INDEX

I Introduction

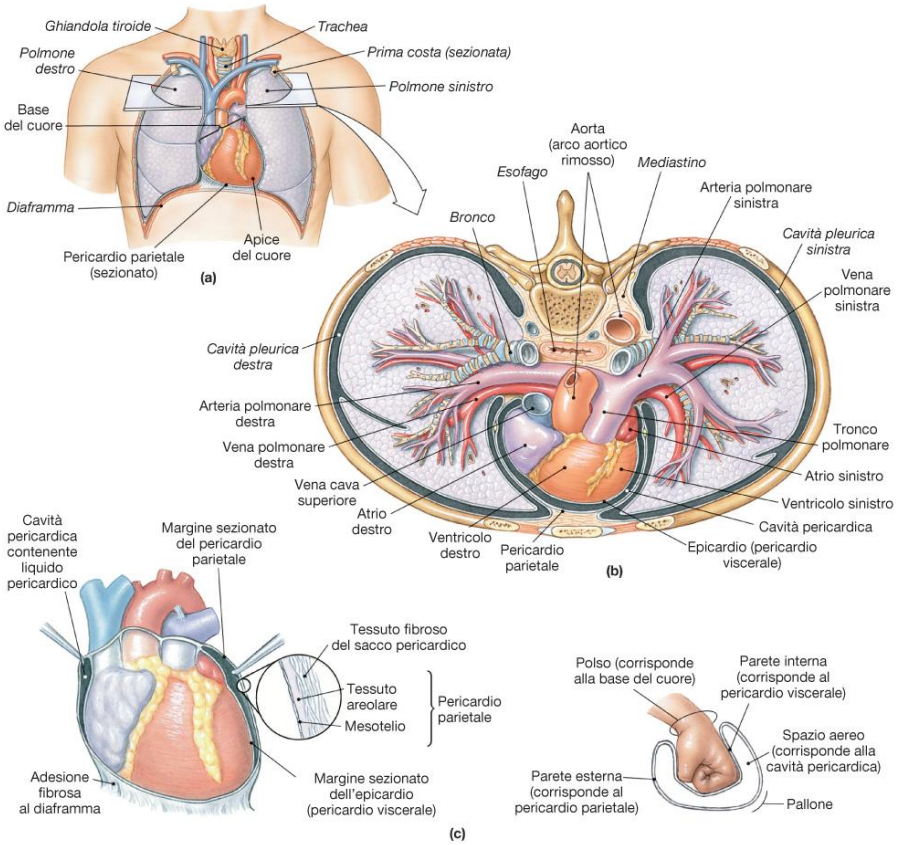
1.1 Heart: gross and microscopic anatomy

The human heart is a pair of valved pumps combined in a single organ. The heart is a hollow, fibromuscular organ of somewhat conical or pyramidal form, with a base, apex and a series of surfaces and borders. Enclosed in the pericardium, it occupies the middle mediastinum between the lungs and their pleural coverings. It is placed obliquely behind the body of the sternum and the adjoining costal cartilages and ribs. Approximately one-third of the mass lies to the right of the midline. The heart is placed obliquely in the thorax. Of the four cardiac chambers, the two atria receive venous blood as weakly contractile reservoirs for final filling of the two ventricles, which then provide the powerful expulsive contraction that forces blood into the main arterial trunks. The atrial and the ventricular septal structures are virtually in line, but inclined forwards and to the left at 45° to a saggital plane. The two pumps are usually described in parallel. In cardiac muscle the contractile proteins are organized into sarcomeres which are aligned in register across the fibers, producing the cross-striations that are visible at the light microscope. The myocardium, the muscular component of the heart, constitutes the bulk of its tissues. It consists predominantly of cardiac muscle cells, each cell has one or two large nuclei, occupying the central part of the cell.

The cells are branched at their ends, and the branches of adjacent cells form a network of branching and anastomosing fibers. Ultrastructurally, cells are bound together by elaborate junction complexes, which means that the fibers of cardiac muscle are not

syncytial cells with a common cytoplasm. Fine fibrocollagenous connective tissue is found between cardiac muscle fibers. It is less regularly organized than skeletal muscle, because of the complex three dimensional geometry imposed by branching cardiac cells. Numerous capillaries and some nerve fibers are found within this layer, coarser connective tissue separates the larger bundles of muscle fibers, and is particularly well developed near the condensations of dense fibrous connective tissue that form the skeleton of the heart. Electron micrographs of cardiac muscle cells in longitudinal section show that the myofibrils separate before they pass around the nucleus, leaving a zone that is occupied by organelles, including sarcoplasmatic reticulum, Golgi complex, mitochondria, lipid droplets and glycogen.

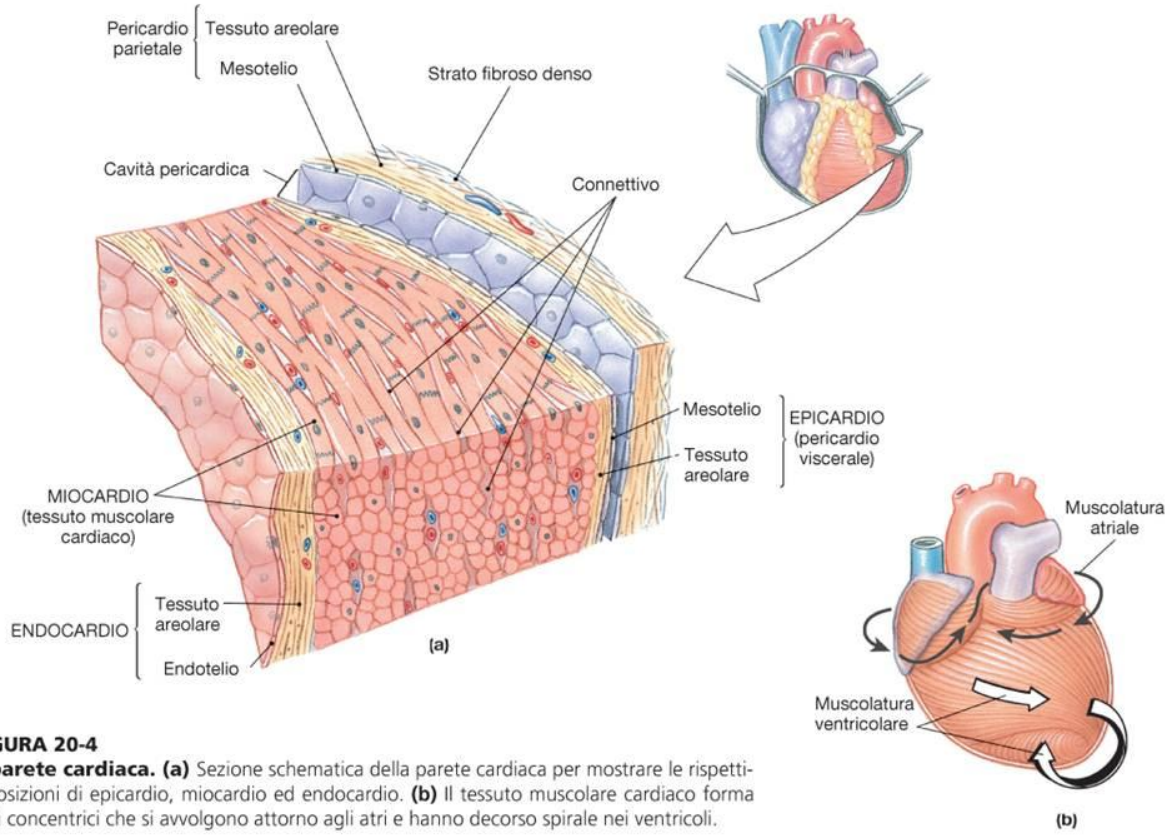
In the ventricular myocardium a precise linear alignment of cardiac myocytes in alternating layers of muscle fibers forms a basket weave of muscle tissue and leads to a muscle fiber alignment designed to propel the blood forward through the outflow tracts. This tissue alignment is evident at multiple levels, from the microscopic scale of sarcomere assembly up to three-dimensional structure of the ventricular chamber (Gray's Anatomy, 2005; Martini et al, 2004).



