TM Polymer-HRP IHC Detection System, Biogenex, San Ramon, CA). Briefly, tissue sections were treated with the Super Enhancer TM Reagent for 20 min at RT , washed in PBS and incubated with SS-HRP polymeric complexes for 30 min at RT. After several washings in PBS, peroxidase activity was detected by incubating tissue sections for 2–3 min with a freshly prepared solution of 3,3'-diaminobenzidine (Sigma Chemicals Co., St. Louis, MO; 40 mg of 3,3'-

diaminobenzidine in 100 ml of PBS containing 100 µl of H2O2). Tissue sections were counterstained with Mayer's haematoxylin (Sigma Chemicals), dehydrated through increased concentration of ethanol, waxed in xilene and then permanent mounted (Canada Balsam, Sigma, Milano, Italy). Negative controls were done by omitting incubation with the primary antibody. Tissue sections were read independently by two investigators. Endothelial cells from venous, arterial and capillary vessels from samples of normal human ovarian cortex were considered as positive controls (*Pasquinelli et al., 2007 a*).

5.12 IDENTIFICATION IN SITU OF A "VASCULOGENIC ZONE": IMMUNOHISTOCHEMICAL STUDIES:

Fresh thoracic aortas and femoral arteries were harvested from 10 heart-beating multi-organ donors (mean age 32 years); after procurement and decontamination arterial samples were routinely processed for histological examination. Details of the procurement and sampling procedures are reported elsewhere (see paragraph 5.1 and 5.4). The immunohistochemical staining here used was similar to that described previously (see paragraph 5.6). Five µm-thick sections from formalin-fixed, paraffin embedded arteries were stained with the monoclonal antibodies (MoAbs) listed below: c-kit (CD117, 1:200, DakoCytomation, Hamburg, Germany), CD34 (1:80, DakoCytomation), von Willebrand Factor (vWF, 1:50, DakoCytomation), CD45 (1:120, DakoCytomation), S-100 (1:5000, DakoCytomation, Hamburg, Germany), CD68 (1:200, DakoCytomation, Hamburg, Germany) and ki-67 (1:200, Novocastra, Newcastle upon Tyne, United Kingdom).

To detect the antigen-antibody reaction, the strept-ABC complex/HRP duet technique was used followed by diaminobenzidine tetrahydrochloride as a substrate solution (DAB, Sigma, Milan, Italy). Negative controls were performed by omitting the primary antibodies. The immunohistochemical

reactions were then observed under a light microscopy (Olympus CX42) with the software *Image proPlus*, connected with a CCD camera (*Pasquinelli et al., 2007 b*).

5.13 ISOLATION AND ESPANSION OF ADULT STEM CELLS FROM FRESH HUMAN THORACIC AORTAS

Human thoracic aortas were harvested from multi-organ donors (n°. 10; mean age 32 years) kept at the Cardiovascular tissue Bank, Policlinico S. Orsola- Malpighi, that weren't considered suitable for cryopreservation and then for the use in arterial reconstructive surgery. Approximately 5 cm-long samples were used for isolation of a putative population of stem cells resident in the vascular wall. The arterial samples after 72 h of decontamination in a sterile solution of RPMI 1640 plus antibiotics (*Pasquinelli et al., 2006 a)*, under sterile conditions, were cut, longitudinally opened, providing an exposed surface area measuring about 25-30 cm^2, and then washed with sterile Hepes Buffer. The harvested pieces of arterial tissue were mechanically minced with a scraper until reaching the adventitial layer and enzymatically digested with 0.1% collagenase type II (Sigma, Milan, Italy) in Hepes Buffer for 30 min at RT (Fig. 18).

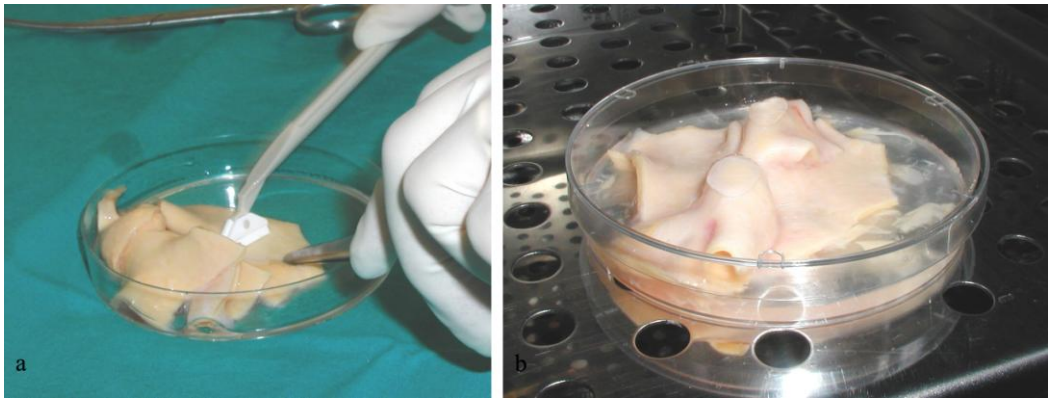

Fig. 18: Mechanics scraper (a) and enzymatic digestion (b) of arterial sample

Minced tissue was subsequently incubated with the same concentrations of collagenase at 37°C in a 5% CO_2 humidified atmosphere for other 30 min. The homogenate was subsequently filtered, centrifuged in Hepes Buffer , pH 7.4 plus 10 % fetal bovine serum (FBS) to block the action of Collagenase. The pellets were washed twice in PBS; the cells were then counted and plated at $1x10^6/cm^2$ in collagen biocoated culture flasks (BD Labware, Franklin Lakes, NJ) in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 20% heat inactivated FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and incubated at 37 °C in a humidified atmosphere with 5% CO_2. The overall number of mononuclear cells recovered from each isolation varied from 1.5×10^6 to 3.5×10^6. After one week the non-adherent cells were removed by replacing the medium supplemented by 10% FBS. After plating, the cells yielded by the primary culture ranged between 0.5 to 1 $x10^6$. When the cultures were near confluence (approximately 1-2 weeks) the cells were detached by treatment with 0.25% trypsin-EDTA (Sigma) and were maintained and subcultured for up to 12-15 passages (*Pasquinelli et al., 2007 b*).

5.14 IMMUNOPHENOTYPING: FLOW CYTOMETRY ANALYSIS

Flow cytometry was used to establish the immunophenotype of cells isolated from fresh human thoracic aortas. Cells taken at passages 3-5 were washed twice with PBS containing 2% fetal bovine serum (FBS) and stained for 20 min at RT using the following MoAbs: CD133-PE (Miltenyi Biotech, Bologna, Italy), CD117-FITC, CD34-PE, CD44-FITC, CD90(Thy-1.2)-PC5, CD105-PE, CD166-PE, CD45-APC, CD146-PE, HLA class I-FITC, HLADR- FITC all from Beckman-Coulter (Beckman-Coulter, Milan, Italy). Because the MSCs from bone marrow are identified for the coexpression of three markers like CD44, CD90, CD105, to study this immunophenotype, the cells were simultaneously incubated with CD105-PE, CD44-FITC and CD90(Thy-1.2)-PC5 MoAbs (Beckman-Coulter).

Negative control samples were run with an appropriate conjugated irrelevant monoclonal antibody . 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) (*Pasquinelli et al., 2007 b*).

5.15 IMMUNOFLUORESCENCE ANALYSIS

For parallel immunofluorescence analysis, the fibroblast-like cells obtained from fresh human thoracic aortas were harvested at the same point of culture for flow cytometry analysis by treatment with 0.05% trypsin-EDTA and then plated at $1x10^3/cm^2$ in collagen biocoated slide chambers (BD Biosciences, Franklin Lake, NJ) and then cultured until near confluence. The samples were then fixed in 2% paraformaldehyde in PBS for 4 min. at RT while to detect intracytoplasmatic protein, 0.01% Triton X-100 was added during the fixation. Unspecific staining were blocked incubating the cells with a solution of 1% BSA (Bovine Serum Albumine) in PBS for 30 min at RT in a humidified chamber. After this, the blocking solution was carefully removed and then the cells were incubated for 45 min at 37 °C in a humidified chamber with the following Monoclonal antibodies: CD44 (1:50, BD Pharmigen, Milan, Italy), CD90 (1:50, BD Pharmigen), CD105 (1:10, BD Pharmigen), CD166 (1:20, BD Pharmigen), ASMA (1:9000, Sigma) and CD45 (1:120, DakoCytomation). Following several washing in PBS (n. 3), the samples were incubated for 60 min at 37° C with FITC-conjugated polyclonal rabbit anti-mouse immunoglobulins (1:250, DakoCytomation, Germany). After this incubation time, the cells were washed several times with PBS to remove the secondary antibodies unlabeled and then the samples were mounted and nuclei counterstained with a commercial kit *"Pro long antifade reagent with DAPI"* (Molecular Probes, Milan, Italy). Negative controls were done by omitting the primary antibodies. Samples were observed under a fluorescence microscope

(Axiovert 40, Carl Zeiss) connected with a CCD camera to performed imagines (*Pasquinelli et al., 2007 b*).

5.16 ULTRASTRUCTURAL ANALYSIS OF CELL : TEM ANALYSIS

For a ultrastructural studies, the cells, at 3-5 passages, were washed with phosphate-buffered 0.15 M, fixed directly in the culture flasks with 2.5% phosphate-buffered glutaraldehyde for 10 min at RT and then scraped, collected in a eppendorf tube and centrifuged for 10 min at 1800 rpm. The pellets were then fixed in 2.5% phosphate-buffered glutaraldehyde for 24 hours at 4°C, and then processed with the same protocol described before for transmission electron microscopy (see paragraph 5.5). The results obtained were compared with the features we found in a study in which we compared the ultrastructural characteristics of mesenchymal stem cells from human bone marrow and term placenta (*Pasquinelli et al., 2007 c*).

5.17 STEM CELLS mRNAs EXPRESSION by RT- PCR

Total RNA was extracted from cells at 3-5 passages isolated from human fresh aortas according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). A human bone marrow derived mesenchymal stromal cells cell, taken from the repository at the Department of Histology, Embryology and Applied Biology, University of Bologna, were used as a positive control. In brief, the cells were homogenized with Trizol reagent, collected in a eppendorf tubes and then incubate for 5 min at RT. To generate the phase separation, an ad hoc quantity of chloroform was added (0.2 ml for 1 ml of Trizol); the samples were then shacked vigorously for 15 seconds and incubated for 2 to 3 min. at RT. The samples were then centrifuged at 12000g for 15 min at 4°C. After this step, the RNA remains

exclusively in the aqueous phase. After collected the aqueous phase in a new tube, the RNA was precipitated by adding isopropyl alcohol (0.5 ml of isopropyl alcohol for 1 ml of initial Trizol reagent). The samples were then incubated at RT for 10 min, centrifuged at 12 000 g for 10 min at 4°C to allowed RNA to precipitate. After removed the surnatant, the gel like pellet RNA was suspended in 75% alcohol for washing (1 ml of 75% alcohol for 1 ml of Trizol), centrifuged at 7500 g for 5 min at 4°C , air-dried, dissolved in Rnase free water (DEPC water) and quantified. The evaluation of the quality was assessed measuring the A260/A280 ratio. Reverse transcription reactions were performed using 2µg of total RNA following the manufacturer's protocol (Invitrogen, Carlsbad, California). The cDNA was stored at –20 °C until PCR reactions were performed. PCR were carried out as follows: 1µl of cDNA was amplified using specific primers in PCR reaction mix (reaction buffer 10x, $MgCl_2$ 2mM, dNTP 0.2mM, primers 0.2 mM and 1.25 U of Taq DNA polymerase). PCR program was:

- denaturation 94°C for 2 min
- denaturation 94°C for 1 min
- annealing: specific for each primer used for 1 min
- extension 72 °C for 1 min
- final extension 72°C for 7 min

Each cycle was repeated for 29 times. Specific primers sequences (Forwards and Reverse), annealing temperatures and amplicon length are reported in the table below (Table 1). PCR products were visualized on a 1.8% agarose gel stained by ethidium bromide.(*Pasquinelli et al., 2007 b*).

gene	T annealing	amplicon length	forward primer	reverse primer
OCT4	60°C	169 bp	5'-CTT GCT GCA GAA GTG GGT GGA GGA A-3'	5'-TGC CCG AAA CCC ACA CTG CAG-3'
BMI-1	57 °C	369 bp	5'-GGA GAC CAG CAA GTA TTG TCC TTT TG-3'	5'-CAT TGC TGC TGG GCA TCG TAA G – 3'
BCRP-1	65 ° C	652 bp	5'-GTT TAT CCG TGG TG T GTC TGG-3'	5'-CTG AGC TAT AGA GGC CTG GG-3'
CD133	62 °C	337 bp	5'-CTG GGG CTG CTG TTT ATT ATT CTG,	5'-GTA CAA CAC TAC CAA GGA CAA GGC GT-3'
KDR	62 °C	555 bp	5' TAT AGA TGG TGT AAC CCG GA-3'	5' TTT GTC ACT GAG ACA GCT TGG-3'
C-Kit	67 °C	275 bp	5' GTC TCC ACC ATC CAT CCA TC-3'	5'CAT ACA AGG AGC GGT CAA CA-3'
IL6	57 °C	388 bp	5'GAG AAA GGA GAC ATG TAA CAA GAG T-3'	5' GCG CAG AAT GAG ATG AGT TG-3'
beta 2µ	58 °C	114 bp	5'-ACC CCC ACT GAA AAA GAT GA-3'	5'-ATC TTC AAA CCT CCA TGA TG-3'

Table. 1: Forward and reverse primers, annealing temperature and amplicon length for each gene are reported.

5.18 ENDOTHELIAL DIFFERENTIATION OF ADULT STEM CELLS FROM HUMAN THORACIC AORTAS

To demonstrate if adult stem cells isolated from human thoracic aortas are able to differentiate into endothelial cells phenotype, we performed an in vitro angiogenesis assay according with Oswald and et. (*Oswald et al., 2004*). Cells , at passage 5, were cultured until near confluence for 7 days in two different condition. The cells induced were plated at the density of 25.000 cells/cm^2 in a T25 culture flasks, and subsequently cultured for 7 days in D-MEM plus 2% FBS and with 50 ng/ml of VEGF (Sigma, Milan, Italy). Control cells were plated at the density of 4.000 cells/cm^2 in a T25 culture flasks and cultured in D-MEM plus 10% FBS without growth factor (VEGF). For both the conditions, the medium was change every 2 days. After this period, the cells were harvested by treatment with 0.05% trypsin-EDTA and then used for different assays.(*Pasquinelli et al., 2007 b*).

5.18.1 In Vitro Matrigel assay :

Matrigel (BD Biosciences), according to the manufacter's instructions, at low temperature is a liquid form while at the high temperature is like a gel formulation. A 96 culture plate was put in ice and then 50 µl/well of cold Matrigel were dispensed. The plate was then incubate at 37°C for 30 min. to allow the Matrigel solution to solidify. The cells of both the conditions after collection from the flasks, were counted and plated at the density of $5x10^3$ in each well above described. Human umbilical vein endothelial cells (HUVEC) were used as a positive control. The plate was then cultured at 37 °C / 5% CO_2 and the formation of capillary like structures were observed in a CKX41 Olympus inverted microscope after 2, 4 , 6, and 20 hours. Each experiments were performed in triplicate.

5.18.2 TEM analysis:

To visualize ultrastructural features of endothelial differentiation, the structures, obtained in Matrigel after 20 h, were fixed "in situ" with buffered 2% paraformaldehyde for 1 h at 4°C. According with the protocol above described for TEM analysis (see paragraph 5.5), fixation, postfixation, dehydration and embedding steps were performed dispensing the appropriate solution into each well. Once the resin polymerized, the blocks were removed from each well and carefully cut until the structures previously observed by inverted microscope were reached. The ultra-thin sections were counterstained and analyzed by an FEI Tecnai 12.

5.18.3Flow Cytomery Assay:

The cells from both the two conditions (induced and controls) , after the Trypsin's protocol, were analyzed at flow cytometry using the following anti-human MoAbs: KDR-APC (RD Systems, Minneapolis, MN) to detect

surface expression of VEGF receptor 2 and vWF (DakoCytomation) to detect the cytoplasmic expression of vWF. For the expression of vWF, the cells were permeabilized with the Intrapep Kit (Beckman-Coulter) and then incubated with vWF MoAb for 1 h at RT. After two washes with PBS, cells were incubated with FITC anti mouse IgG (Beckman Coulter) for 30 min at RT. Samples were then washed twice over and incubated for 20 min with normal mouse Ig (Sigma), to saturate free anti mouse IgG sites. CD105 PE was added and incubated for 20 min and washed twice. Analyses and data collections were performed as described above (see paragraph 5.14).

5.18.4 In situ Immunofluorescence assay:

To visualize the expression of vWF, the cells, conditioned and controls , were seeded (5000 cells/cm2) on glass coverslips placed in 6-well plates and cultured. After fixation with 2% parafomaldehyde plus 0.1 % Triton X 100 to permebilize the cells membrane , the samples were stained with a MoAb against vWF (1:50, DakoCytomation). For the immunofluorescence staining, the same protocol described above was used (see paragraph 5.15). The mean percentage of vWF+ cells was calculated by counting positive cells on digital images taken from 10 randomly selected fields at a magnification of 40 x. At least 100 DAPI+ cells were analyzed each sample.

5.18.5 CD133 and KDR mRNAs expression by RT-PCR

A molecular assay , using the same protocol specified above (see paragraph 5.17) was done to verify whether VEGF treatment could modify the expression of KDR and CD133 mRNA

5.18.6 Quantification of NOS mRNAs expression by Real Time PCR

The assay for NOS isoforms mRNA expression was optimized for the Light Cycler version 3.5 (Roche Molecular Biochemicals, Mannheim, Germany), with a RT-PCR in two steps. RNA extraction was performed using a High Pure RNA Isolation Kit and reverse transcription using Transcriptor First Strand cDNA Synthesis Kit (all reagents from Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. eNOS mRNA expression was quantified relative to the β-actin housekeeping gene. The primer sequences were as follows: β-actin, sense 5'-TTG GCA ATG AGC GGT TCC-3' and antisense 5'-AGC ACT GTG TTG GCG TAC-3'; PCR fragment of 148 bp. eNOS, sense 5'-GGA CAT TTT CGG ACT CAC ATT G-3' and antisense 5'-GCT GTC GCT CCT GCA AAG A-3'; PCR fragment of 77 bp. Real-time PCR was performed in Light Cycler capillaries using a master mix containing TaqMan DNA polymerase and the respective probes for each NOS type mRNA (Light Cycler TaqMan Maste, Roche Molecular Biochemical). DNA fragment amplification was performed after the addition to the master mix of primers (final concentration: 0.5 μM) and template cDNA (10 to 100 ng). The protocol was the following: 1 cycle at 95 °C for 15 min for the initial enzyme activation, then 45 cycles of denaturation (94°C for 10 s), annealing (60 to 63°C for 10 s) and extension (72°C for 6 to 15 s). In order to verify the purity of the products, a melting curve was produced after the completion of each PCR amplification by increasing the temperature of the reaction mixtures up to 95 °C, by 0.1 °C/s, starting at 60 °C for 60 s. The Roche software uses the second derivative maximum method to calculate the fractional cycle numbers where the fluorescence rises above background at the fastest rate (crossing point, C_p). The amount of mRNA was calculated according to Pfaffl (*Pfaffl, 2001*) relative to the housekeeping gene β-actin. All the values are normalised to the corresponding mRNA of NOS isoforms expressed by HUVECs at passages between 3 and 6

6. RESULTS

6.1 FRESH AND CRYOPRESERVED ARTERIAL TISSUE: HISTOLOGY ANALISYS

At the beginning of this study, we want to evaluate the structural integrity and viability of Smooth Muscle Cells (SMC), the effectors of blood vessel contractile responses, in cryopreserved and unfrozen human aortas harvested from five multi-organ donors (mean age 40 years; 4 male and 1 female), thanks to a collaboration with Cardiovascular Tissue Bank, Policlinico S.Orsola- Malpighi, Bologna, Italy. At light microscopy analysis, fresh human thoracic aortas were normal except for a patchy distribution of luminal endothelium (Fig 19) ;after thawing, the histological picture was similar to that of unfrozen arteries, even if endothelial cells were little represented. Focal changes, including nuclear polymorphism, hypercromasia and nuclei collapse were seen in SMCs (Fig. 20a); small bar-like areas of tissue clearings were found close to SMCs (Fig. 20b)(*Pasquinelli et al., 2006*).

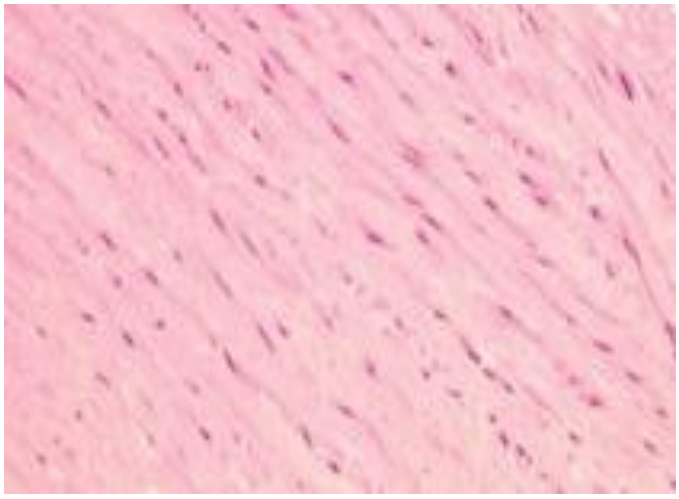

Fig.19: Light Microscopy 10X. Fresh Human Thoracic Aorta from multiorgan donor. Particular of the media: no degeneration's signs, the media appeared well preserved. (*Pasquinelli et al., 2006*).

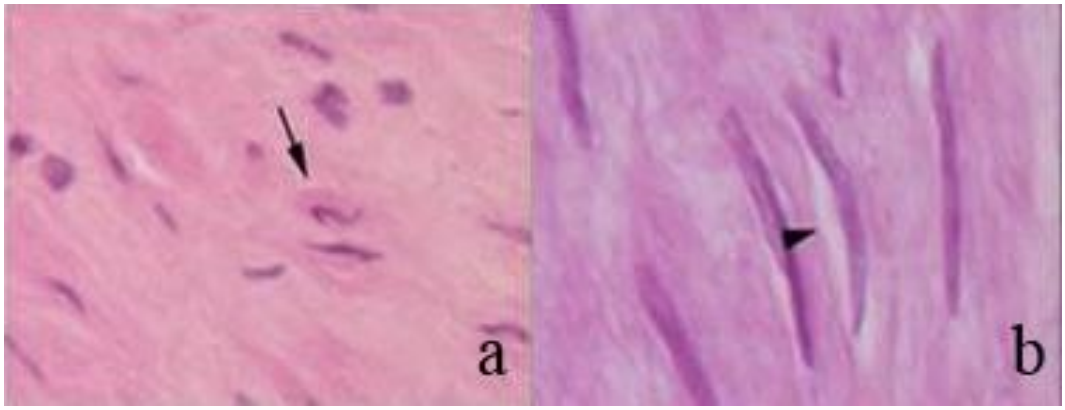

Fig. 20: Light Microscopy 20X: Human Thoracic Aortas after cryopreservation: LM shows a normally looking media with nuclear polymorphism and hypercromasia (➨ , a) and with focal perinuclear clearings close to SMCs (▶, b) . (*Pasquinelli et al., 2006*).

6.2 FRESH AND CRYOPRESERVED ARTERIAL TISSUE : TEM ANALYSIS

The ultrastructural analysis performed on fresh human arterial tissue revealed minimal feature of SMC injury including vesicular nuclei and oedematous mitochondria. This kind of findings could be correlated with the time of cold ischemia existed between the explants and the arriving at the Cardiovascular Tissue Banking. (Fig.21).

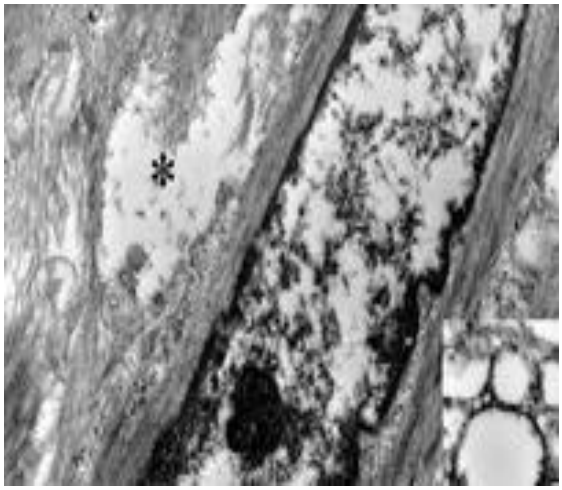

Fig 21: Transmission Electron Microscopy. Fresh Human Thoracic Aortas from multiorgan donor. Minimal feature of SMCs damage; particularly of edematous mitochondria.(6000X)

After cryopreservation, ultrastructural analysis showed significant alterations nuclei-cytoplasmatic in the SMC. The analysis revealed smooth muscle cell's nuclei with condensed chromatin and dark stained cytoplasm (Fig. 22 a); foci of contractile filament loss, small collections of lipid droplets and oedematous mitochondria were a common finding; the areas of tissue clearings observed at LM were located between the SMC plasma membrane and the innermost portion of the contractile cytoplasm; they appeared as empty cytoplasmic zones with granular and filamentous remnants (Fig. 22 b) (*Pasquinelli et al., 2006*).

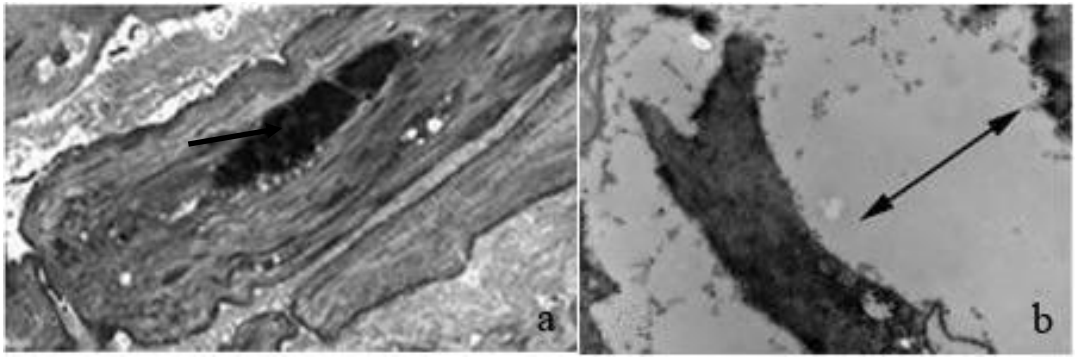

Fig. 22: Transmission Electron Microscopy : Cryopreserved human arterial tissue: a) a smooth muscle cell (SMC) with homogeneous chromatin clumping (single arrow), and dense cytoplasm. 3600X; b) subplasmalemmal structureless area (double arrow) of SMC cytoplasm corresponding to the clearings seen at LM. 6900X.(*Pasquinelli et al., 2006*).

This results confirmed that cryopreservation induce several damage at the vessel's wall, in particular in the luminal endothelial cells, but also demonstrate that, cryopreservation procedures induced different damage to the SMC, the principal effectors of blood vessel contractile responses.(*Pasquinelli et al., 2006 a*).

6.3 IN SITU CELL PRESERVATION : IMMUNOHYSTOCHEMISTRY RESULTS

In order to evaluate the in situ cell preservation of arterial tissue before and after cryopreservation, we performed an immunohistochemical studies on paraffin sections using monoclonal antibodies to detect endothelial cell CD34 antigen and smooth muscle actin (ASMA) . CD34 is a transmembrane protein that defined ˜ 1% of normal bone marrow mononuclear cells including hematopoietic precursors/stem cells and normal endothelial cells. ASMA is a alpha-Smooth muscle (alpha-sm)

actin, an isoform typical of smooth muscle cells (SMC) and present in high amounts in vascular SMC, where was localized in microfilament bundles. In this studies, CD34 immunohistochemical staining showed a reduction in the number of luminal endothelial cells after cryopreservation, even if a positive CD34 immunostaining was found in the endothelial cells lining the vasa vasorum adventitial. (Fig 23)

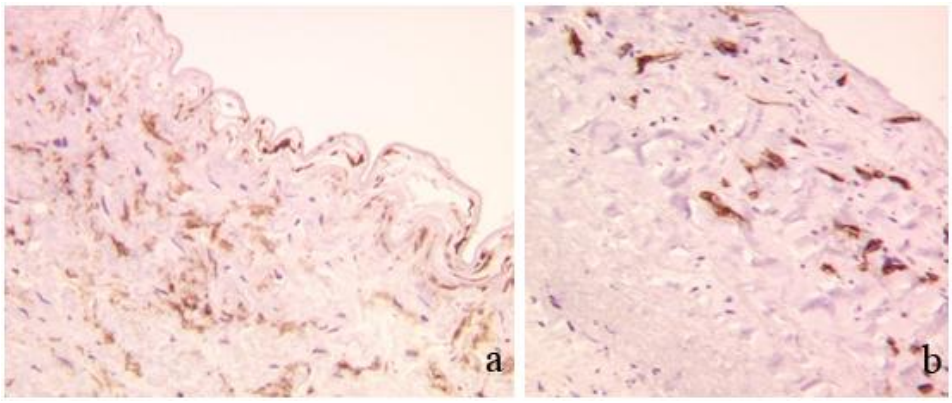

Fig. 23: Human Thoracic Aortas: CD34 staining; a) unfrozen arterial sample; b) Cryopreserved arterial sample

The general pattern expression of ASMA in cryopreserved aortas was similar to the expression pattern seen on unfrozen arteries, except for some unreactive zone in the media layer. (Fig 24)

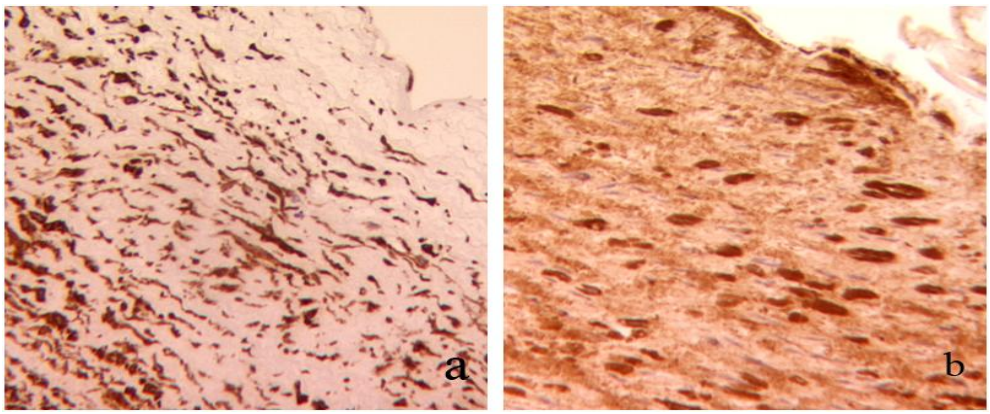

Fig.24: Human Thoracic Aortas: ASMA staining; a) unfrozen arterial sample; b) Cryopreserved arterial sample.

6.4 IN SITU CELL DEATH: TUNEL ASSAY

The in situ cell death detection was investigated in unfrozen and frozen arterial tissue using the TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) assay, which reveals "in situ" DNA strand ruptures. This assay revealed directly in situ the apoptotic cells, is a precise, simple and fast method without the use of radioactive substances. Cleavage of genomic DNA during apoptosis may yield double-stranded, low molecular weight DNA fragments(mono-and oligonucleosomes) as well as single strand breaks " nicks" in high molecular weight DNA. Those DNA strand breaks can be identified by labeling free 3'-OH termini with modified nucleotides in a enzymatic reaction. In the " In situ Cell Death Detection Kit" the DNA strand breaks were labeled with Terminal deoxynucleotidyl transferase(TdT), which catalyzes polymerization of labeled nucleotides to free 3'-OH DNA ends in a template-independent manner. The incorporated fluorescein was detected by anti-fluorescein Fab fragments from sheep, conjugated with horse-radish peroxidase (POD). After substrate reaction, stained cells can be analyzed under a LM.

For this experiments, we analyzed n. 6 human thoracic aortas before the procedures of cryopreservation and n. 10 of human thoracic aortas after the cryopreservation protocol. The reactions were observed at light microscope, using a specific software (Image pro plus) connected to a CCD camera. Light microscopy analysis showed that the principal type of cells which resulted positive at the staining was the smooth muscle cells, principal component of the arterial media layer. (Fig 25)(*Pasquinelli et al., 2006*).