•FIGURA 20-2
Sede del cuore nella cavità toracica. Il cuore è situato nella parte anteriore del mediastino, immediatamente al di dietro dello sterno. **(a)** Veduta anteriore della cavità toracica aperta che mostra la posizione del cuore e dei grossi vasi in rapporto al polmone. **(b)** Veduta dall'alto del cuore e degli altri organi mediastinici; il tessuto polmonare è stato rimosso per mostrare i vasi sanguigni e i bronchi. *(c)* Rapporto del cuore con la cavità pericardica; confrontare con l'esempio del pugno nel pallone. ATLANTE Figura 7.4a, 7.6a,b

Martini
Fondamenti di Anatomia e Fisiologia
EdiSES

Fig.1.1



●**FIGURA 20-4**
La parete cardiaca. (a) Sezione schematica della parete cardiaca per mostrare le rispettive posizioni di epicardio, miocardio ed endocardio. **(b)** Il tessuto muscolare cardiaco forma strati concentrici che si avvolgono attorno agli atri e hanno decorso spirale nei ventricoli.

Martini
Fondamenti di Anatomia e Fisiologia
EdiSES

Fig 1.2

The view of the heart as muscular pump has dominated cardiovascular science and medicine for over a century. However, the heart is clearly more than muscle, with a panoply of diverse cardiac, smooth muscle, valvular, pacemaker, and endothelial cell types with discrete contractile , electrical, and vascular roles. To form a fully functional heart organ, a set of embryonic precursor cells must give rise to these distinct cell types, which must ultimate assemble and align within specific heart compartments to form ventricular chambers, coronary artery, and the conduction system (Chien et al., 2008).

1.2 Heart morphogenesis and development

The cardiovascular lineages are generated from mesoderm, one of the three primary germ layers formed from uncommitted epiblast cells as they traverse a structure known as the primitive streak (PS). During gastrulation, epiblast cells ingress through the PS to form mesodermal and endodermal tissues. The first generated mesodermal cells exit the posterior region of the PS and contribute predominantly to heamatopoietic and vascular lineages of the blood islands in the yolk sac.

Cardiac mesoderm is derived from epiblast cells that move through a more distal region of the PS slightly later in time. These mesodermal cells migrate to the anterior part of the embryo where they organize as an epithelial layer to form a structure known as cardiac crescent, that once formed fuses at the midline to form the heart tube consisting of an outer layer of myocardium and an inner layer of endothelial cells, a population known as endocardium. Finally the tube begins to loop and ultimately forming the four-chambered heart (Kattman et al., 2007).

The formation of organs and body parts proceeds by sequential gene regulatory steps that dictate cell fates and organize specialized cell types into complex three-dimensional structural and functional units.

The heart is the first organ to form during mammalian development and that becomes functional in the vertebrate embryo. The heart in vertebrates is derived from a subpopulation of mesodermal precursor cells that become committed to a cardiogenic fate in response to inductive cues from adjacent cell types. The precardiac mesoderm forms a tubular hearts which stars beating at about 3 weeks of gestation in man. After this period it undergoes complex events of looping morphogenesis, chamber specification, septation, and diversification as well as integration with vascular system and it maintains its physiologic pumping function while it is continuously remodeled until the four chambered organ is formed (Zaffran & Frasch, 2002).

The heart is an asymmetric structure which receives its polarity from three body axes, undergoes multiple morphogenetic processes, which are governed by these axes. Several cell populations that have their origin outside the heart field migrate into the heart and provide additional cell types and play a role in modeling the embryonic heart (Schlüter, Piper, 1999).

Cardiac progenitor cells, which originate in the primary and secondary heart fields, are subject to a rapidly changing environment because of cell migration and changes in three dimensional architecture of the primitive heart. The development of cardiac progenitor populations is subject to modulation by non-cell-autonomous signaling molecules that originate from neighboring endothelial, endocardial, and other mesodermally-derived cells in the primitive embryonic heart.

Studies of heart development and its genetic underpinnings in simple models have exposed an evolutionarily conserved gene regulatory network consisting of functional interconnections between myogenic transcription factor, their downstream target genes, and signaling pathways that direct cardiac cell fate, myocyte differentiation, and cardiac morphogenesis (Olson, 2006).

1.3 Gene regulatory networks

Heart morphogenesis is a complex process, endoderm-derived signals (FGF2, BMP2) (Brand, 2003) act as inducers of cardiac mesoderm formation, characterized by the induction of a core set of evolutionary conserved cardiogenic transcription factors, such as NKX2.5, GATA factors, myocardin, that coordinately control cell fates, the expression of genes encoding contractile proteins and the morphogenesis of cardiac structures (Park et al., 1998).

Members of GATA and MEF2 families of transcription factors play key roles in cardiomyocite differentiation, the ability of these factors to directly interact also provides mechanisms for cooperative activation of target genes that contain binding sites for only one member of these families of transcription factors. In addition to their roles in activation of cardiac structural genes, GATA and MEF2 factors have been implicated in the early step of morphogenesis of the heart tube (Cripps & Olson, 2002; Koutsourakis et al. 1999).

Three members of GATA family, GATA4, 5, 6, zinc finger transcription factor, are expressed in the cardiac lineage at various stages of development, and GATA 4 and 6 continue expression in the adult cardiac myocytes (Pikkarainen, 2004). These three GATA genes

are expressed in heart in vertebrates, they share homology in two zinger domains that mediate DNA binding and cofactor interactions.

GATA4 is required for proper embryonic folding and heart tube formation, overexpression of GATA4 in embryonic stem cells displays enhanced cardiac differentiation (Reiter et al., 2001; Grépin et al.,1997; Nemer & Nemer, 2002). Functional analysis of GATA4 suggested a role as mediator of cardiomyocite differentiation, proliferation and survival, even if when endoderm induction was blocked, cardiac marker expression increased, suggesting a role for this GATA factor within the induced myocardial cells themselves. GATA5 was similarly active in this study leaving open the possibility that was required a more general activity of GATA family. However GATA4 is expressed throughout development and also in adult heart, clinical studies supports a role in apoptosis and adult cardiomyocite survival during hypertrophy which may provide insights into their role during embryogenesis (Peterkin et al., 2005).

GATA5 has also been implicated in cardiac gene expression. In particular, a specific function of GATA5 in endocardial differentiation has been proposed recently: its role for endoderm formation has been described in zebrafish. Furthermore overexpression of GATA5 in embryo leads to ectopic Nkx2.5 expression and formation of ectopic beating cell clusters in the fish embryo (Reiter et al., 2001).

GATA6 is essential for mesoderm formation during gastrulation, even if its role has not analyzed fully, but little information on the role of GATA6 during cardiogenesis has emerged from a study with GATA6 null mice: it appears that GATA 6 is required at the earliest stage of development (Nemer & Nemer, 2002). GATA6 expression in heart precursors decreases as cardiac machinery gene expression starts. Elevating GATA6 beyond this time delayed the onset of terminal

differentiation, once degradation of the exogenous mRNA had occurred, maturation proceeded, resulting in an increased thickness in the myocardium. These data indicated that GATA6 may hold the cardiac precursors in a progenitor and proliferative state, needing to be downregulated for heart cells to mature. More recent experiments have shown for the first time that GATA6 is required for differentiation of cardiac lineage during embryogenesis (Grépin et al.,1997).

Promoter studies reveal a mutually reinforcing regulatory network of Nkx2.5 and GATA transcription factors during cardiogenesis. GATA4 and Nkx2.5 directly interact at the protein level to regulate the expression of the ANF, α-cardiac actin promoters, they were shown to be mutual co-factors in its synergistic activation, this interaction causes a conformational change exposing the Nkx2.5 activation domains (Brand, 2003).

GATA5 can substitute for GATA4 in this interaction but GATA6 cannot, suggesting that the interaction with Nkx2.5 can convey functional specificity to GATA factors during development (Peterkin et al., 2005).

Nkx2.5 is expressed in the cardiogenic mesoderm in concomitance with specification of the lineage: it is regulated by a complex series of positive and negative regulatory modules. Two independent enhancers have been shown to direct transcription in subtly different patterns within the cardiac crescent, later these enhancers and others come to demarcate distinct transcriptional territories, both early cardiac enhancers contains tandem GATA binding sites that are required for cardiac expression (Cripps & Olson, 2002).

Myocardin is a transcriptional coactivator that regulates cardiac and smooth muscle gene expression by associating with serum response

factor, which has also been shown to stimulate expression smooth and cardiac muscle genes in association with a variety of homeodomain proteins as GATA transcription factor.

Myocardin and GATA factors both interact with SRF and participate in cardiac gene expression modulating each other's activities; in particular GATA4 augments the activity of myocardin on some genes, such as the cardiac homeobox Nkx2.5. This modulating activity is mediated by the direct physical interaction between the factors (Oh et al., 2005).

The MADS-box family of transcriptional regulators, myocite enhancer factor-2 (MEF-2) proteins, have been widely implicated in cardiomyocite maturation and differentiation. The first Mef2 family members to be expressed during cardiac development are Mef2B and Mef2C. Regulation of Mef2 function is complex, including protein-protein interactions with other transcription factors and/or transcriptional regulators, phosphorylation by ciclin-dependent kinases, interactions with histone deacetylases, control by Ca2+ - dependent signaling cascade and interaction and phosphorylation by mitogen-activated protein kinases (MAPK) (Olson et al. , 1995). In particular p38 MAPK has an important role in the regulation of Mef2 transcriptional activity during development and differentiation. p38 plays a role also in the regulation of cardiomyocite proliferation, hypertrophy, apoptosis and growth response, although the precise role in cardiogenesis is still unclear (Hernández-Torres et al., 2008).

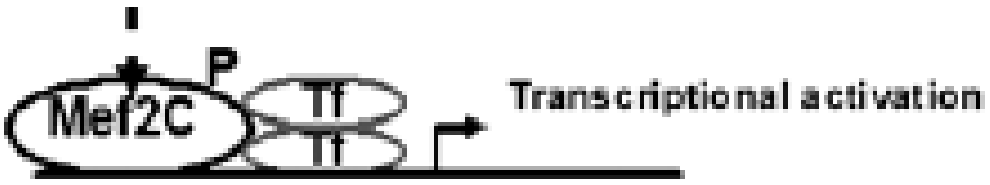


Fig. 1.3 Cooperation between Mef2c and other transcription factor for transcriptional activation of cardiac specific genes.

Myocardin is a serum response factor (SRF) cofactor. In addition to activating cardiac gene expression and the cardiogenesis program, myocardin is also a potent transactivator for smooth muscle cells (SMC) differentiation and SM-related gene expression (Parmaceck, 2003). Although myocardin lacks intrinsic DNA-binding ability, it forms a stable ternary complex with SRF to potently activate muscle-specific genes through the consensus sequence CC(A/T)6GG, known as a CArG box.

Myocardin transactivation is also positively and negatively regulated by p300 and HDAC5, suggesting an additional layer of regulation at the chromatin level (Oka et al., 2007).

GATA4 represses or activates myocardin-mediated transactivation, depending on specificity of target genes.

Many studies showed a role of Bone morphogenetic proteins (BMP) in regulating myocardin expression and activity to control gene expression, BMP are growth and differentiation factors of the transforming growth factorβ (TGFβ) superfamily. BMP signaling was also able to induce expression of myocardin, suggesting a potential positive-feedback mechanism. This mechanism could be used where both myocradin and BMP initiate early cardiac gene expression, whereas myocardin is later used for the maintenance of the cardiac program (Callis et al., 2005).

1.4 Extracellular matrix

The extracellular matrix (ECM) is an integral part of the growth and development of the heart as well as its response to pathophysiologic stimuli. The dynamic interaction of the ECM with the various cellular components of the heart is a fundamental process in cardiac remodeling. In the heart, extracellular matrix is composed of a macromolecular complex that functions to communicate with the specific tissue cells (Borg et al., 2000; Goldsmith et al., 2001).

The main cellular components of the heart are myocytes and fibroblasts, other cell types as mast cells, macrophages and lymphocytes may be present and can play a role in the secretion of ECM components that can influence cardiac function.

Cardiac fibroblasts, the predominat cell type of the heart by number but not by volume, are organized into a three-dimensional network in the heart. This organization follows the endomysial weave network that surrounds group of myocytes. This structure, described as laminae of two to five myocytes in thickness, is fundamental to the organization and function of the heart (Goldsmith et al, 2004).

These laminae are surrounded by a weave system of endomisial collagen. The fibroblasts are responsible to synthesize it, and express several types of collagens, MMPs, growth factor and their role in cytokines signaling. Fibroblasts have been termed sentinel cells because of the many function they mediate in the heart. They show three types of of connections: 1) fibroblast-fibroblast contacts via log filapodia, 2) cell- ECM interactions, 3) fibroblast- myocyte interactions (Banerjef et al., 2006).

The transmission of mechanical force is one of the principal functions of the connective tissue network in the heart. The organization of this

network has been well described in normal hearts and in a variety of cardiac disease models (Borg et al., 2000).

The dynamic interaction between the ECM components determines cellular growth and function.

The main components of ECM consist of structural proteins such as collagens and elastin, adhesive proteins such as laminin, fibronectin, type VI collagen, anti-adhesive proteins such as tenascin, thrombospondin and osteopontin, proteoglycans, noncollagenous glycoproteins, growth factor and cytokines, extracellular proteases, hormones and ECM receptor (Goldsmith et al., 2001).

A well organized extracellular matrix is necessary also to maintain strength and organization of cardiac tissue and is involved in communication between different cardiac cells. There are a group of matrix proteins that modulate cell function but do not appear to have a direct structural role in the extracellular matrix of the heart. These matrix proteins have been termed matricellular proteins to highlight their role in modulating cell function (Schellings et al., 2004).

These proteins are a group of ECM binding proteins, like osteopontin, osteonectin, thrombospondin-1, that shown their higher expression during embryogenesis, with a strong decrease after birth, and their expression becomes low to absent during normal adult life. Matricellular proteins exert their function by binding to matrix proteins, cell surface receptors or molecules such as cytokines.

Cardiac remodeling results from a complex balance between cell proliferation, hypertrophy and cell loss by programmed cell death. Cardiac hypertrophy is characterized by an increase in protein content per cell, and multiplication of non muscle cell. The process involves changes in the expression of a number of genes and among them, those coding for the ECM proteins, resulting in an overall increase in

ECM synthesis accumulation and cardiac fibrosis. On the other hand, the synthesis and secretion of growth factors is induced and maintained by the hemodynamic changes associated to hypertension and cardiac overload. Growth factors such as FGF2 contribute to the maintenance of the phenotype of adult cardiac myocyte and participate both in cell hypertrophy process and interstitial fibrosis (Corda et al., 2000).