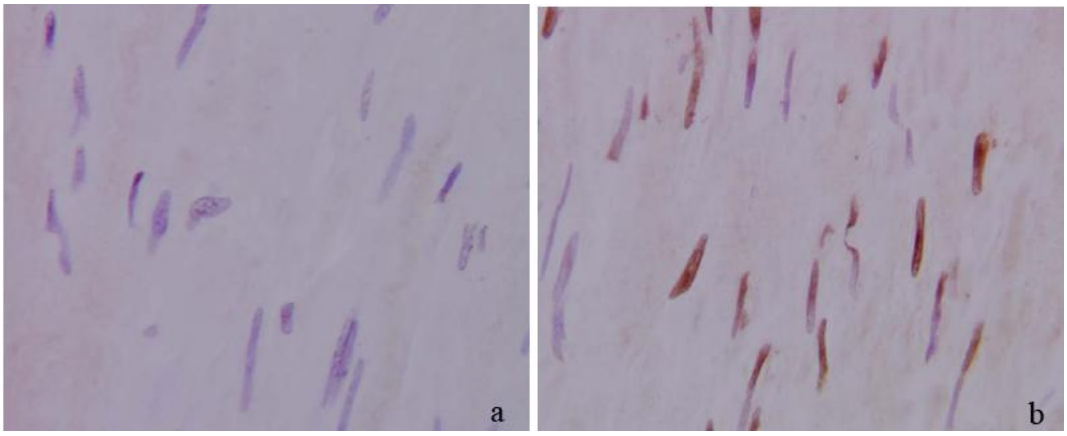

Fig 25: TUNEL ASSAY, Light Microscopy 10X. a) Unfrozen arterial tissue; b) Cryopreserved arterial tissue : SMC resulted positive staining (brown nuclei) (*Pasquinelli et al., 2006*).

In order to perform a semi quantitative analysis of the positive cells, we took series pictures of the total area for each samples and subsequently the imagines were evaluated by Image Pro Plus software. The results, after counting the number of positive cells, revealed that percentage of TUNEL stained SMCs ranged from 0.3 to 6.2% (average of 4.2% labeled SMCs) in unfrozen human thoracic aortas, while after cryopreservation the percentage increased from 2.8 to 16.5% with an average of 8.6% (Fig. 26)(*Pasquinelli et al., 2006*).

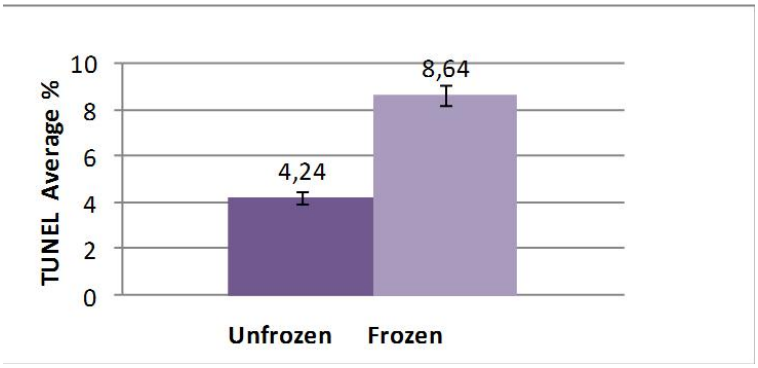

Fig. 26: Average of TUNEL positive cells in unfrozen and frozen human arterial tissue.

This results confirmed that cryopreservation procedures induce loss and death of SMCs.(*Pasquinelli et al., 2006*).

6.5 ISOLATION CELLS FROM CRYOPRESERVED ALLOGRAFTS: FLOW CITOMETRY STUDY

We tried to isolate cells from cryopreserved human allografts using an enzymatic and mechanically methodology/approach, in order to assess in vivo cell viability. On the primary isolated obtained, we performed a flow citometry 7-ADD staining, a nuclear dye that is excluded by viable cells but penetrate cell membranes of dying or dead cell and it is generally used to identify necrotic and late apoptotic cells, and also we used flow cytometry to establish the phenotype of cells isolated from cryopreserved allografts (CD34 and ASMA staining). Flow cytometry 7-AAD staining of cryopreserved homograft isolates documented that an average of 49 ±16% cells were unlabeled thus suggesting that a reduced cell fraction was still alive at the time of analysis. As shown in Table 2, in three cases the percentage of unlabeled cells was less than 50%, while two samples showed more satisfactory results, i.e., 56 and 75%. Flow cytometry immunophenotyping showed that SMCs were the major cell component present in the primary isolates (an average of 76 ±12% cells were ASMA positive); the CD34 positive endothelial cells were a minor fraction only (percentages of CD34+ positive cells ranged from 3.9 to 0.01%). (Table 2). Since ASMA staining requires cell permeabilization, we were not able to determine the absolute 7-AAD unstained fraction of ASMA-positive cells. This date demonstrate that the cryopreservation procedures induced an important loss of viability for all type of cells, e.g. ECs and SMCs (*Pasquinelli et al., 2006*).

DONORS	Z.I.	L.U.	D.B.	R.U.	N.U.
VIABLE CELLS	56%	37%	75%	37%	43%
ASMA+	66%	68%	93%	84%	71%
CD34+	3.9%	2.28%	0.01%	1.5%	2.1%

Table 2: The table schematizes the mean percentages of 7-AAD unlabeled (line 1), ASMA positive (line 2), and CD34 positive (line 3) cells found in primary isolates recovered from cryopreserved human thoracic aortas (n. 5 samples harvested from heart beating donors, first letters of each individual donor' name [i.e., ZI, LU etc.] are indicated. Flow cytometry assay. 10,000 events per analyses. The percentage is a mean of a triplicate examination /each donor. 7-AAD dye unlabeles structurally viable cells; ASMA monoclonal antibody stains smooth muscle cells and CD34 monoclonal antibody stain endothelial cells (*Pasquinelli et al., 2006)*.

6.6 ORGAN CULTURE: LM E TEM

Since the results described before confirmed that the cryopreservation/thawing procedures altered the structure and the viability of the principal cells that reside in the arterial wall, e.g. SMC and EC, we want to investigate if this cells showed the ability to recover cryopreservation/ thawing damage in a in vitro organ cultures. Little fragment of fresh and cryopreserved human allograft were cultured in a RPMI 1640 with L-Glutamine, 10% FCS and 1% Penicillin/Streptomycin in a 37 °C incubator with 5% CO_2. After culturing for 4, 8, and 15 days tissues were processed for LM and TEM analysis. LM did not reveal significant differences between unfrozen and cryopreserved samples in organ cultures taken at 4 days and 15 days (Fig. 27 a-b) .TEM analysis

showed at 15 days of culture, some signs of SMC's degeneration like the presence of chromatin clumping. (Fig.27 c)(*Pasquinelli et al.,2006*).

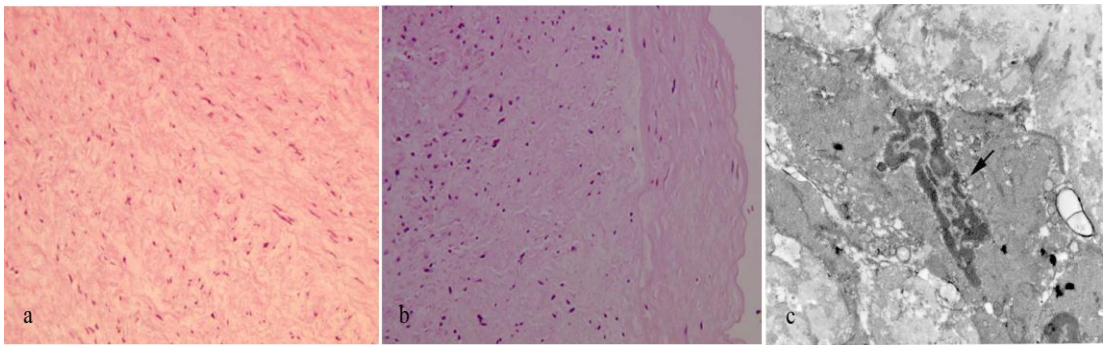

Fig. 27: Organ culture of an unfrozen Human Thoracic Aortas. At LM no significant decrease in the number of SMCs is seen during culture conditions (a, 4 days; b, 15 days); after 15 days SMCs shows peripheral chromatin clumping (arrow) (c, TEM, 3600X))(*Pasquinelli et al., 2006*).

However, a progressive reduction in the number of SMCs from day 4 to day 15 associated with degenerative ultrastructural changes were found in cryopreserved samples when compared to controls (Figs. 28 and 29) (*Pasquinelli et al., 2006*).

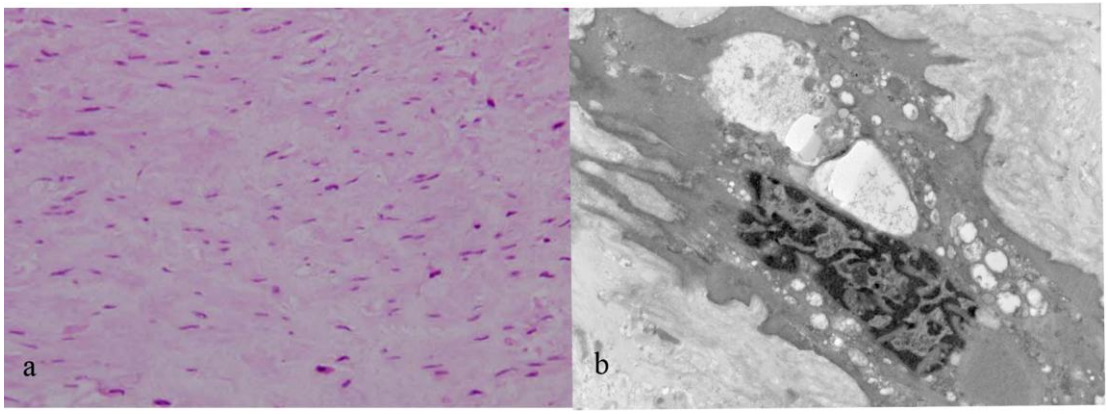

Fig.28: Organ culture 4 days of a cryopreserved HTA. a) reduction in the number of SMCs during culture conditions is seen by LM (10X) ; b) TEM shows progressive loss of SMC structural integrity (3600X)(*Pasquinelli et al., 2006*).

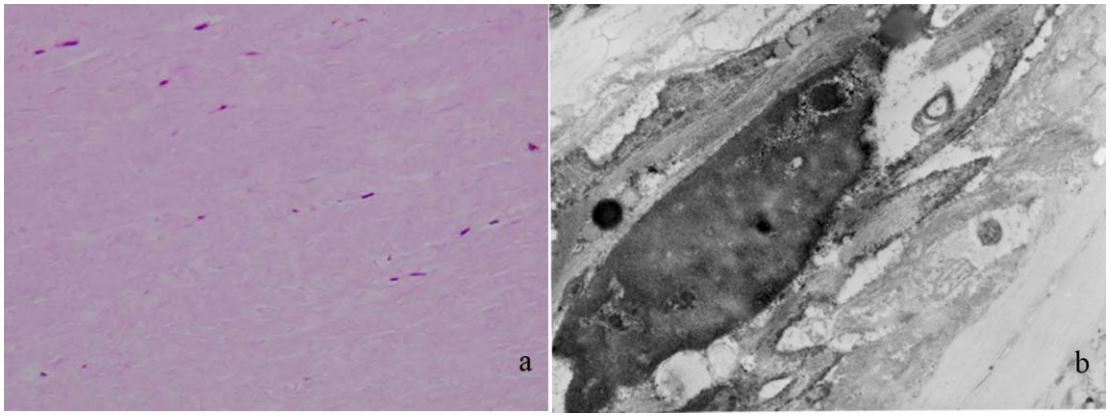

Fig. 29: Organ culture 15 days of a cryopreserved HTA. a) loss of SMC in the media layer is seen by LM (10X); b) TEM snows ultrastructural degeneration of SMC (3600X)(*Pasquinelli et al., 2006)*.

However, even if this results demonstrated that the damage of parietal SMC could only partially recovered by organ colture " in vitro", in this experiments we were able to see, by LM and TEM, just at day 4, that the zone between media and adventitial layer, which represents the zone of nutritionals changes, was well preserved with some vasa vasorum vital (Fig. 30).

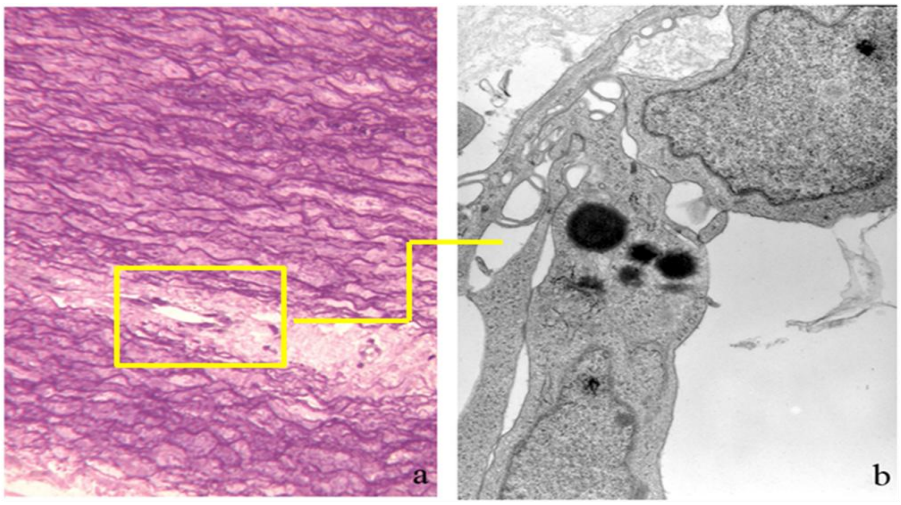

Fig. 30: Organ culture 4 days of a cryopreserved HTA. a) LM showed the presence of well preserved vasa vosorum located in the adventitial layer. b) Ultrastrctural features of adventitial vasa vasorum vital by TEM.

6.7 ANTIGENICITY PRESERVATION: IMMUNOHISTOCHEMICAL STAINING FOR HC-10 E NAMB-1:

Subsequently, we tried to establish whether the cryopreservation procedure can modify arterial homograft antigenicity. So we performed an immunohistochemical study on fresh and cryopreserved human arterial homografts in order to visualize the expression of HLA class I heavy and light chains ‘‘in situ’’ by using the HC-10 and Namb-1 monoclonal antibodies. To standardize immunostaining, flow-cytometry indirect immunofluorescence analysis was performed on HUVEC (Fig.31 a-d); immunohistochemistry of human ovarian cortical vessels was performed as an additional positive control, where endothelial cells showed an intensity of HC-10 (Fig. 31) and Namb-1 (Fig.31f) immunostaining which was comparable to that previously seen in ovarian and submandibolar gland tissues. Negative controls were performed by omitting tissue incubation with primary antibodies. The HC-10 antibody recognizes an epitope expressed on virtually all b2-microglobulin- free HLA-B heavy chains and on b2-microglobulin- free HLA-A10, HLA-A28, HLA-A29, HLA-A30, HLA-A31, HLA-A32 and HLA-A33 heavy chains. The Namb-1 antibody recognizes both free and HLA class I heavy chain-associated human b2-microglobulin (*Pasquinelli et al., 2007 a*).

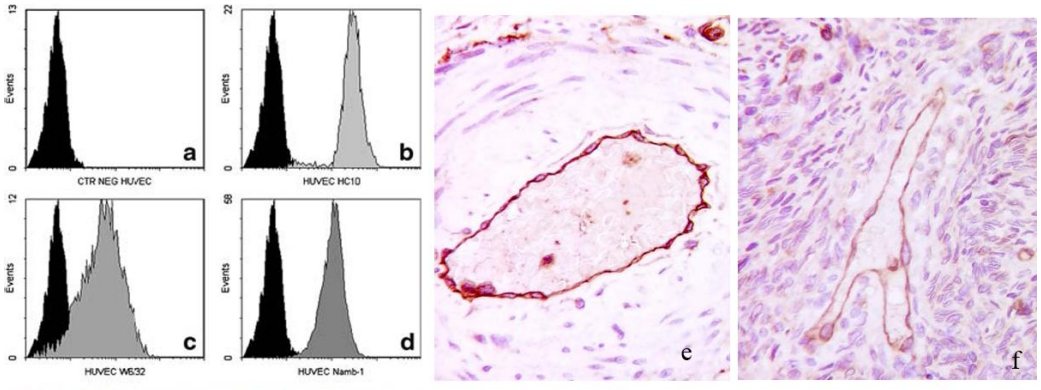

Fig 31: a-d) Representative examples of flow-cytometry of unfixed HUVEC : (a) negative control; (b) HC10 MoAb; (c) W6/32 MoAb; (d) Namb-1 MoAb. e–f) Immunohistochemistry analyses performed on human

ovarian cortical vessels, used to standardize immunostaining with HC-10 and Namb-1 MoAbs. (e) HC-10 MoAb; (f) Namb-1 MoAb. Original magnification (e, f) 250X.(*Pasquinelli et al., 2007 a*).

The immunohystochemical results obtained on fresh human arterial tissue showed that, in the intima, HLA class I antigens were markedly expressed by luminal endothelial cells; the positivity was intense and found both on cell membrane and in the cytoplasm; in the media smooth muscle cells showed a diffuse and moderate staining with HC-10; in the adventitia we found a well-developed network of vasa vasorum whose endothelial cell lining was intensely stained with a pattern similar to that of luminal endothelium; when compared to endothelial cells, smooth muscle cells of the same vessels showed a lower expression of the HLA class I antigens. The cell staining pattern of Namb-1 was similar to that of HC-10. Representative results are shown in Figs. 32 a, c, e and 33 a, c, e.