Intercellular communication is maintained by gap-junctional channels that connect neighboring cells and allow electrical and metabolic communication, thus forming a functional syncytium. In the heart, gap-junctional communication contributes to the biophysical properties of the tissue, playing a role in the immuno-inflammatory pathology. The connexins (Cx) comprise a gene family of 21 members in humans. Cx37, Cx40, CX43, Cx45 are those most expressed in the cardiovascular system; in human cardiac disease, Cx43 expression is often reduced, so that it is possible to detect a change in the distribution gap junctions (Dhein & Jongsma, 2004).

The accumulation of ECM components in the heart and in the blood vessels contributes to cardiac failure (Corda et al., 2000).

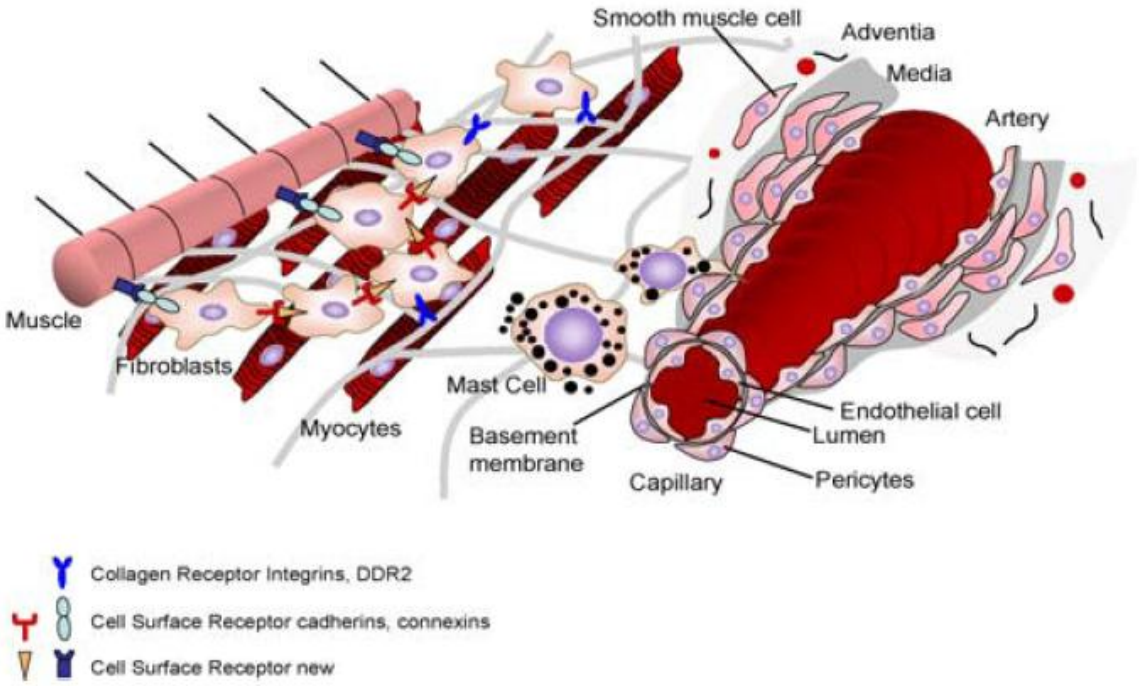


FIGURE 1. Model of interactions between cells and extracellular matrix in the heart.

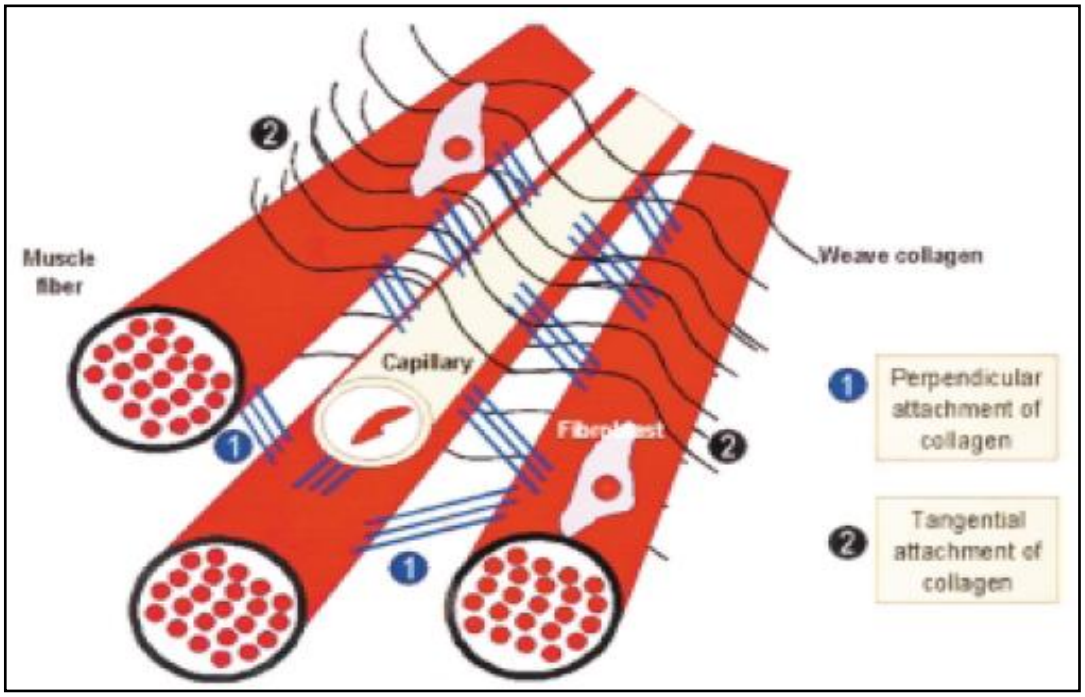


Fig. 1.5 Models of collagen fibers.

1.5 Response to adult heart disease- cardiac repair

Adult heart disease can arise from either congenital abnormalities associated with a defect in one or more cardiac developmental process, or due to acquired disorders such as long-standing hypertension, ischemia/myocardial infarction associated with coronary artery disease, valvular insufficiency, myocarditis, etc.

The majority of these disease predisposing stimuli, first induce a phase of cardiac hypertrophy in which individual myocytes grow without proliferation (Klein et al., 2003; Lips et al. 2003).

Many of the transcription factors just described are re-employed in the adult heart in response to a disease state, where they are thought to mediate the re-expression of the "fetal program" and of genes involved in growth. In most cases, these transcription factors are activated by signal transduction pathways initiated by membrane bound receptors in response to neural-humoral agonists.

The adult cardiac hypertrophic growth program is a complex biologic process that involves the expression of many genes, a group of which represent genes with important developmental functions as GATA4, Mef2, Nkx2.5, SRF. There is a partial conservation in function whereby genes that promote embryonic and fetal heart growth are re-employed in the adult heart in response to disease stimuli. (Oka et al., 2007).

Cardiac injury result in acute loss of a large number of myocardial cells. The human heart has low regenerative ability. Cardiomyocite death triggers a reparative response that ultimately results in formation of a scar and is associated with dilative remodeling of the ventricle. Cardiac injury activates innate immune mechanisms initiating an inflammatory reaction (Frangogiannis, 2008).

The inflammatory response and cytokine release from the myocardium are essential components of the host response to acute myocardial infarction, and play a crucial role in cardiac repair.

Healing of an infraction can be divided into three phases: the inflammatory phase, the proliferative phase and the maturation phase. (Nah & Rhee, 2009).

The acute repair process is mediated by cytokines and inflammatory cells in the infarcted myocardium. There is an involvement of multiple overlapping regulatory mechanisms controlling various proinflammatory pathways activated in the infarcted myocardium. Many proteins play a role in these mechanisms (as CD44 a cell surface glycoprotein involved in cell-cell interaction and cell adhesion and migration) (Nah & Rhee, 2009; Anzalone et al., 2009).

Extensive experimental evidence suggested that infiltrating leucocytes and inflammatory mediators may induce negative effects on cardiomyocytes in the infarcted heart, attempts to mitigate inflammatory injury in clinical practice have been in general unsuccessful. Even if interventions targeting the inflammatory response do not reduce cardiomyocyte death, modulation of the reparative process in order to optimize the mechanical and functional properties of the infarcted heart remains an interesting direction (Frangogiannis, 2008).

Today there's an evolving concept of the cardiac repair.

Cardiac myocytes are thought to be terminally differentiated cells unable to regenerate and replace damaged myocardium. This concept was challenged by recent evidences suggesting that a fraction of cardiomyocites may be able to re-enter the cell-cycle, and/or that limited cardiac regeneration may occur through recruitment of resident and circulating stem cells (Nadal-Ginard et al., 2003; Wollert

& Drexler, 2005). Cellular cardiomyoplasty may be a new hope in heart failure, it's based on the transformation of non –myogenic into contractile cells or attempts to induce the cardiomyocyte to re-enter the cellular cycle (Hagège & Menasché, 2000).

Reversing the process of heart disease progression is fundamental in understanding of the many lives of heart cell, with a primary focus on the emerging links between cardiogenesis and heart stem cell biology (Martin-Puig et al., 2008; Urbanek et al., 2005; Anversa et al., 2002).

1.6 Evidences for the presence of Adult Cardiac Stem Cells

In the past few years it has been established that the mammalian heart is in continuous turnover and has intrinsic regenerative potential.

Many studies demonstrate that the heart contains a reservoir of stem and progenitor cells. These cells express various stem/progenitor cell markers (Barile et al., 2007; Ohnishi et al, 2007). The identification of different classes of cardiac progenitor cells suggest that the heart is not a terminally differentiated, postmitotic organ, but an organ regulated by a stem cell compartment. The possibility has also been raised that stem cells are present in the normal and diseased heart (Bearzi et al., 2007).

The detection of human heart stem cells requires the identification of interstitial structures with the characteristics of stem cell niches and the recognition of the mechanisms of stem cell division that regulate niche homeostasis and the self-renewing properties of stem cells.

The main function of stem cells in postnatal life is to repair and regenerate the tissue in which they reside. Stem cells have the ability to self-renew and to differentiate into at least one mature cell type (Zuba-Surma et al., 2009).

Many study indicate the presence of a stem cell compartment in adult organ, for example in the dog heart, in particular canine cardiac stem cells have been shown to be self-renewing, clonogenic and multipotent. In this model the differentiation of these primitive cells into myocytes and coronary vessels repairs the infarcted area, restores local wall motion, improves ventricular hemodynamics, and positively interferes with pathologic ventricular remodeling (Linke et al., 2005).

An interesting work of Ivanova and colleagues investigated and compared global gene profiles of various type of stem cells and determined the molecular similarities and differences among five distinct stem cell populations, human fetal, murine fetal and adult human stem cells derived from diverse organs. The similarities should help to define a common genetic program or molecular signature (Ivanova et al., 2002).

Many studies demonstrate that MSC (Mesenchymal Stem Cells) which were first isolated from bone marrow, adhere to plastic in vitro and expand in tissue culture, with a finite lifespan of 15-20 population doublings (PD), but recently Zeng et al demonstrated that human, mouse and rat postnatal bone marrow contains primitive progenitors termed multipotent adult progenitor cells (MAPC).

MAPC can be expanded under defined conditions for more than 100 (for human) and 400 (for rat) PD, without telomere shortening or karyotypic abnormalities (Zeng et al., 2006; Wang et al., 2007).

DNA repair maintains genomic stability and the loss of DNA repair capacity results in genetic instability that may lead to a decline of cellular function. In adult tissues, cells maintain a delicate balance between proliferation and apoptosis. Adult stem cells are extremely important in the long-term maintenance of tissues throughout life, their longevity is dependent on careful control of gene expression,

proliferation and cell cycle, and differentiation signal (Kenyon & Gerson, 2007).

So it is of great importannce to evaluate the proliferative capacity of expanded adult stem cells to maintain long-term regeneration before re-infusion (Bonab et al., 2006): evidences showed indeed substantial physiological benefit derived from transplanting cells into the infarcted heart. Interestingly almost every cell type tested seemed to be equipotent: benefit is derived from cardiomyocytes, skeletal myoblasts, hematopoioetic stem cells, mesenchymal stem cells (Murry et al., 2006).

1.7 Mesenchymal Stem Cells

Mesenchymal Stem Cells (MSC) populations have been detected in several human organs, derived from all of the germ layers. In vivo and in vitro studies performed models have suggested that MSC obtained from different tissues can differentiate into unrelated ones, even crossing the lineage boundaries. These cells can differentiate into distinct types of mesenchymal cells including osteoblasts, chondroblasts, adipocytes and myoblasts, which contribute to the formation of mesenchymal tissues (bone, cartilage, muscle, marrow stroma, ligament, tendon, fat, dermis and connective tissue) (Păunescu et al., 2007).

Examples of transdifferentiation are present in nature in differentiated cells and are remarkable because they ultimately demonstrate that this cells are not fixed but rather retain the ability to undergo genetic reprogramming (Thowfeequ et al., 2007). For stem cell this property is named stem-cell plasticity (Beltrami et al., 2003; Beltrami et al., 2001).

Mesenchymal stem cells (MSC), first isolated from bone marrow (BM-MSC) as stromal cells supporting hematopoiesis, and early described as capable of differentiating toward adipocytes (Tremain et al., 2001; Javazon et al., 2004), are pluripotent cells capable of long-term self-renewal and able to contribute to the regeneration of mesenchymal tissues such as connective tissues (cartilage, bone, tendons, ligaments, and adipose) and nervous tissue (Davani et al., 2005).

These cells, after isolation, may have initial lag phase after isolation and then undergo rapid divisions, population doubling rate depending on the donor and the initial density (Chamberlain et al., 2007; Troyer, Weiss, 2008; Rubio et al., 2008).

Bone marrow represents the main source of MSCs, but also fetal-associated sources contain populations of MSC. Umbilical cord blood, for example, is a known source of stem cells for experimental and potentially clinical purposes, but also Wharton's Jelly, the primitive connective of the human umbilical cord represent a main source for MSC. The human umbilical cord is embriologically derived at day 26 of gestation, and it grows to form a 30-50 cm long helical organ at birth, given this expansion there must be a mesenchymal precursor cell population within the UC that give rise to the Wharton Jelly connective tissue (Sarugaser et al., 2005).

MSCs express a number of markers that are also shared by several differentiated phenotypes. In fact, analysis of the gene expression profile of MSC demonstrated the presence of transcripts typical of osteoblasts, chondrocytes, endothelial cells (EC), epithelial cells, and neurons (Grogan et al., 2009; Warejcka et al., 1996; Anversa et al., 2007).