In cryopreserved arterial allografts the expression of HLA class I antigens was comparable to that observed in fresh, unfrozen tissues; however, as a consequence of surface endothelium damage induced by cryopreservation we found a decreased expression of HLA class I antigens in the luminal intima; no difference of HLA class I and b2-microglobulin expression was found in the adventitia with respect to fresh arterial tissue. Representative results are illustrated in Figs. 32 b, d, f and 33 b, d, f. This data confirmed a previous study of our group, which demonstrate that cryopreserved allograft, like the fresh counterpart, induced an immunological response in the donors (*Mirelli et al., 2005*), and also demonstrated that the endothelial cells in the adventitial vasa vasorum are the principal cells that induced an immunological response (*Pasquinelli et al., 2007 a*).

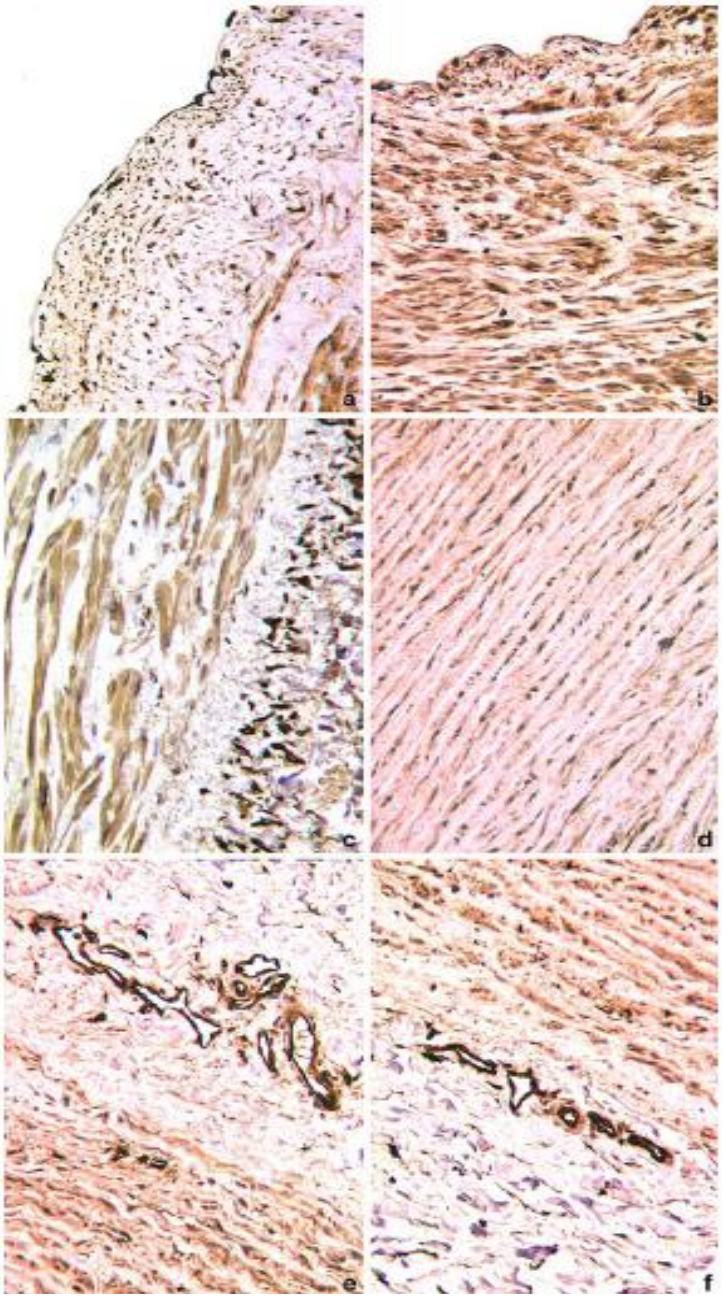

Fig. 32: Representative examples illustrating the HC- 10 immunoreactivities seen in the different layers of fixed and routinely processed samples from fresh (a, c, e) and cryopreserved (b, d, f) human arterial allografts. Note that no significant difference of HLA I heavy chain antigen expression was found between fresh and cryopreserved arterial allografts. HC-10 immunostaining is intense in endothelial cells lining the surface intima (a) and adventitial vasa vasorum (c, e, f), moderate and diffuse in medial smooth muscle cells (b, c, d). Original magnification (a–f) 100x (*Pasquinelli et al., 2007 a*).

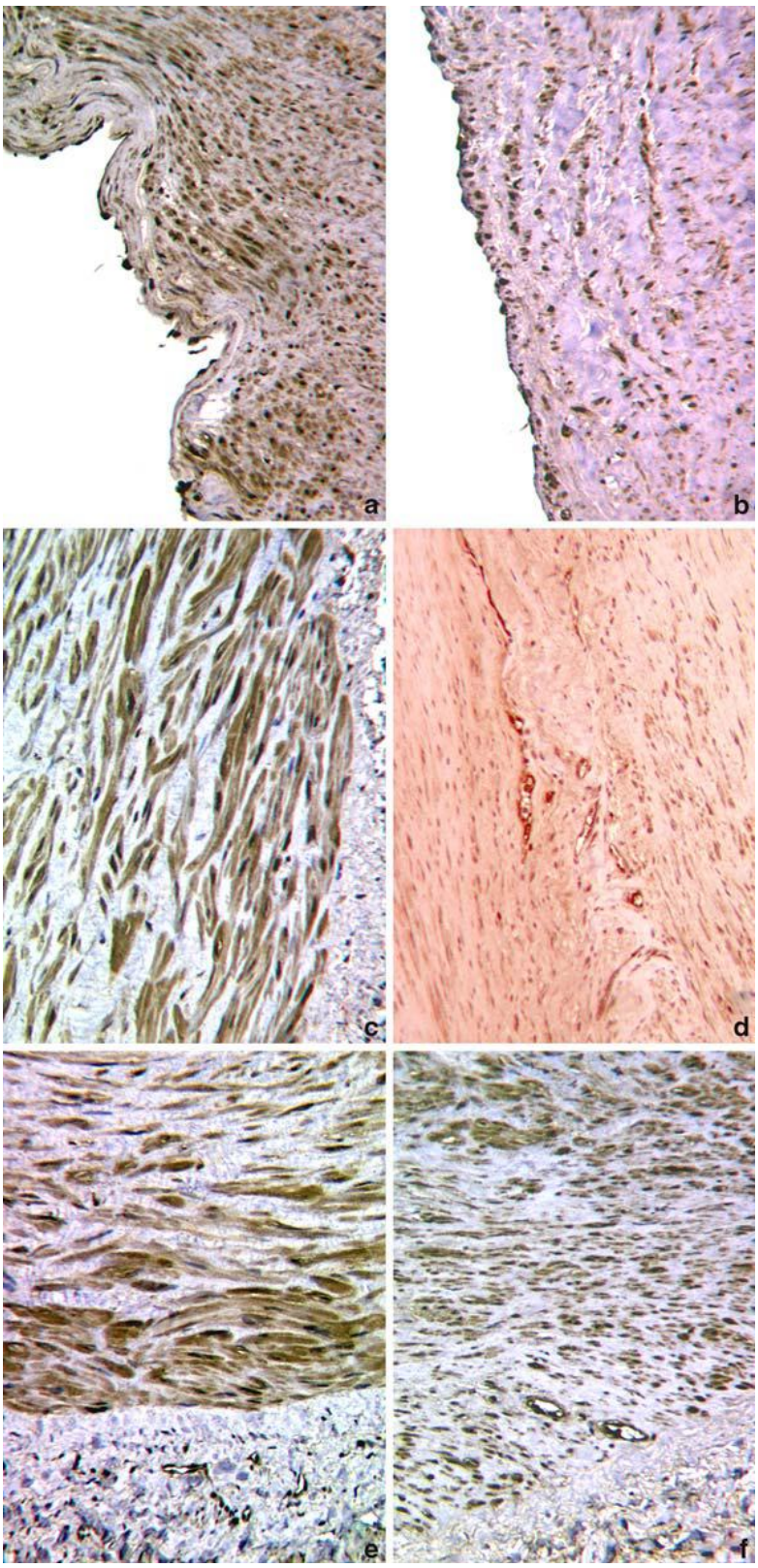

Fig. 33: Representative examples illustrating The Namb1immunoreactivitie is seen in the different layers of fixed and routinely processed samples from fresh (a, c, e) and cryopreserved (b, d, f) human arterial allografts. Alike Fig.16 no significant difference of HLA I light chain antigen expression was found between fresh and cryopreserved arterial allografts. Namb- 1 pattern of immunostaining is comparable to that of HC-10. Original magnification (a–f) 100 X·(*Pasquinelli et al., 2007 a*).

6.8 IDENTIFICATION IN SITU OF A "VASCULOGENIC ZONE": IMMUNOHYSTOCHEMICAL STUDIES:

In according with the previous results that identified, in human thoracic aortas, a zone between the media and the adventitial layers in which are present cells that remain vital, proliferate in organ culture system and maintain the expression of HLA-class I after cryopreservation procedures, we performed an immunohystochemical studies to better characterize this cells population. Therefore, in order to identified a vasculogenic zone in situ where the adult stem cells could be present, the immunohystochemical studies, were performed on fresh human femoral arteries and thoracic aortas harvested from multiorgan donor, collected at the Cardiovascular tissue banking, S.Orsola-Maplighi Hospital, Bologna, Italy. It's now that arteries are constituted of an endothelial layer (intima), while the tunica media, the middle layer of the walls of arteries, is composed of smooth muscle and elastic fibres. For this studies, we used different type of monoclonal antibodies to characterized the cell populations presents in the arterial wall, like:

- CD34: a transmembrane protein that defined ˜ 1% of normal bone marrow mononuclear cells including hematopoietic precursors/stem cells and normal endothelial cells

- vWF: Factor VIII related antigen is more appropriately know as the von Willebrand factor; it is a glycoprotein and is complexes with factor VIII related antigen in plasma. Factor VIII is also present in the endothelial cells where is shows a granular pattern of reactivity.

- CD45: recognizes a family of proteins known as the leukocyte common antigen exclusively expressed on the surface of almost all haematolymphoid cells and their progenitors

- S-100: normally present in cells derived from the neural crest (Schwann cells, melanocytes, and glial cells), chondrocytes, adipocytes, myoepithelial cells, macrophages, Langerhans cells, dendritic cells, and keratinocytes. S100 proteins have been implicated in a variety of intracellular and extracellular functions, are involved in regulation of protein phosphorylation, transcription factors, Ca++ homeostasis, the dynamics of cytoskeleton constituents, enzyme activities, cell growth and differentiation, and the inflammatory response.

- CD68: higly glycosylated lysosomal glycoprotein present in the cytoplasmatic granules of monocytes, macrophages, neutrophils, basophils and large lymphocytes; it also can be expressed in the cytoplasm of non-hematopoietic tissue

- C-Kit: C-kit is a proto-oncogene, which encodes for growth factor receptor protein KIT (Cd117), the receptor for stem cell factor. It is expressed in hematopoietic stem cells, tissue mast cells, basal cells line of the skin, epithelial cells of the breast, melanocytes, germ cells..

- Ki-67: a marker of cell proliferation which recognizes a nuclear protein expressed in the G1, S, M, and G2 phases of the cell cycle.

The immunohystochemical results showed a distinct CD34+ and Ckit+ cell population between the media and adventitia layers.(Fig.34)(*Paquinelli et al., 2007 b*).

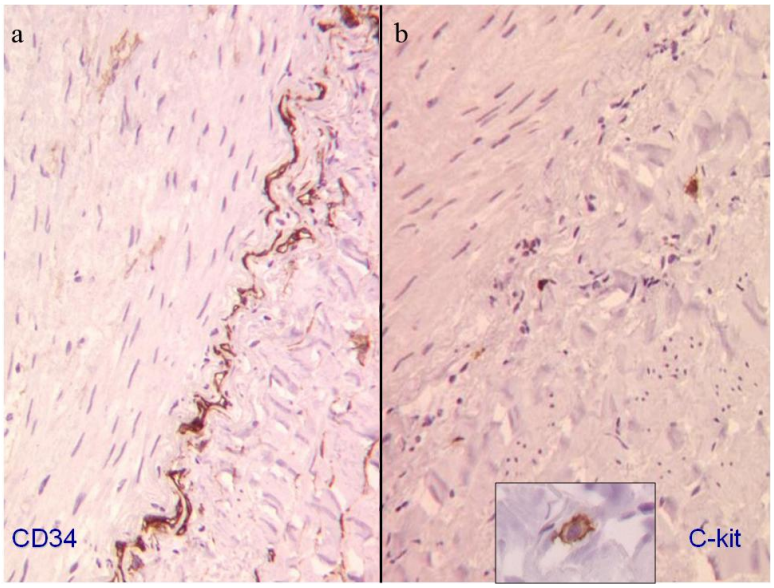

Fig.34: " Vasculogenic zone" at the border between adventitia e media layers. a) CD34 positive cells; b) C-kit positive cells

Unlike femoral arteries which show a thin layer of CD34+ capillary-like structures at the border between the media and adventitia (Fig.35A), in the human thoracic aortas , we observed a more complex network of CD34+ cells which also extended into the adjacent media and adventitia layers. The CD34+ cells were arranged both as single elements and capillary structures with evident lumina. (Fig. 35 B). The immunostaining for vWF (a mature endothelial markers) only stained some of the larger vascular channels present in the adventitia layer. Besides, CD34+ vascular channels were also seen in the media, which adjacent tissue sections showed to be vWF- (Fig.35 C-D)(*Pasquinelli et al., 2007 b*).

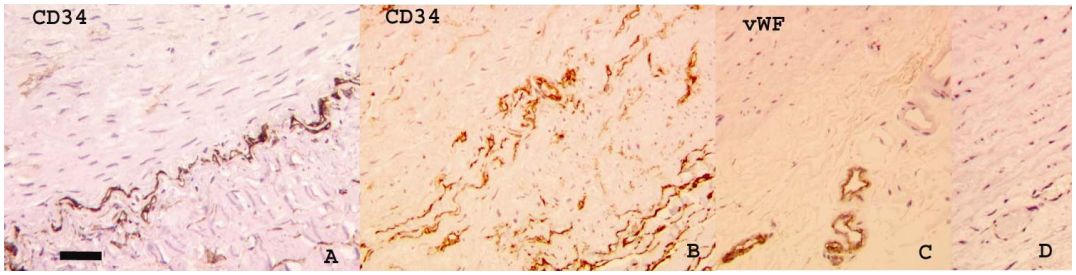

Fig. 35 : IHC results for CD34 and vWF staining A) Femoral artery, B-D) Thoracic aorta, Scale Bars are 50 μm.

CD45+ were seen in association with the CD34+ cell network and it could be connected with a inflammatory cells near the vasa vasorum adventitial. Further, rare CD68+ and S-100 + elements cells were found were found in the adventitial layers connected with vasa vosorum (Fig.36)

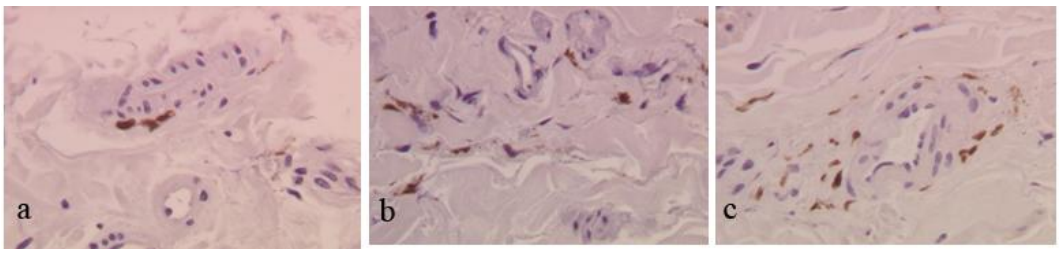

Fig.36: Immunohystochemicals reactions performed on fresh human thoracic aortas for CD45, CD68, S-100. Rare positive elements were found in the advential layers a) CD45 staining; b) CD68 staining; c) S-100 staining

The second population of round stromal cells, C-Kit + were observed in the adventitia close to the richly vascularized border. Only in the thoracic aortas, we found rare C-kit + elements in the subintimal layers,

adventitial vasa vasorum and some stromal elements. This cells were present in cluster, above all in couple. This cells are CD34, CD68, S100 negative; some were CD45+ (Fig. 37 A-D).