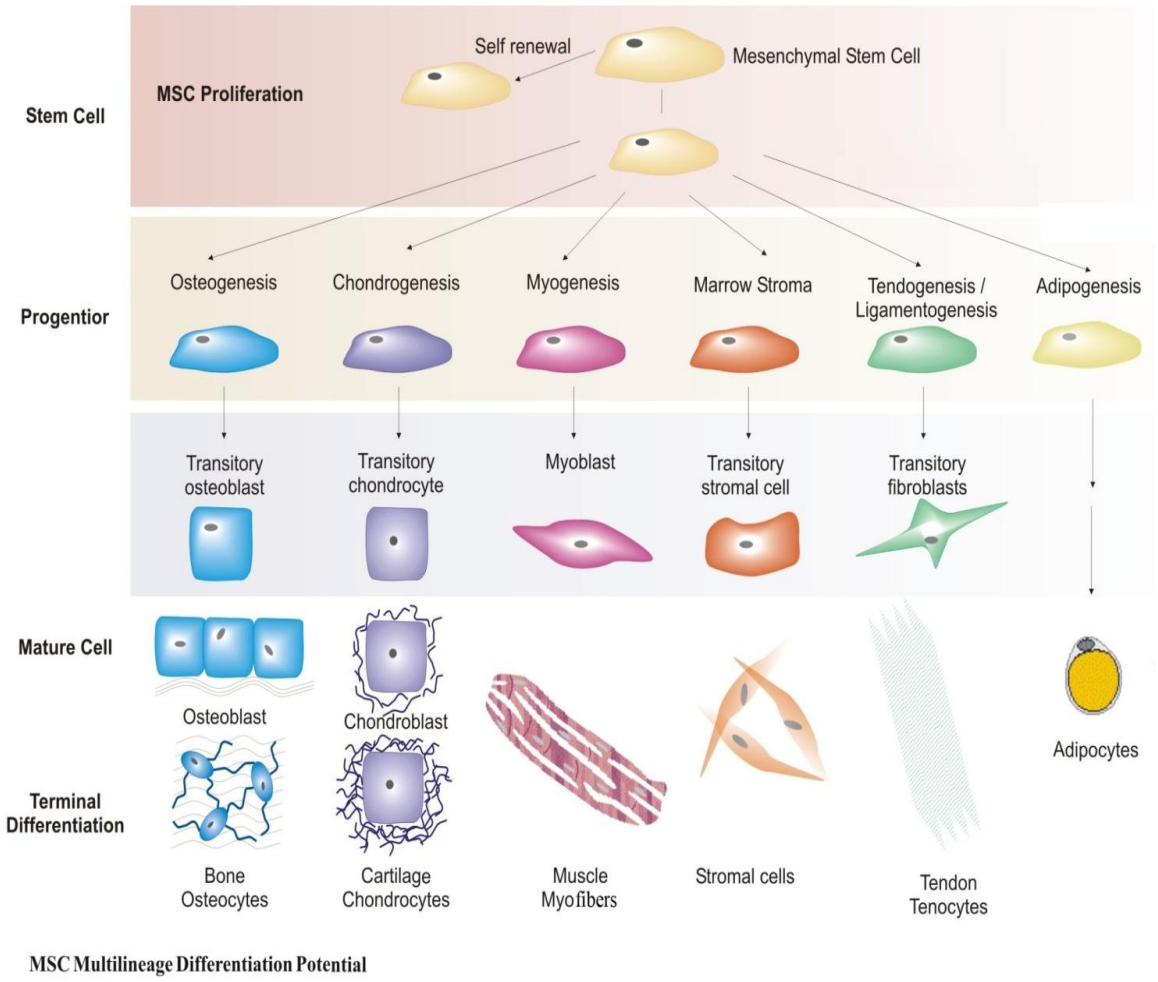


Fig. 1.6 The process of mesengenesis, by which the differentiation of mesenchymal stem cells produces the mature cytotypes of mesenchymal origin.

Several authors have contributed to the definition of markers useful for BM-MSC immunophenotyping. Among the "core" markers, CD44, CD73, CD90, CD105, CD166 are reproducibly found expressed in different preparations of BM-MSC. Other markers for which a general consensus exists are: CD49e, CD51, CD54, CD59, CD71, while it is widely accepted that CD117 is not expressed in human BM-MSC, although being reported in other MSC populations (Chamberlain et al., 2007; Laflamme et al., 2002; Reffelmann & Kloner, 2003).

BM-MSC do not express typical markers of endothelial/hematopoietic cells such as CD31, CD14, CD34, CD45, CD79, CD86, and glycophorin A (CD235a). In addition, these cells express molecules of class I major histocompatibility complex (MHC), but not class II (such as HLA-DR).

The immune phenotype of MSCs widely described as MHCI+, MHCII-, CD40-, CD80-, CD86-, is regarded as non-immunogenic and therefore transplantation into an allogenic host may not require immunosoppression. MHC class II may activate T cells, but with the absence of costimulatory molecules, a secondary signal would not engage, leaving the cell anergic (Di Nicola et al., 2002).

Many reports have also described MSC as having immunosuppressive properties, specifically indicating that MSC can modulate many T-cell functions including cell activation (Bartholomew et al., 2002; Ballard & Edelberg, 2007), or impairing maturation and function of dendritic cells. However, a part of the mechanisms by which MSC exert their immunosuppressive effects that must be still understood, there is some evidence that these in vitro observations may translate to the in vivo setting (Chamberlain et al., 2007; Anzalone et al., 2010).

MSCs have been isolated from several compartments of the umbilical cord, umbilical cord blood, umbilical vein subendothelium, and the Wharton's Jelly (WJ).

Recent data suggest that Wharton's Jelly mesenchymal stem cells can differentiate into ectodermal, mesodermal and endodermal cellular lineages and successfully be expanded ex vivo and cryopreserved.

WJ-derived MSC (WJ-MSC) have a gene expression profile similar to BM-MSC, even if expressing also additional markers as CD117. (Quiao et al., 2008; Can & Karahuseyinoglu, 2007). BM-MSC and WJ-MSC express several stem cell markers, including c-Kit, Nanog, Oct4 (Marcus, Woodbury, 2008).

Markers	*BM-MSC*	*WJC*
CD10	+	+
CD13	+	+
CD14	−	−
CD29	+	+
CD31	−	−
CD33	−	−
CD34	−	−
CD44	+	+
CD45	−	−
CD49e	+	+
CD51	+	+
CD54	+	NA
CD56	+	−
CD59	+	NA
CD68	NA	+
CD71	+	NA
CD73	+	+
CD79	−	NA
CD80	−	+
CD86	−	−
CD90	+	+
CD105	+	+
CD117	−	+
CD163	NA	−
CD166	+	+
CD235a	−	NA
CK-7	NA	−
CK-8	NA	+
CK-18	+	+
CK-19	+	+
Connexin-43	+	+
GATA-4	+	+
GATA-5	NA	+
GATA-6	NA	+
GFAP	+	+
HLA-A	+	+
HLA-B	+	+
HLA-C	+	+
HLA-DR	−	−
HLA-G	+	+
HNF-4α	NA	+
Nanog	+	+
Nestin	+	+
NSE	+	+
Oct3/4A	+	+
α-SMA	+	+
Vimentin	+	+

Table 1.1: Comparison of markers expressed by BM-MSC and WJ-MSC; n.a.: not applicable. La Rocca et al, Histochem Cell Biol, 2009. Anzalone et al, Stem Cells And Dev, 2009.

Injury models have shown that systemic administration of MSC is followed by migration and engrafment into the damaged sites, whereas in the absence of injury they engraft into a plethora of nonhematopoietic tissues. Besides their regeneration abilities, MSC possess immunomodulatory functions, being able to suppress immune reactions both in vitro and in vivo inhibiting several functions of naïve and memory T cells, suppress the develop of monocyte-derived dendritic cells. MSCs also alter the phenotype of NK cells and suppress proliferation, cytokine secretion and cytotoxicity against HLA-class I expressing targets. (Sotiropoulou et al., 2006; Puymirat et al., 2009; La Rocca et al., 2009a).

Immune response of the host is essential to understand how these cells can be applied in regenerative medicine (Rizzino, 2007; La Rocca et al., 2009b).

In clinical practice, the delivery of MSC to infarcted heart has been attempted as a method for myocardial regeneration. But currently there are few evidences that the amelioration of patients' conditions derive actually by the transdifferentiation of MSCs towards myocytes (Li et al., 2007).