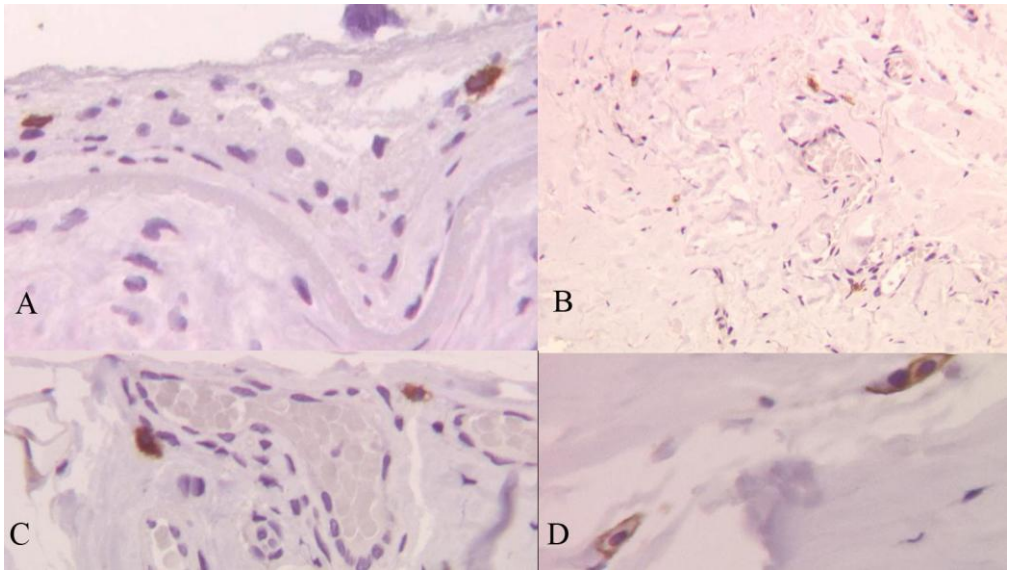

Fig. 37: Immunohystochemical analysis for Ckit performed on human thoracic aortas. A) positivity cells in the sub-intimal layer B) positivity cells in the adventitia layers; C) perivascular ckit+ cells; D) Stromal Ckit+ cells

This type of cells, C-kit, which is commonly associated with the more primordial cardiac stem cells is also expressed by mastzellen; however, at the histology analysis the CKIT+ cells showed eosinophil cytoplasm, elevated ratio nucleo/cytoplasm and eccentric nucleo; ultrastructural examination of the same samples ruled out this possibility, demonstrating that this cells are indifferentiated cells (results not shown).

Stem cells are quiescent cell that, under internal and external signals, undergo to a asymmetrical divisions. Ki-67 is a marker of cell proliferation and is able to recognize a nuclear protein expressed in the all phase of the cell cycle, except the G0 phase. The

immunohistochemical assay for Ki67 exclusively stained individual endothelial cells and stromal cells located between the media and adventitia. (Fig 38-39). This data indicated that cells with high growth potential are located in a restricted area of the aortic wall corresponding to the site of CD34+ and ckit + cell residence. (Fig.34)(*Pasquinelli et al., 2007 b*).

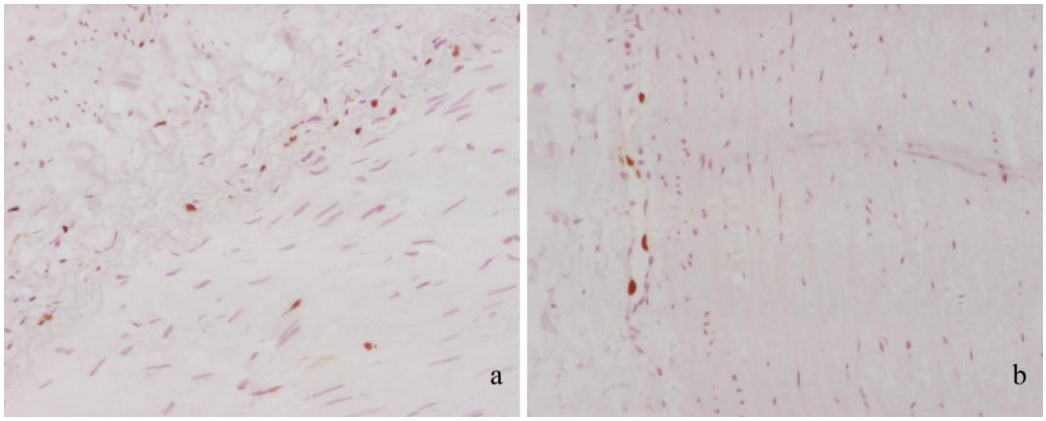

Fig.38: Ki67 immunostaining. a) endothelial cells and stromal cells in the zone between the media and the adventitia.; b)adventitial vasa vorum with Ki67 + cells.

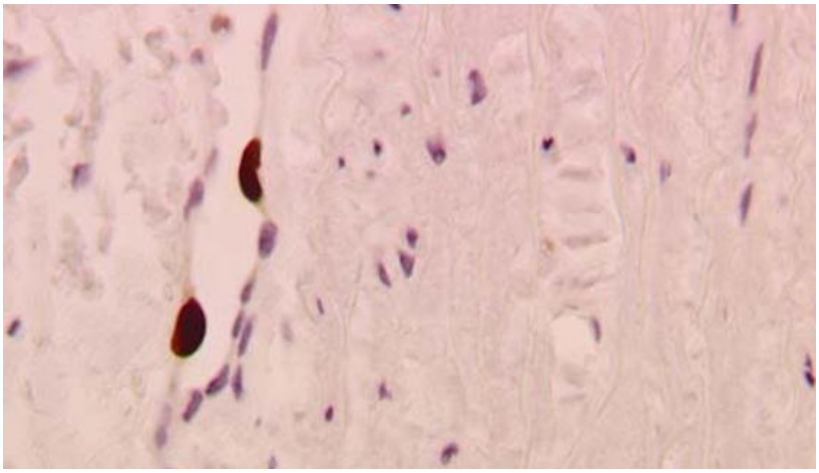

Fig.39. Particular of Ki67+ cells integrated in a little vessel.

6.9 ISOLATION AND ESPANSION OF ADULT STEM CELLS FROM FRESH HUMAN THORACIC AORTAS

Taken together the previous data presupposed the presence of resident adult stem cells within the human arterial wall. The successive step was tried to isolation this kind of cells from fresh human thoracic aortas. Like described in materials and methods section, we isolated the cells from a 25 cm2 of tissue, using a combination of enzymatic and mechanical procedures. Cell isolation was carried out successfully in 3 of the 10 cases. In the remaining 7 cases cell cultures were discarded due of a fast growth of contaminant bacteria and fungi. This type of contamination didn't derived from biological contamination of media, solution or incubator but, the microbiological assays performed on the solution used for delivering arterial grafts to the Cardiovascular Tissue Bank facility revealed the presence of the bacterial species and yeasts listed in Table n.3.

Staphilococcus Epidermidis	4/7
Enterococcus Faecalis	3/7
Staphilococcus Aereo	3/7
Candida Albicans	3/7
Enterobacter Aerogenes, Klebsiella Pneumonie, Proteus Mirabilis, Pseudomonas Aeruginosa, Staphilococcus Hominis	2/7
Propionibacterium Acnes, Serratia Marcescens	1/7

Table n.3: Contaminant microorganisms responsible for unsuccessful cell isolation and culture (7 out of 10 cases).

Started from 25-30 cm^2 of human thoracic aortas, after the procedures of isolation, the total number of mononuclear cells obtained, counted with Methyl Violet in a Neubauer-counting chamber, varied from 1,5 x 10^6 to 3,5 x10^6. The cell colonies began to appear approximately 3-4 days after

initial plating, and so, considering a 36 hours of doubling time of the isolated cells, the number of resident mesenchymal stromal cells present in the primary isolate from a 25 cm^2 sample of aorta can be estimated around 0.5 ~ 2.0 x 10^4 cells. At the beginning of the cell culture, the cells showed two distinct morphologies: one having a fibroblastoid appearance and the other one with a more rounded appearance (Figure 40 a). The rounded cells could be a contamination of endothelial cells, data confirmed also in flow cytometry analysis performed on primary isolated (data non shown). After passaging, these rounded cells rapidly disappeared from culture and the elongated cells continued to proliferate, even after numerous passages (Figure 40 b) and their appearance was morphologically indistinguishable from that of MSCs isolated from the human bone marrow.

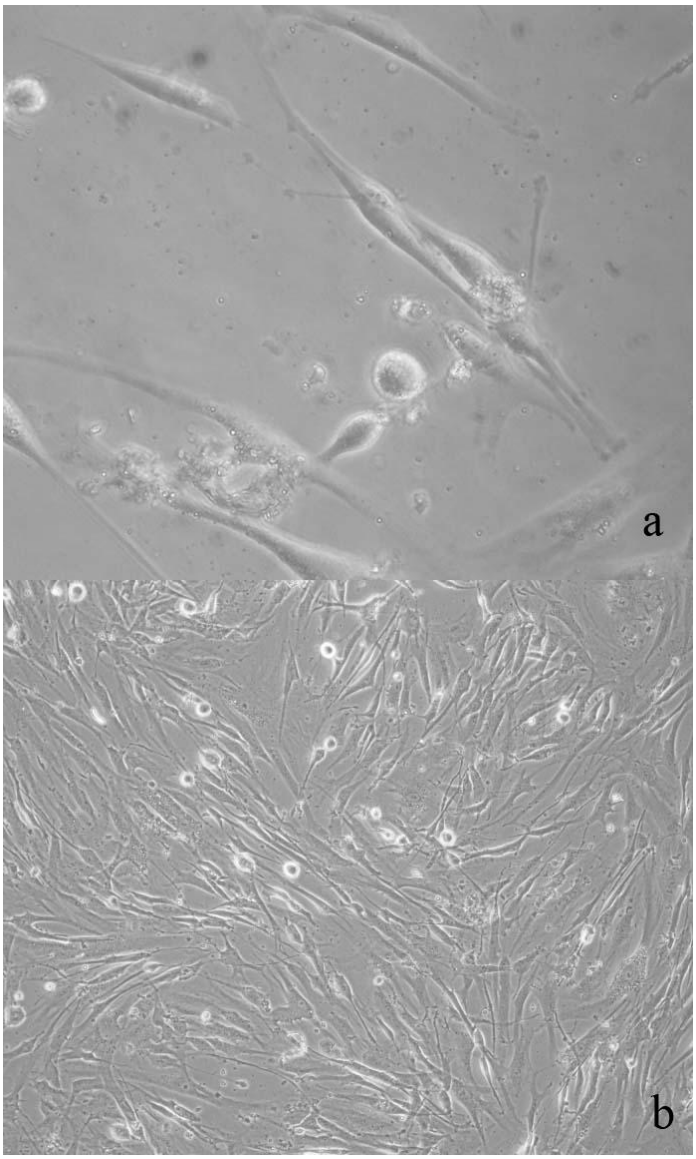

Fig. 40: MSCS from arterial tissue. a) two distict type of morphologies after 3 days of culture, bar =20 µm; b) Typical phenotype of mesenchymal stromal cells after 3 passage in culture

The cells were expanded in culture for up to 12-15 passage; they were cultured in collagen type 1 flasks in D-MEM plus 10 % FBS, like the culture of MSCS isolated from human bone marrow (*Pasquinelli et al., 2007 b)*. To ruled out a cellular transformation or a viral infection, virological assays for CMV and EBV as well as cytogenetic analyses resulted negative (results not shown).

6.10 IMMUNOPHENOTYPING: FLOW CYTOMETRY ANALYSIS

Because the morphology of the cells isolated from human thoracic aortas was identical at the morphology of mesenchymal stem cells isolated from human bone marrow, we characterized our cells, in a flow cytometry assay, using the panel of antibody used to characterized the MSCs. Triple flow cytometry immunostaining of the cultured cells taken at passages 3-5 revealed that these cells coexpressed molecules commonly found in MSCs, i.e., CD44, CD90, CD105. In particular 85% of the cell populations were CD44+/CD90+; 95% of CD44+/CD90+ also expressed CD105. Considering the results altogether, more than 80% of the overall cell population simultaneously expressed CD44, CD90 and CD105 molecules (Fig.41).Cells taken at 3-5 passages were analyzed for the single expression of CD166, a markers used to identified MSCs, the negativity for hematopoietic stem cells markers (CD34, CD45), endothelial marker (vWF, CD146) and for the expression of KDR, CD133, C-Kit. Besides we evaluated the expression for HLA class 1 and HLA-DR. Single labeling experiments also showed that at least 80% of the cultured cells were CD166+; KDR, CD34 and CD133 were found expressed in negligible cell fractions (0.1 to 5 %) whereas c-kit+ cells were not detected at all (data non shown). Cells were also negative for lineage markers, i.e., CD45, which is expressed in mature hematopoietic cells, CD146 and vWF which are markers of differentiated endothelium. Immunologically the cells expressed HLA class I antigens and were negative for HLA-DR. In summary, the phenotype of the isolated cells was CD44+, CD90+, CD105+, CD166+, KDRlow, CD133low, CD34low, c-kit -, CD45-, CD146-, vWF-, HLA classI + and HLA-DR -.(Fig.42) (*Pasquinelli et al., 2007 b*).

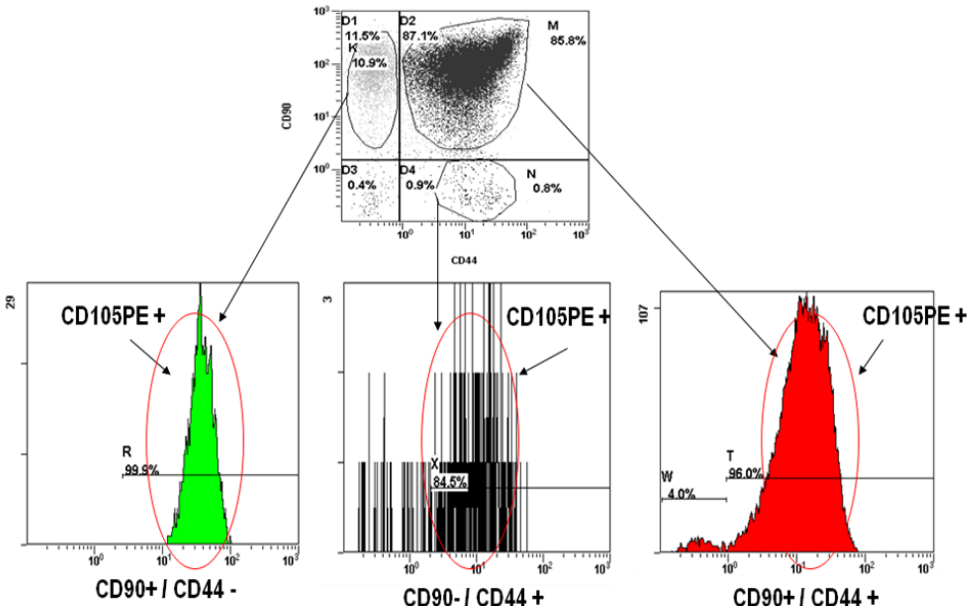

Fig. 41: At flow cytometry thoracic aorta MSCs co-express CD44, CD90 and CD105 surface molecules. Percentages and cytograms from a representative experiment.

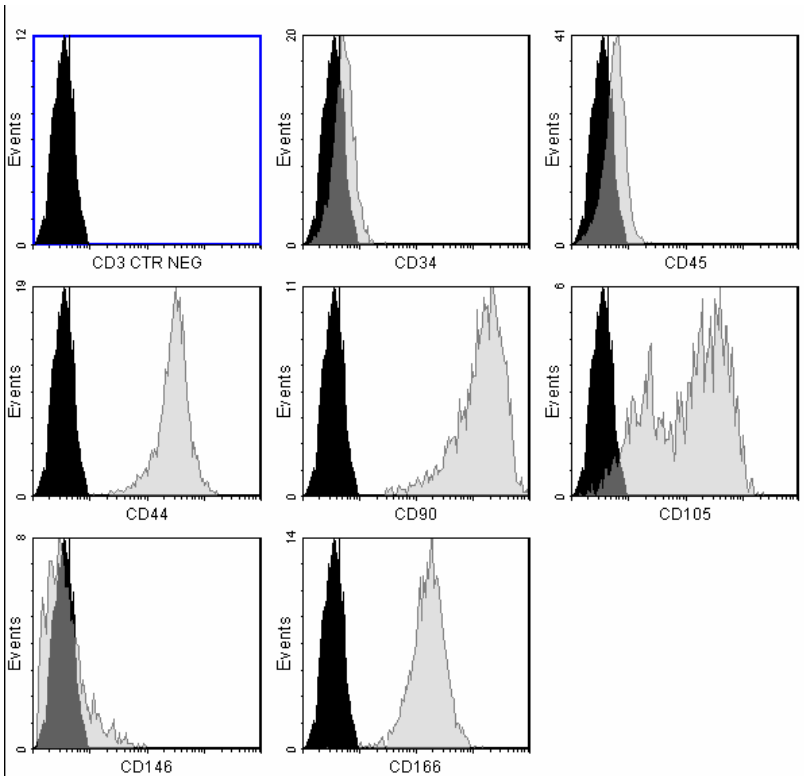

Fig. 42: Immunophenotype of thoracic aortas MSCs at flow cytometry.

6.11 IMMUNOFLUORESCENCE ANALYSIS

The in situ single immunofluorescent staining performed on cells cultured in chamber slides confirmed the results obtained at flow cytometry. The cells with a fibroblast-like morphology resulted positive for CD44, CD90, CD105, CD166, some cells with a smooth muscle phenotype resulted positive for the ASMA staining while rare elements with a rounded appearance resulted positive for CD45 labeling (Fig. 43)(*Pasquinelli et al., 2007 b*).

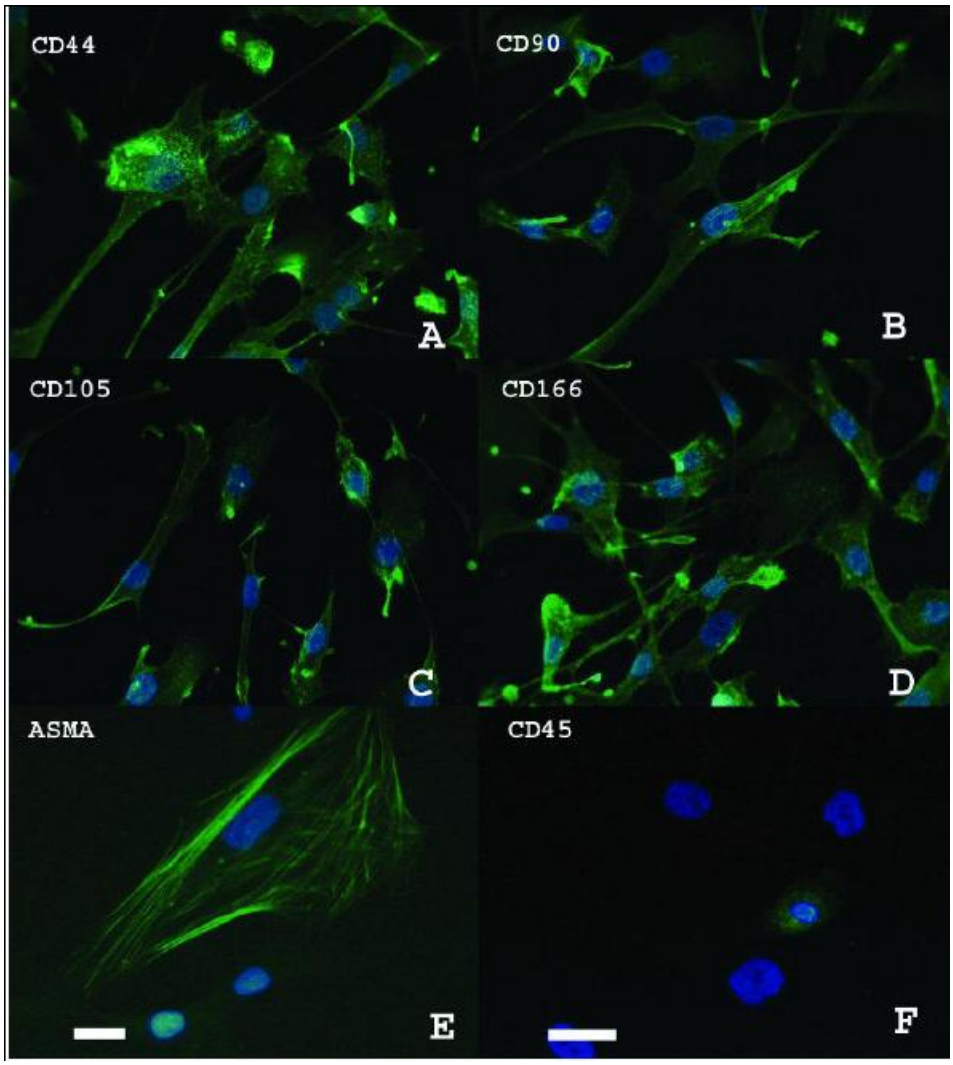

Fig. 43: Immunofluorescence characterization of MSCs cultured on slide chambers. Cells were stained with monoclonal antibodies directed against CD44 (A), CD90 (B), CD105 (C), CD166 (D), ASMA (E) and CD45 (F). Scale Bars are 10 μm.

6.12 ULTRASTRUCTURAL ANALYSIS OF CELL : TEM ANALYSIS

Cells taken at 3-5 passages in culture were processed for ultrastructural analysis. Ultrastructural analysis showed cells with high ratio nucleo/cytoplasm, large euchromatic nuclei, prominent nucleoli and abundant cytoplasm containing numerous organelles, e.g., mitochondria and dilated cisternae of rough endoplasmic reticulum, a few lipid droplets, and peripheral cytoplasmic collections of clear vesicles, vacuoles and blisters (Fig. 44) (*Pasquinelli et al., 2007 b*).

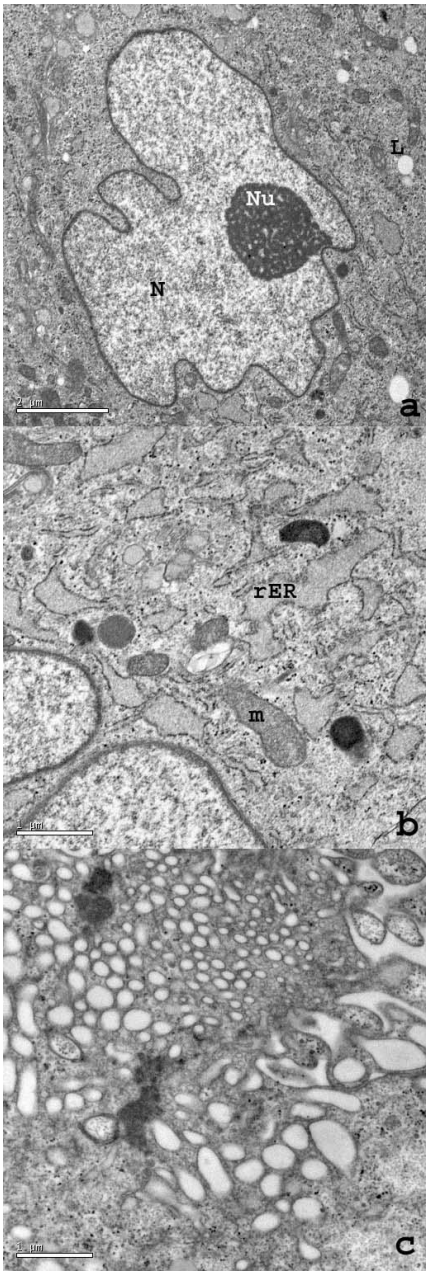

Fig. 44: Ultrastructural analysis performed on MSCS isolated from human thoracic aortas taken at 3-5 passages. A) nuclei (N) with eucromatin finely disperse and prominent nucleoli (Nu). The cytoplasm contained some lipids droplets. Bar: 2 μm B) abundant cytoplasm containing numerous organelles, e.g., mitochondria(m) and dilated cisternae of rough endoplasmic reticulum (rER).; C) peripheral cytoplasmic collections of clear vesicles, vacuoles and blisters (bar=1 μm.)

All of this ultrastructural features, especially the multivacuolar appearance, were described in a previous study of our group , where we compared the ultrastructural characteristic of mesenchymal stem cells derived from human bone barrow and term placenta (amnios and chorion membrane)(*Pasquinelli et al., 2007 c*).

In this study, electron microscopy analysis performed on BM-hMSCS showed cells with surface filopodia and prominent blebs; the cytoplasm contained intermediate filaments, contractile filaments, prominent rough endoplasmic reticulum cisternae, lipids and glycogen. The most distinctive finding observed was the presence of wide collections of clear vacuoles, channels, and vesicles giving the periphery of the cytoplasm a labyrinthine appearance (Fig. 45) (*Pasquinelli et al., 2007 c*).

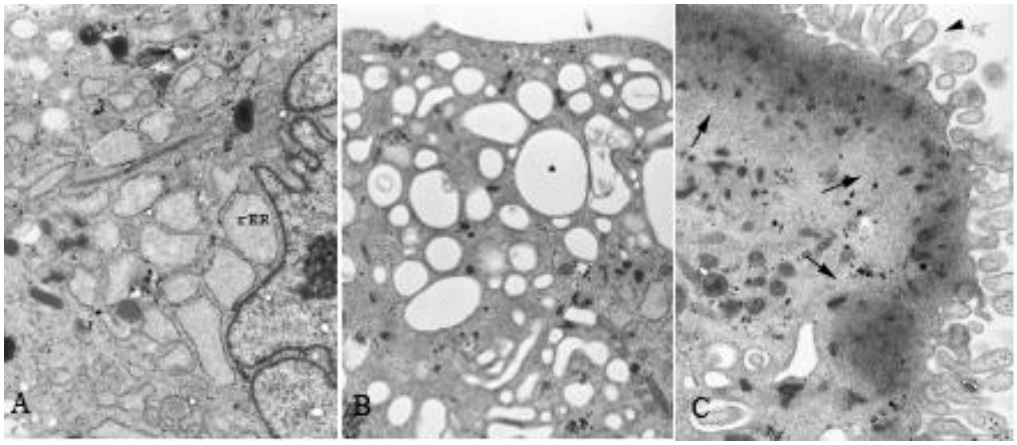

Fig. 45: TEM analysis of BM-hMSCs. Distinctive features were the presence of dilated cisternae of rER (A), clear blisters and vacuole located at the periphery of the cell cytoplasm (B); and bands of contractile filaments (arrows) with dense bodies were seen in association with surface blebs (arrowhead) (C).

Even if ultrastructural differences exist among phenotypically similar hMSCs derived from different human tissues that can be revelatory of their in vitro differentiation potential, some identical features like dilated cisternae of rough endoplasmic reticulum and the presence of peripheral collection of multiloculated clear blisters are present. The last features could be invaginations of the cell membrane that reflect some kind of cell memory effect of the original environment in which the MSCs originally grew in different type of tissue. Besides, the elevating proliferating activity is a typical property of stem cells.

6.13 STEM CELLS GENES EXPRESSION by RT- PCR

The RT-PCR performed on MSCs isolated from human thoracic aortas and MSCs isolated from human bone marrow, as a positive control, showed that our cells constitutively expressed transcripts associated with stem cells. The MSCs isolated from human thoracic aortas expressed the embryonic stem cell marker, Oct4. During embryonic development, Oct4 is expressed initially in all blastomeres. Subsequently, its expression becomes restricted to the inner cell mass and down regulated in the trophectoderm and the primitive endoderm. Thus, it has been proposed that Oct4 is a key regulator of stem cell pluripotency and differentiation (*Pan et al., 2002*). Moreover, Human thoracic aortas MSCs expressed some transcripts involved in stem cell critical regulatory pathways, i.e., c-kit, that is involved in the proliferation and differentiation of HSC; IL-6 and BCRP1, that maintaining the undifferentiated state of embryonic stem cells; BMI-1 , that regulated self-renewal of hematopoietic and neural stem cells and KDR, receptor type 2 of VEGF, as well as, to a lower extent, hematopoietic stem cell transcripts, e.g., CD133 (Figure 46). The positive control, BM-MSCs, expressed all the molecules involved in stem cells conditions, like CD133, Oct-4, IL-6, BCRP-1,

BMI-1 while they didn't expressed the hematopoietic stem cells molecules Ckit and KDR.(*Pasquinelli et al., 2007 b*).

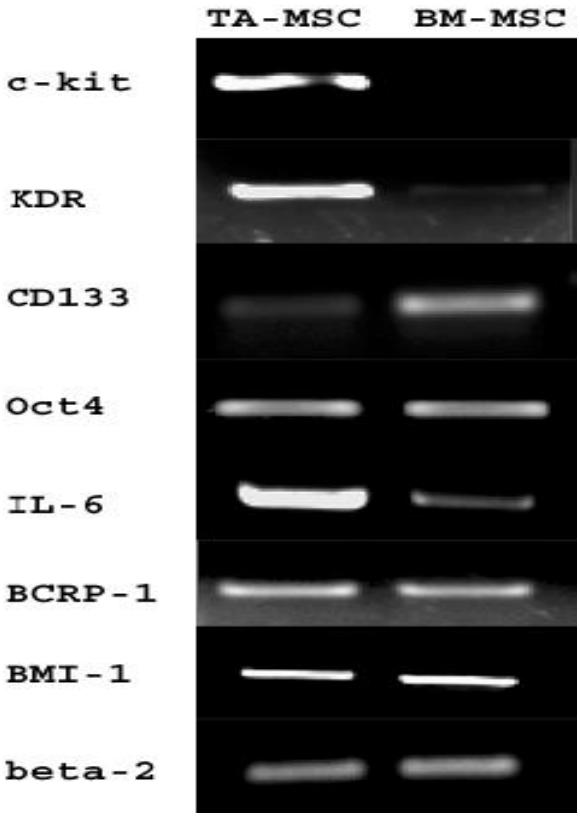

Fig.46: RT-PCR analysis of mesenchymal stromal cells derived from the human thoracic aorta. The left lane shows transcript expression found in cells isolated from the thoracic aorta (TA-MSCs). The right lane indicates the expression intensity seen in parallel experiments performed on mesenchymal stromal cells derived from the human bone marrow (BM-MSCs). The data representative of a set of at least three experiments. β2μ was used as a house-keeping gene. Amplicon lengths: OCT4: 169 bp, BMI-1: 369 bp; BCRP-1: 652bp; CD133: 337 bp; KDR: 555 bp; C-kit: 275 bp; IL6 : 388 bp; Beta 2: 114 bp;

6.14 ENDOTHELIAL DIFFERENTIATION OF ADULT STEM CELLS FROM HUMAN THORACIC AORTAS

Thoracic aorta mesenchymal stromal cells isolated from 3 multiorgan donors, according with Oswal et al., showed the capacity of differentiating in vitro into endothelial cells when cultured confluent in the presence of 2% FBS and 50 ng/ml VEGF for 7 days. This results was obtained using different technique able to demonstrate this differentiation potential.(*Pasquinelli et al., 2007 b*).

6.14.1 ***In Vitro Matrigel assay:***

After 7 days of induction with VEGF, the cells were seeded on Matrigel, a matrix solution, in order to visualize the formation of capillary like structures. After 2 and 4 hours of Matrigel incubation, no morphological changes were seen in the 2 experimental conditions investigated. After 6 hours of incubation, the cells that had previously been conditioned with VEGF showed, evident morphological changes like a more elongated shape and distinct, thin cytoplasmic projections sprouting from the cell periphery. After 20 hours, the cells appeared to be connected by thicker projections, thus forming an evident capillary-like network. These features were not observed with unstimulated cells. (Fig.47) As expected HUVEC spontaneously aggregated in a capillary-like network when seeded on Matrigel (data non shown).

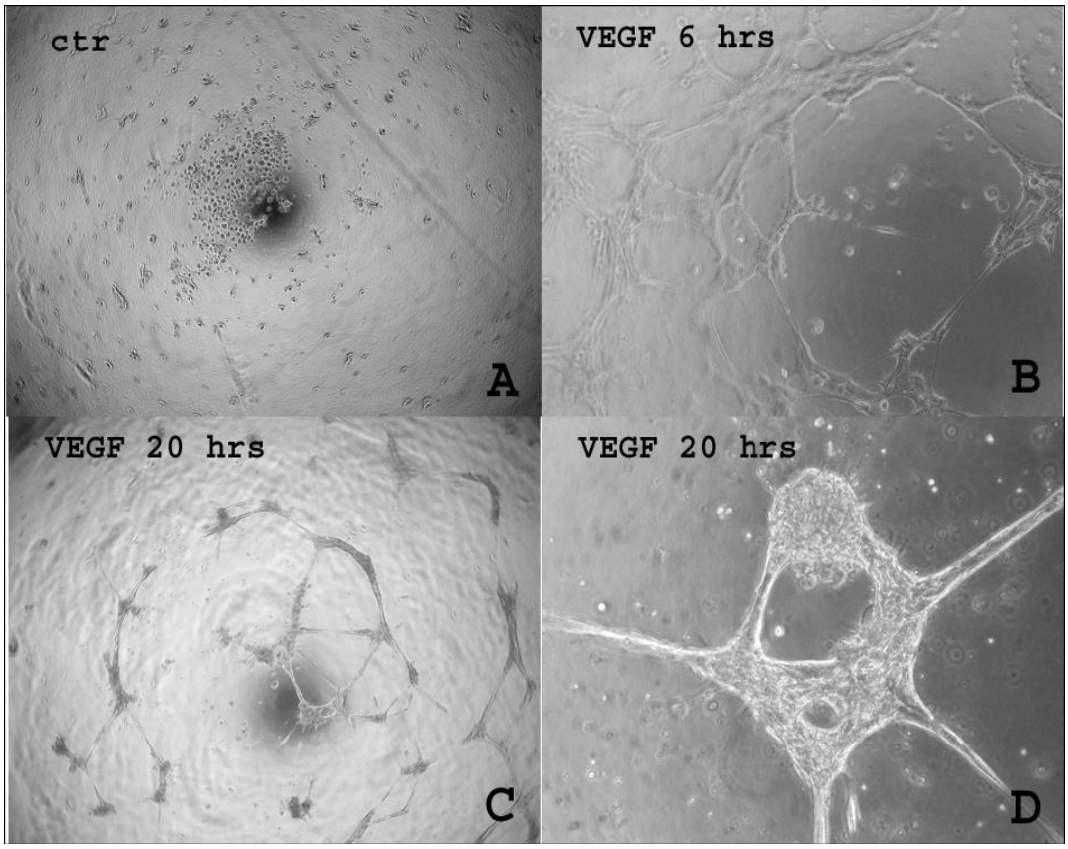

Fig. 47: MSCs aggregated in capillary-like structures when seeded on Matrigel. Cells were cultured for 7 days in the absence (A) or presence (B-D) of VEGF. B corresponds to 6 hours from Matrigel seeding; C, D to 20 hours from Matrigel seeding (*Pasquinelli et al., 2007 b*).