Matrix remodelling, paracrine effects, neo-angiogenesis, have been proposed as alternative mechanisms.

Different cell types, either resident in the myocardium, or trans-differentiated from MSCs, should be the cause of the improvement of symptoms (Gojo et al., 2003).

II Objectives

Due to the critical importance of the presence of a stem cell compartment in adult human heart, which could be activated in response to injury, there is the need to set up strategies to isolate and fully characterize these cells, in order to use them for regenerative medicine applications.

Between the stem cells subtypes, mesenchymal stem cells emerged as reliable cells with a great plasticity. Their presence in human heart, and their possible role in repair processes following injury, have not yet been clarified.

Therefore, the aim of the present project is to isolate and characterize MSC populations from adult human hearts explanted from patients undergoing transplantation due to chronic heart failure. The technique applied will be similar to that used in our laboratory to obtain MSC from the human umbilical cord, and will be based on enzyme-free techniques.

The obtained cells will be subcultured and expanded in standard culture media, and will be characterized for the expression of markers of the MSC lineage and of mature cytotypes, by means of RT-PCR and immunocytochemistry. In particular, we will investigate the presence of molecules classically defining the MSC phenotype, as well as those normally not expressed in such cells. Particular attention will be dedicated to the characterization of novel molecules expressed by these cells, which can contribute to better define their in vivo phenotype and function.

In addition, since one of the key characteristics of MSC is the potential to differentiate towards mesenchymal tissues (bone, adipose, cartilage), we will perform differentiation experiments using specific

culture media to differentiate HSE-MSC towards osteoblast-like, chondroblast-like and adipocyte-like cells. The success of differentiation will be assessed both by specific histological stains and the expression of key molecules of the differentiated cytotype.

Furthermore, due to the importance of immunomodulation as a feature of MSC populations, potentially favoring the engrafting of transplanted cells in organ, we will perform ICC analyses aiming to determine the expression of molecules potentially involved in immune recognition, as the type I and II HLAs and the non-classical type I HLA-E.

III Materials and methods

3.1 Tissue samples and cellular isolation and protocol

Tissue samples: hearts of patients affected by chronic heart failure (n=4) for the cellular isolation from the sub-endocardial layer and infarct scar.

The heart wall sections were washed in warm HBSS (Gibco), and then were cut in small pieces (about 0.5 cm length).

Different incisions were made with sterile scalpel to increase area exposed to the contact with medium, composed by DMEM low glucose (Sigma), supplemented with 10% FCS (fetal cow serum, PAA), 1x NEAA (non-essential aminoacids, Sigma), 1x antibiotics-antimycotics (Gibco), and 2mM L-glutamine (Sigma). This isolation protocol does not make use of enzymes to dissociate subendocardial cells from the embedding matrix, but is based on their migratory ability. After 15 days of culture, the cells adhered to the plastic surface, and were cultured until confluence.

3.2 Cell culturing and passaging

After reaching confluence, the cells were removed from flasks with triple select (Invitrogen) and cultured for up to 15 passages corresponding to about 45 population doublings. For immunocytochemical analysis, the cells were plated in 8-well chambers slides and were used after reaching 90% confluence. For DNA and RNA extraction, cells were cultured either in 6-well tissue culture plates or in 25cm^2 tissue culture flasks (Corning).

3.3 Immunocytochemical analysis

Immunocytochemistry detect the expression of specific antigens recognized by primary antibody which are bound by secondary antibody.

The cells were culture in chamber slides (BD Falcon). After removal of the medium, the cells were washed with PBS, fixed and dehydrated with methanol for 20 minutes at -20C. After a rinse with PBS, the cells were treated for 3 minutes with Triton X-100 0,1% in PBS 1X. Then, the cells were washed two times with PBS and after were treated with solution 0,3% of hydrogen peroxide to inactive endogen peroxidases. After 20 minutes with blocking solution, the cells were incubated with specific primary antibodies for 1hour and 30 minutes at room temperature. After another washing with PBS, the cells were incubated with species-specific secondary antibodies, for 10 minutes. Subsequently, it was added streptavidin- peroxidase (DAKO-Cytomation) was added a followed by the 3.3'-diaminobenzidine (DAB chromogenic substrate solution, DAKO). At the end, Hematoxylin (DAKO) was added to stain the nucleus of the cells.

The antibodies used in the present study, with indications of the working conditions used, are listed in Table 3.1.

Table 3.1 List of antibodies used in the present study

Antigen	**Host**	**Manufacturer**	**Diluition**
CD10	Mouse	Dako	1:50
CD13	Mouse	Chemicon	1:50
CD31	Mouse	Dako	Pre-diluted
CD34	Mouse	Dako	Pre-diluted
CD45	Mouse	Dako	Pre-diluted
CD79	Mouse	Dako	1:100
CD117	Rabbit	Stressgen	1:200
Connexin-43	Mouse	BD Laboratories	1:50
GATA-4	Mouse	Santa Cruz	1:200
α-SMA	Mouse	Dako	Pre-diluted
Nestin	Mouse	BD Laboratories	1:50
Vimentin	Mouse	Santa Cruz	1:100
Von Willebrand factor	Mouse	Dako	1.50
Pancitokeratina	Mouse	Dako	1:100
CD68	Mouse	Novo Castra	1:200

3.4 Total RNA extraction

Total RNA extraction from cultured cells was performed trough RNAspin mini RNA isolation Kit (GE Healthcare).

The cells were lysed by adding 350µl of Buffer RA1 and 3.5 µl of β-mercaptoethanol, and were mixed vigorously. The lysate obtained was filtered through RNAspin Mini Filter Unit (supplied by the kit) and was centrifuged for 1 minute at 10000 rpm. After centrifugation, the mini filter was discarded and the filtrate was transferred to a new 1.5 ml tube where were added 350µl of 70% ethanol. After mixing the lysate was pipetted 2-3 times and was transferred to a RNAspin Mini column, placed in a 2ml tube and centrifuged at 8500 rpm for 30 second. After centrifugation, the column was placed in a new tube, and desalted . Each sample was incubated for 15 minutes with 95µl of a mixture containing DNase (10µl of DNase in 90 µl DNase buffer), to avoid a possible DNA contamination. Following washes were carried out with specific buffers supplied by the kit (RA2 and RA3), the column was transferred into a tube 1.5 ml (nuclease-free) provided by the kit. The RNA was eluted from the filter in 100µl of RNase-free H2O (by centrifugation at 10.000 rpm for 1 minute).

The extracted RNA was stored at 20°C until use. The concentration of RNA extracted was determined by spectrophotometer with a wavelength of 260nm. Moreover, to detect a possible protein contamination, the extent of this contamination can be determined by the ratio DO to 260 nm / D.O. at 280 nm (where D.O. indicates the optical density). This value should be between 1.8 and 2, lower values indicate the presence of contaminating proteins.

3.5 RT-PCR (Reverse Transcription Polymerase Chain Reaction)

Qualitative RT-PCR was performed using Phusion High-Fidelity RT-PCR kit (Finnzymes).

RT-PCR consists of two phases: retro-transcription where RNA is converted in DNA complementary (cDNA) and amplification of cDNA. After treatment with DNase, at 2 µg of RNA were added oligo dT and oligo N so that to select only mRNA fro total one. Subsequently was added 5µl of Buffer Phusion 10x, 1µl dNTP mix, 1µl of RNase inihibitor, 1µl of AMV reverse transcriptase and Water until final volume of 50µl. The reaction comprised a reverse transcription step of 50 minutes at 42 C° and an inactivation phase of 5 minute at 92 C°. Then, to 2 µl of DNA obtained were added 10pM of specific primers, 4µl of 5x Phusion Buffer, 0,4µl 10mM dNTP, 0,6 µl DMSO, 0,2 µl of Phusion DNA Polymerase and water until final volume of 20µl. The amplification reaction was performed according to five steps. The initial denaturation of 30 second at 98C°, the denaturation step of 10 second at 98 C°, the annealing phases of 30 second at specific-primers temperatures, the extension step of 30 second at 72C°, and finale extension for 10 minutes at 72 C°.