6.14.2 ***TEM analysis:***

The capillary like structure obtained in the Matrigel assay after 20 h of incubation was fixed and observed with a transmission electron microscopy. The in situ ultrastructural analysis of Matrigel matrices showed cell aggregates having features consistent with an early endothelial phenotype; these characteristics included the presence of loosely arranged vimentin-like intermediate filaments, collections of micropinocitotic vesicles and caveolae, tight junctions; around the Golgi area, moderately

electron-dense oval-shaped granules possibly representing immature Weibel-Palade bodies were also seen (Figure 48); this feature was only observed when cells had been treated with VEGF and not in the control (results not shown).

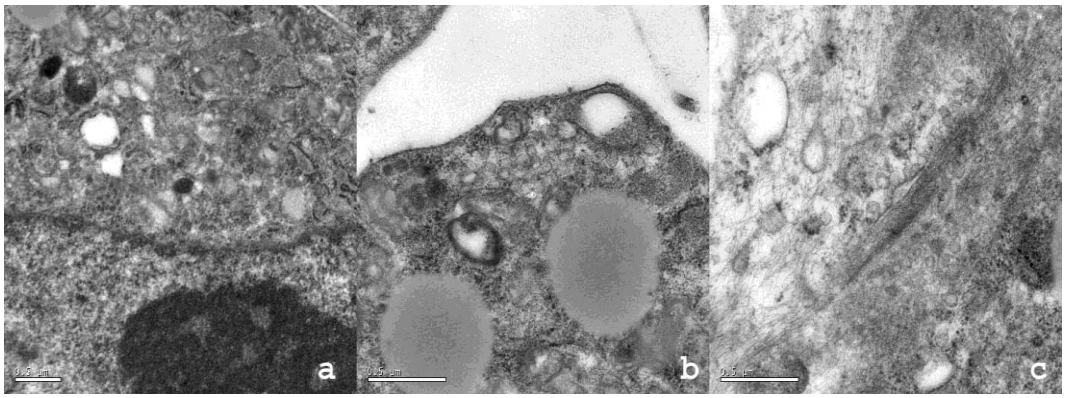

Fig. 48: Ultrastructural aspects of thoracic aorta MSCs cultured in the presence of VEGF and then seeded on Matrigel matrices for 20 hours. a) The cells show oval-shaped electron dense granules in the Golgi area close to the nucleus (black arrowhead); these granules possibly represent immature Weibel-Palade bodies. b) A cluster of micropinocytosis vescicles is seen close to the plasma membrane (arrow). c) A detail of the cell periphery shows a tight junction, vesicles, caveolae and loosely arranged intermediate filaments (white arrowheads). a,b, c bars = 0.5 μm. (*Pasquinelli et al., 2007 b*)

6.14.3 ***Flow Cytomery Assay:***

VEGF display differential interactions with tree related receptor tyrosine kinases (VEGF-R1/ Flt-1, VEGF-R2/ KDR/ Flk1, VEGFr3/ Flk-4). VEGFr1 and VEGF r2 are restricted largely to vascular endothelium. In vitro, VEGF seems to have the ability to induce endothelial cells

proliferation as well as migratory and sprouting activity, and to help endothelial cells to form tubule like structures; these effects seem to be mediated largely by KDR. So, in parallel assays, indirect immunofluorescence flow cytometry revealed that KDR expression was significantly increased when cells had been conditioned with VEGF for 7 days (Figure 49 A). Moreover, single (Figure 49B) and double (Figure 49 C) labeling experiments demonstrated that VEGF promoted the vWF cytoplasmic expression (marker of mature endothelium) in more than 50% of the CD105+ cell population. vWF expression was exclusively observed after cells had been treated with VEGF. (Figure 49 is representative of three different experiments) (*Pasquinelli et al., 2007 b*).

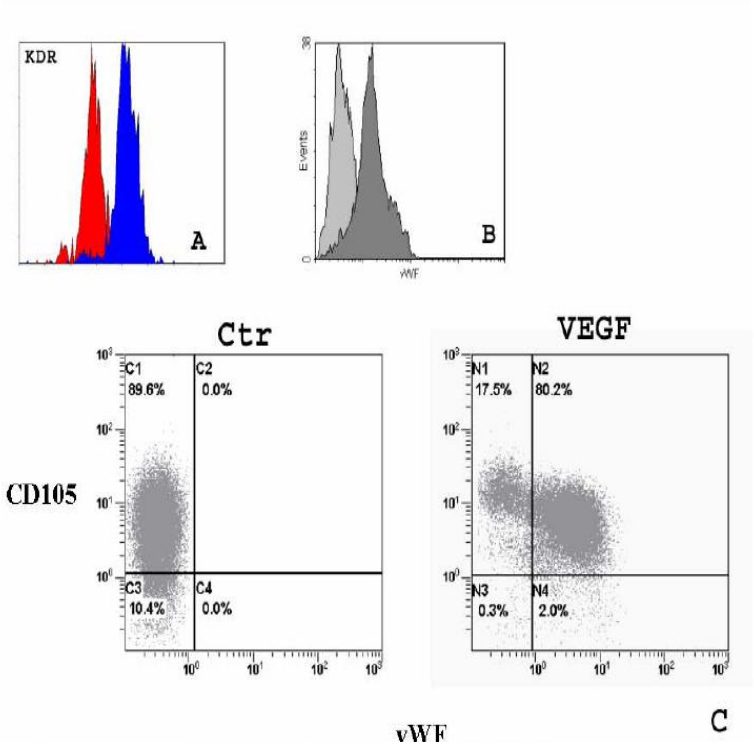

Fig. 49: VEGF promotes endothelial differentiation in MSCs. Flow cytometry analysis of KDR (A, single labeling staining) and vWF (B, single labeling staining) expression in MSCs before and after VEGF incubation; double labelingstaining demonstrates cytoplasmic expression of vWF in CD105+ cells (C) (*Pasquinelli et al., 2007 b*).

6.14.4 In situ Immunofluorescence assay:

The expression of vWF was confirmed by immunofluorescence staining of cells cultured on glass coverslips. In these experiments, 12 ± 3% of cells revealed intense cytoplasmic staining for vWF when cultured in the presence of VEGF; vWF expression was associated with a rounded morphology of the positive cells (Figure 50 b); control HUVEC showed an intense vWF dot-like staining (Figures 50 a)(*Pasquinelli et al., 2007 b*).

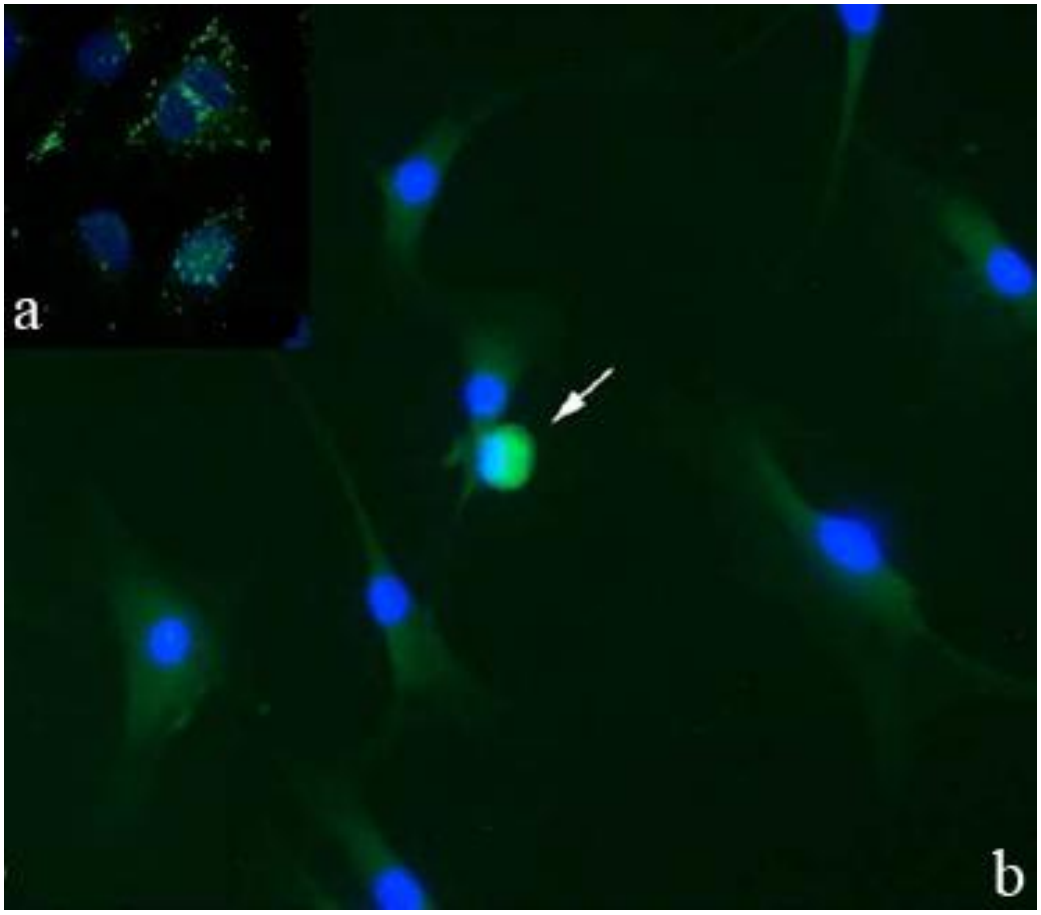

Fig 50: Immunofluorescence staining shows vWF expression (white arrow) in VEGF-treated MSCs (b); vWF dot-like staining in control HUVEC (a)

6.14.5 ***CD133 and KDR mRNAs expression by RT- PCR***

RT-PCR showed CD133 (a marker of stemness) down-regulation after VEGF exposure (Fig. 51), indicating an acquisition of a more differentiated phenotype. On the other hand, after VEGF treatment, the mRNA expression by RT-PCR for KDR increased as expected from the flow cytometry results. (Fig.51)(*Pasquinelli et al., 2007 b*).

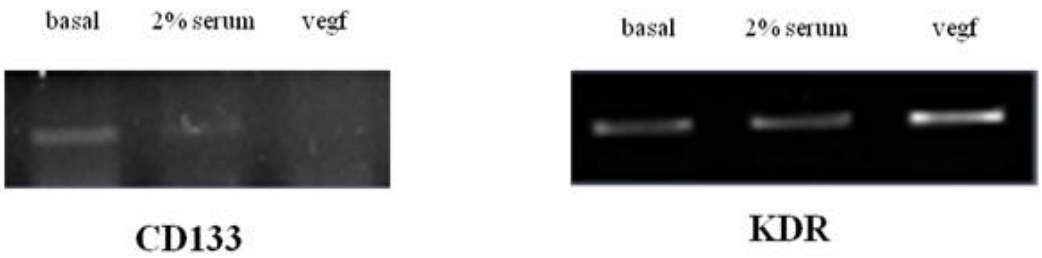

Fig. 51: RT-PCR possibly documenting CD133 down-regulation and KDR up-regulation as a consequence of VEGF exposure.(*Pasquinelli et al., 2007 b).*

6.14.6 ***Quantification of eNOS mRNAs expression by Real Time PCR***

Real time PCR results for mRNA-eNOS quantification in unstimulated cells respect the VEGF-treated cells demonstrated that MSCs from thoracics aorta showed a basal expression of eNOS in the undifferentiated cells. (Data not published).This data may due to a fraction of already differentiated vasculogenic progenitors in the MSCs population. Anyway, NO seems to be important to maintain the bone-marrow stem cells in the undifferentiated state (*Liu et al., 2007*) .After 7 days of VEGF exposure , the mRNA eNOS expression by Real Time PCR was significantly ($p < 0,05$) expressed as showed in Fig.52. May be this results must be connected with the up-regulation expression of KDR (Fig.52) after VEGF exposure, in fact

seemed that, in endothelial cell, VEGF-A induced an upregualtion of eNOS through KDR (*Kroll et al., 1998*).

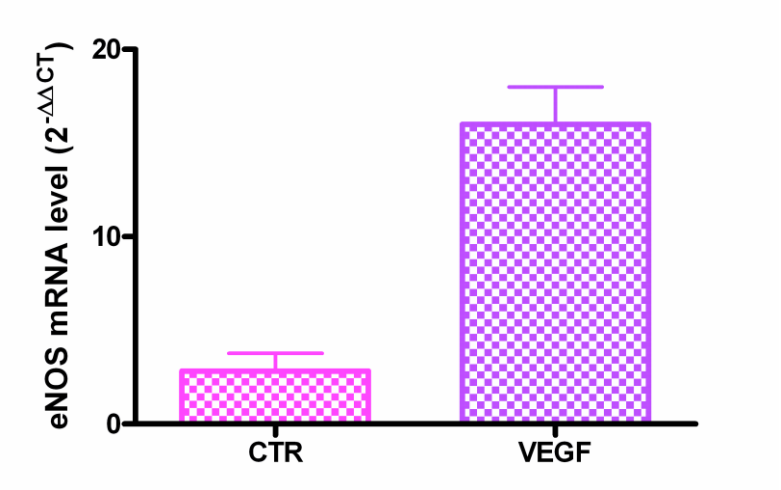

Fig. 52: eNOS expression after the VEGF exposure of aortas MSCS by Real Time PCR after 7 days of differentiation ($p<0,05$). CTR (Control) are unstimulated cells. VEGF are the cells treated with VEGF (50 ng/ml). The amount of mRNA was calculated according to Pfaffl (2001)(*Pfaffl, 2001*) relative to the housekeeping gene β-actin. All the values are normalised to the corresponding mRNA of NOS isoforms expressed by HUVECs at passages between 3 and 6. The data were expressed as mean of 2 experiments (n=2).

6. DISCUSSION

There is some evidence that the arterial wall is much more dynamic than ever before believed. Hu et al. (*Hu et al., 2004*)recently found abundant vascular progenitor cells in the adventitia of ApoE-deficient mice; these progenitors contributed to experimental atherosclerosis and did not originate from the bone marrow. Moreover, human mature endothelial cells have been unexpectedly found to contain a subpopulation of hierarchically organized EPCs (*Ingram et al., 2005a*). Thus, apart from the unquestionable link with the hematopoietic system, some progenitors seem to be located within the adult vessel wall. Consistent with this view, constitutively resident vascular progenitor cells have been isolated from the thoracic and abdominal aortas of healthy adult mice (*Sainz et al., 2006*) and from the internal thoracic arteries of humans (*Zengin et al., 2006*). Recent studies have indicated that the human arterial wall contains a distinct subtype of resident progenitor cells with vasculogenic properties (*Zengin et al., 2006*). These cells, termed vascular wall-resident progenitor cells, extend the spectrum of the known endothelial progenitors, which until now included the bone-marrow resident EPCs (CD133+, CD34+, and KDR+) and their circulating counterpart EPCs (CD34+, KDR+).EPCs are promising candidate for clinical applications aimed to restore blood supply in ischemic damaged tissues (*Asahara et al., 1999 a*). However, the number and function of progenitors significantly decrease with age (*Riha et al., 2005,Sethe et al., 2006*) and disease (*Hristov et al., 2003,Fadini et al.,2006*). Circulating EPCs are extremely low in number (*Hristov et al., 2003*). Recent studies have indicated that EPCs may play a minimal role in neovascularization of tumors, vessel repair or normal vessel growth and development (*Zentilin et al., 2006,Ingram et al., 2005 b, Gothert et al., 2004, Stadtfeld et al., 2005*). Moreover, Zenging et al. hypothesized the

presence of a vasculogenic zone within the arterial wall that apparently contains different cell populations, like mesenchymal stem cells (*Zengin et al., 2006*). All these evidences justify the search for adequate alternative sources of these cells for autologous and allogenic use.

The present work started in 2005 in collaboration with Emilia Romagna Cardiovascular Tissue Bank, S.Orsola-Malpighi Hospital, which cryopreserved vascular homografts harvested from brain-dead multiorgan donors (aged 15-55). We focused our attention on thoracic aortas from heart-beating, healthy and young multiorgan donor as new possible source for adult stem cells. The possibility to isolate adult stem cells from human thoracic aortas was realized thanks to the first results obtained from our group. In 2006, we demonstrated, that the cryopreservation procedures of arterial homografts used for clinical application damaged aortic SMC. Even so, the immunohystochemistry analysis, performed on fresh and frozen human thoracic aortas, revealed the presence of a vasculogenic zone, located between the media and the adventitial layer, in which a well preserved plexus of CD34 positive cells, lining the endhotelium of adventitial vasa vasorum, was found (*Pasquinelli et al., 2006*) . Moreover the organ cultures revealed the presence of vital adventitial vasa vasorum in the cryopreserved tissue since 4 days even so this approach was unable to recover *in vitro* the SMC's damage after cryopreservation. The ultrastructural analysis showed the presence of well preserved vasa vosorum located in the adventitial layer. This result seemed to demonstrate the existence of resident vascular progenitor cells able to resist at the cryopreservation protocol and able to induce angiogenesis in ex vivo culture experiments. Subsequently, in another study we demonstrated that cryopreserved arterial allografts are immunogenic as their fresh counterparts. To this purpose, we performed an immunohystochemical study on fresh and cryopreserved human arterial tissue to visualize the

expression of HLA-class I heavy and light chains using " in situ" the HC-10 and Numb-1 Monoclonal antibodies. HC-10 recognizes an epitope expressed on virtually all β2-microglobulin-free HLA-B heavy chains and on selected β2-microglobulin-free HLA-A heavy chains whereas Namb-1 recognizes human free- and heavy chain associated-beta 2-microglobulin. This study demonstrated that the in situ expression of HLA-I antigens is not altered by cryopreservation, in particular, the fact that both epitopes were found expressed in cryopreserved arteries could lead to the hypothesis that HLA class I molecules might be present in their native conformation (*Pasquinelli et al., 2007 a*). This data confirmed the previous serological results described in clinical studies. Immunological follow-ups of patients treated with cryopreserved arterial allografts unequivocally demonstrated that both humoral and cellular specific responses are raised in the recipients against donor cells within 30–60 days from surgery (*Mirelli et al. 2005*). Besides surface endothelium, smooth muscle cells and vasa vasorum stained positive with HC-10 and Namb-1 MoAbs. An impressive finding was the high numbers of vasa vasorum found in our samples. Arterial allografts are prepared by removing adventitia from media; however this surgical procedure is not sufficiently adequate to remove the vasa vasorum which are intimately connected to the medial layer. In our arterial samples vasa vasorum were found between media and adventitia and in the outermost portion of the media; these vessels were lined with continuous endothelium highly expressing HLA class I antigens Taken together these results demonstrated that the vasa vasorum network, lined with highly HC-10 positive endothelial cells, is expected to be one of the major targets of the ''in vivo'' immunological response(*Pasquinelli et al., 2007 a*). As demonstrated by these results, a vasculogenic zone with hard-wearing and immunological cells seemed to be present in the postnatal aorta tissue. The successive step was to performed tissue studies, using different antibodies, on fresh human thoracic aortas in order to identify the cell populations

present in the arterial tissue. Our tissue studies showed that two distinct cell populations, one composed of CD34+ cells and the other of c-kit+ cells, are present in human thoracic aortas. It is worth noting that both surface markers are expressed in cells with stem cell capabilities. Immunohistochemical tissue studies showed a well developed plexus of CD34+ small vessels at the border between the media and adventia layers; some of the cells lining these vessels were also vWF+, thus showing they had been employed in mature endothelium; this area, also containing CD45+ inflammatory cells, topographically corresponds to the CD34+ / CD31- cell layer, recently observed in human inner thoracic arteries and demonstrated to be highly vasculogenic through ring assay studies (*Zengin et al., 2006*). Rare S100 and CD68 elements were found in the same zone, near adventitial vasa vasorum. In addition to the vasculogenic area, CD34+ cells were also seen as elongated elements in adjacent portions of the media and adventia. CD34+ vascular channels, which adjacent sections showed to be vWF- , could also be found embedded within the media(*Pasquinelli et al., 2007 b*). A similar vascular organization, composed of cells highly expressing Human Leukocyte Antigen class I antigen (*Pasquinelli et al., 2007 a*), was also before discussed and may therefore suggested that distinct patterns of CD34+ cell aggregation existed in elastic and muscular arteries. The fact that such CD34+ cells can be organized into evident vascular structures did not conflict with the possibility that they might be progenitors; in fact vascular wall-resident EPCs are believed to be the most likely recruitable cells contributing to new vessel formation (*Ingram et al., 2005 b*). This view is in accordance with the concept of angiogenesis is defined as the sprouting of new blood vessels from the differentiated endothelium of pre-existing vessels (*Carmeliet, 2000*). A novel finding was the observation of cells expressing the stem cell surface marker c-kit which is commonly associated with the more primordial cardiac stem cells (*Anversa et al., 2006*).This cell subpopulation was found to be scattered

round c-Kit + cells, and even if c-Kit is associated also with mast cells, ultrastructural examinations of the contiguous samples ruled out this possibility. Tissue immunostaining for Ki-67, cell proliferation marker, documented that cells with high growth potential are located in a restricted area of the aortic wall corresponding to the site of CD34+ and ckit + cell. Extensive observations showed that Ki67 exclusively stained individual endothelial cells and stromal cells located in the vasculogenic zone, while other cells composing the arterial wall proved negative. It remains to be ascertained whether these cells could have angiogenic potential and whether they could be perivascular pericytes (*Howson et al., 2005*) or an equivalent of the mouse and chicken mesoangioblast (*Cossu et al., 2003*).

According to these results, we tried to isolated adult stem cells from the thoracic aortas of multiorgan donors using a combined procedure: mechanical and enzymatic digestion with Collagenase type II. Under sterile conditions, we scraped the aorta wall until reaching the adventitial layers, where adult stem cells seems to be located. After enzymatic digestion, the minced tissue was filtered and the cells obtained, after counting with Methyl Violet, were seeded in culture cell plate with D-MEM 10% FBS and then cultured at 37°C 5% $CO_{2.}$. After removing the non adherent cells , the attached cells shared many properties with Mesenchymal stromal cells like: plastic adherence; fibroblast-like morphology; high proliferation rate in vitro (*Dominici et al., 2006*). We are able to achieved adult stem cells only from 3 out of 10 specimens. The reasons of this unsuccessful case were caused by fast growth of bacteria and fungi at initial plating, due to the solution used for arterial graft delivery to the Cardiovascular Tissue Bank. Moreover, the arterial graft disinfection protocols (72 hours in an antibiotic mixture composed of mefoxin 240 mg/ml, lincomycin 120 mg/ml, colimycin 100 mg/ml, vancomycin 50 mg/ml) are not adequate for establishing cultures of arterial wall progenitors cells. As above reminded,

the cells isolated from human thoracic aortas showed a fibroblast like morphology, indistinguishable from that of MSCs isolated from the human bone marrow. Flow cytometry assay showed that more than 80% of the cells coexpressed molecules commonly found in MSCs such as CD44, CD90, and CD105. Although performed as a single staining CD166 was found expressed in most cells. Further, the immunophenotype profile of our cells was: CD44+, CD90+, CD105+, CD166+, KDRlow, CD133low, CD34low, c-kit -, CD45-, CD146-, vWF-, HLA classI + and HLA-DR -. The ultrastructural analysis showed some features that we have previously observed in the MSCs derived from human bone marrow, like multivacuolar appearance and high synthetic competence (*Pasquinelli et al., 2007 c*). Moreover, gene expression analysis by RT-PCR documented that Aorta-MSCs expressed gene correlate with stemness like: C-Kit, involved in the proliferation and differentiation of hematopoietic stem cells; IL6 and BRP-1, important for maintaining the undifferentiated state of embryonic stem cells; BMI-1, crucial for the self renewal capacity of hematopoietic and neuronal stem cells. In addition, Aorta-MSCs expressed OCT4 transcripts that is involved in the governance of pluripotency and cell fate determination of embryonic stem cells. Further, the expression of BCRP-1 has been described in our study; this protein is linked to the specific ability of stem cells to exclude dyes such as rhodamine and Hoechst. This property identifies a small subset of stem cells termed the "side population" (SP) .Therefore the existence of MSCs in adult arteries is supported by a recent paper documenting the isolation of an SP of stem cells from adult aortas in healthy mice (*Sainz et al., 2006*). Interestingly, these cells were able to differentiate into endothelial and smooth muscle cells but failed to give rise to hematopoietic lineages. Moreover, considering their capacity to form vascular-like structures in vitro, these cells could play a major role in vasculogenisis and collateral growth. The presence of adult vascular wall resident MSCs has been postulated in

humans too (*Zengin et al., 2006*). The authors proposed a hypothetical scheme of the so-called "vasculogenic zone" sketching a complete hierarchy of resident stem cells niched in the arterial wall . Within that proposed network of progenitors we believe that the cells we have isolated could correspond to MSCs of the "vasculogenic zone". At present, we do not know whether the MSCs we have isolated are multipotent; this aspect of their biology is under investigation; however, it should be noted that "ectopic" tissues, e.g., cartilage, bone, and fat can be seen especially in atherosclerotic arteries during routine histopathological observations (*Abedin et al., 2004*) and MSCs isolated from the human saphena vein are able to differentiate in vitro into osteoblasts, chondrocytes and adipocytes (*Covas et al., 2005*). Moreover, calcifying vascular cells isolated from the bovine aortic media display multilineage potential in vitro (*Tintut et al., 2003*). Recently, it has been reported that human vascular adventitial fibroblasts from pulmonary arteries contain a cell population that has strong ability to differentiate into mesenchymal lineages, even so is not clear if this cells contains multipotent stem cells or is a mixture of committed progenitors cells with restricted single-potential(*Hoshino et al., 2008*). If the multi-lineage differentiation capacity of our cells is documented, the arteries from multi-organ donors may serve as an important source of multipotent MSCs for clinical needs and tissue engineering. (*Pasquinelli et al., 2007 b*).

In according with Oswald et al, we tested in vitro if cultivating confluent aorta MSCs in the presence of low-serum culture supplemented with VEGF-A (50 ng/ml) for 7 days, they were able to differentiate in vitro into endothelial cells like BM-MSCs (*Oswald et al., 2004*).VEGF-A provided the first example of a growth factor specific for the vascular endothelium . The VEGF- A through the interaction with its receptors (VEGF-R1/Flt-1, VEGF-R2/KDR/Flk-1) have the ability to induce cell proliferation as well

as migratory and sprouting activity, and to promote capillary like structure formation. These effects seems to maintained largely by VEGF-R2 or KDR. To assay in vitro angiogenesis, the cells, after 7 days of induction, were seeded in a three dimensional Matrigel semisolid matrix . After 20 hrs of culture, the MSCs were able to form capillary like structures but this ability was strictly depended on VEGF induction; controls always proved negative. Electron microscopy performed on this samples showed features consistent with a basic endothelial cell employment, i.e., collections of micropinocytotic vesicles and caveolae, tight junctions, and immature Weibel-Palade bodies. VEGF induction was accompanied by increased cell expression of KDR, as documented by flow cytometry; more importantly, we found that more than 50% of CD105+ cells co-expressed vWF following VEGF induction as indicated by double labeling experiments performed at flow cytometry. At immunofluorescence, vWF intensely stained the cytoplasm of cells with a rounded morphology (12 ± 3). Moreover, RT-PCR showed an increased mRNA expression of KDR after stimulation with VEGF, while the CD133, stemness marker, decreased after induction indicating an acquisition of a more differentiated phenotype. CD133 was first isolated from hematopoietic stem cells .CD133 or Prominin-1 is expressed on different types of stem cells, but it is not known if it plays a significant role in key stem cell functional features like self-renewal and differentiation. Although the biological function of Prominin-1 is not well understood, the AC133 epitope currently serves as a useful marker for the isolation of hematopoietic and endothelial progenitor cells (*Shmelkov et al., 2005*), and recently Mesenchymal Stem Cells Derived from CD133-Positive Cells in Mobilized Peripheral Blood and Cord Blood showed Proliferation, Oct4 Expression, and multilineage differentiation like BM-MSC (*Tondreau et al., 2005*). Besides, CD133 seems to be a marker associated with more primitive stem cell phenotype than CD34 (*Handgretinger et al., 2003*). By real time PCR we evaluated the

expression of eNOS before and after VEGF exposure. Nitric Oxide (NO) is a potent vasodilatator and it is synthesized through the enzymatic conversion of L-Arginine and molecular oxygen to L-citrulline by nitric oxide synthases (NOS). There are 3 type of NOS: nNOS or neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). The eNOS is the membrane-bound isoform first found in vascular endothelial cells (VEC), which play a critical role in the functioning of endothelial cells. eNOS is essential in the signaling for vascular endothelial growth factor (VEGF), which is required for the development and function of endothelial cells (*Liu et al., 2007*).Moreover, eNOS is critically important in remodeling, hemostasis, angiogenesis, and mobilization of endothelial progenitors cells (*Fish et al., 2006*). VEGF is a key regulator factor in blood vessels development, endothelial biology and vascular disease, and now day the relationship between VEGF and eNOS/NO becomes increasingly defined. The quantification of eNOS mRNA by real time PCR revealed that , even if our cells expressed high level of eNOS in the basal situation, after VEGF stimulation, eNOS increased the expression. This data may due to a fraction of already differentiated vasculogenic progenitors in the MSCs population. Anyway, NO seems to be important to maintain the bone-marrow stem cells in the undifferentiated state (*Liu et al., 2007*) . Recently, Krumenacker et al demonstated that nNOS and eNOS were present in the undifferentiated mouse embryonic stem cells and when ESCc were induced to differentiate, nNOS expression quickly decreases within 1 day, whereas eNOS expression increase significantly after 5 days *(Hefler et al., 2001)*. Finally in our results, the cells, after VEGF exposure for 7 days, showed an increased eNOS expression by real time PCR. Maybe, these results must be connected with the up-regulation expression of KDR after VEGF treatment, in fact seemed that, in endothelial cell, VEGF-A induced an upregualtion of eNOS through KDR (*Kroll et al., 1998*).

In conclusion, the possibility of obtaining angiogenic MSCs from the thoracic aortas of multi-organ donors paves the way for creation of an allogenic bank of vascular progenitors of aortic origin. As already indicated, the fundamental scarcity of EPCs in the hematopoietic system is their main limitation when it comes to clinical application. Ex vivo expansion of EPCs cultured from the peripheral blood of healthy human volunteers yields about 5.0 x 10^6 cells per 100 ml of blood, while the amount of autologous bone marrow blood aspirated for therapeutic neovascularization is reported to be approximately 500 ml per person (*Tateishi-Yuyama et al., 2002*). These figures suggest that at least 30-45 x 10^7 EPCs are required in a clinical setting. Thus the volume of blood required to extract an adequate number of progenitors for transplantation represents a practical limitation for the use of same. Under our experimental conditions, it can be calculated that 0.5-2.0 x 10^4 progenitors are originally present in the mononuclear That fraction obtained from a 5-cm-long segment of human thoracic aorta; these cells expand quickly and a week after initial plating their expansion yields about 0.5 to 1 x 10^6 cells. Considering the rate of expansion from the initial number of cells yielded, ideally the number of cells that could be obtained from a few in vitro passages might be about 1 x 10^8 cells, a reasonable amount for a clinical therapeutic approach to angiogenic disorders.

In summary, our findings indicate that thoracic aortas from heart-beating, multi-organ donors are highly suitable for obtaining MSCs with the ability to differentiate in vitro into endothelial cells Even though their differentiating potential remains to be fully established, it is believed that their angiogenic ability is in itself a useful property for allogenic use. These cells can be expanded rapidly, providing numbers which are adequate for therapeutic neovascularization; again, being recovered from young and

healthy donors, they can be cryostored in appropriate cell banking facilities for later use (*Pasquinelli et al., 2007 b*).

Finally, the possibility to use human aortas from multiorgan heart-beating donors offers the possibility to study the existence of precursors in adult tissue, e.g mesangioblasts. Moreover, the Cardiovascular Tissue Bank offers the facility of different human blood vessels (veins, a. femoral, a. iliac and so on) in order to verify if a heterogeneity of resident stem cells in the blood vessels anatomically distinct exists. This topic could be important also to understand the pathogenesis of several peripheral vascular diseases and could give some indications for new therapeutic approach.

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