The specific primers pairs used in this study are listed in table 3.2.

Table 3.2-A List of PCR primers used for the present study

Name	Product size	Primers	
Actin, beta	350bp	Forward	5-AAACTGGAACGGTGAAGGTG-3
		Reverse	5-TCAAGTTGGGGGACAAAAAG-3
Actin alpha2	321	Forward	5-TGATCACCATCGGAAATGAA-3
		Reverse	5-GCTGGAAGGTGGAAATGAA-3
CD29	186	Forward	5-CTGATTGGCTGGAGGAATGT-3
		Reverse	5-TTTCTGGACAAGGTGAGCAA-3
CD31	309	Forward	5-CCATGCACCCTCATACACAG-3
		Reverse	5-CTGTGCTTGTTCCACCTTCA-3
CD44	282	Forward	5-TCTCAAGGGCGTAACTCTGG-3
		Reverse	5-GCCAATTCTACCAGGCTTGA-3
CD73	308	Forward	5-CCTGCTCAGCTCTGCATAAGTA-3
		Reverse	5-CCCTATTTTACTGGCCAAGTGT-3
CD80	259	Forward	5-AGGGCCTCCTTAGATCCCTA-3
		Reverse	5-TTAGCTGCCATGAGATGTGC-3
CD86	250	Forward	5-TCCTGGCTGAGAGAGGAAGA-3
		Reverse	5-AGACTGCCCCATCCCTTAGT-3
CD90	265	Forward	5-TTTGGCCCAAGTTTCTAAGG-3
		Reverse	5-AGATGCCATAAGCTGTGGTG-3
CD105	179	Forward	5-TCCAGCACTGGTGAACTGAG-3
		Reverse	5-TGTCTCCCCTGCCAGTTAGT-3
CD106	340	Forward	5-TGGAGGAGTTCCTTGATCTG-3
		Reverse	5-CTGAAAGTCAACCCAGTGCT-3
CD117	268	Forward	5-ACTTCAGGGGCACTTCATTG-3
		Reverse	5-ACGTGGAACACCAACATCCT-3

Table 3.2-B List of PCR primers used for the present study

CD133	255	Forward	5-GCATGCAAAAGCCATCATAG-3
		Reverse	5-ATCCATGCTGGACACCAGA-3
CD166	283	Forward	5-TGGTGTGGGAGATCAAAGGT-3
		Reverse	5-TGTGGCTGCCATTAAACAAG-3
GATA-4	270	Forward	5-CCAGAGATTCTGCAACACGA-3
		Reverse	5-ATTTTGGAGTGAGGGGTCTG-3
GATA-5	259	Forward	5-GAATGGCCGGTGATGTATGT-3
		Reverse	5-TGAAGCTGATGCCAGACAAC-3
GATA-6	259	Forward	5-ACTAACCCACAGGCAGGTTG-3
		Reverse	5-GGTACAAAACGGCTCCAAAA-3
HLA-A	262	Forward	5-TGGGACTGAGAGGCAAGAGT-3
		Reverse	5-ACAGCTCAGTGCACCATGAA-3
HLA-DR-B1	349	Forward	5-GCACAGAGCAAGATGCTGAG-3
		Reverse	5-AGTTGAAGATGAGGCGCTGT-3
Cytokeratin 8	216	Forward	5-TCTGGGATGCAGAACATGAG-3
		Reverse	5-AGACACCAGCTTCCCATCAC-3
Cytokeratin 18	263	Forward	5-CTGCTGCACCTTGAGTCAGA-3
		Reverse	5-GTCCAAGGCATCACCAAGAT-3
Cytokeratin 19	295	Forward	5-ATGAAAGCTGCCTTGGAAGA-3
		Reverse	5-CCTCCAAAGGACAGCAGAAG-3
Adiponectin	164	Forward	5-GCTGGAGTTCAGTGGTGTGA-3
		Reverse	5-ACCAACCTGACGAATGTGGT-3
Nanog	209	Forward	5-CTCCATGAACATGCAACCTG-3
		Reverse	5-CTCGCTGATTAGGCTCCAAC-3
Nestin	275	Forward	5-TATAACCTCCCACCCTGCAA-3
		Reverse	5-AGTGCCGTCACCTCCATTAG-3

Table 3.2 C List of PCR primers used for the present study

Oct-1	297	Forward	5-GCAACCCTGTTAGCTTGGTC-3
		Reverse	5-CTCTCCTTTGCCCTCACAAC-3
Oct-2	286	Forward	5-AGGCCTCAGCGTTCTCTTTT-3
		Reverse	5-TGCCAGTCCCTTCTCTCTTC-3
Oct-4 A	273	Forward	5-AGTGAGAGGCAACCTGGAGA-3
		Reverse	5-GTGAAGTGAGGGCTCCCATA-3
Oct-4 B	194	Forward	5-TATGGGAGCCCTCACTTCAC-3
		Reverse	5-CAAAAACCCTGGCACAAACT-3
Osteonectin	296	Forward	5-TGATGATGGTGCAGAGGAAA-3
		Reverse	5-GGGGGATGTATTTGCAAGG-3
Periostin	185	Forward	5-TGGAGTTAGCCTCCTGTGGT-3
		Reverse	5-ACAAGGCTCGGTCTTTTCAA-3
Vimentin	345	Forward	5-AGATGGCCCTTGACATTGAG-3
		Reverse	5-TCTTGCGCTCCTGAAAAACT-3
vWF	317	Forward	5-GGGGTCATCTCTGGATTCAA-3
		Reverse	5-CAGGTGCCTGGAATTTCAT-3
ABCG2	255	Forward	5-ATGGTGTATAGACGCCTGA-3
		Reverse	5-GGGACAGGTATGTGAAAAGC-3
MYL-2	250	Forward	5-CAAGGAGGAGGTTGACCAGA-3
		Reverse	5-GCAAAGAAGATGGAGGTGGA-3
NKX 2.5	316	Forward	5-CATGGTATCCGAGCCTGGTA-3
		Reverse	5-GAGCTCAGTCCCAGTTCCAA-3
RYR-2	332	Forward	5-CCCCATATGCTCCTGCTATT-3
		Reverse	5-CTGATCACAGGTGGCTGAAA-3

Table 3.2 D List of PCR primers used for the present study

MEF2C	430	Forward	5-AGGACCCCCAAATGTCACT-3
		Reverse	5-AGCGGCAGCCTTTTACAAT-3
Myocardin	193	Forward	5-CTCGGCTTCCTTTGAACAAG-3
		Forward	5-CTTCCCAGAGAATCCATCCA-3
CD40	406	Forward	5-TCCATCCAGAACCACCCACT-3
		Reverse	5-TTGGAGAAGAAGCCGACTGG-3
FABP4	252	Forward	5-CATCAGTGTGAATGGGGATG-3
		Reverse	5-GTGGAAGTGACGCCTTTCAT-3
Connexin 43	240	Forward	5-CTTCAAGCAGAGCCAGCAG-3
		Reverse	5-TACCCCATACACCCCCAGT-3
ANF	450	Forward	5-CGCAGACCTGATGGATTTCA-3
		Reverse	5-GCAGCTTAGATGGGATGATCAC-3
MYBPC3	220	Forward	5-CAGCAAGCAGGGAGTGTTG-3
		Reverse	5-GAGGGGTTTCCCCAACTTC-3
TNNI3	244	Forward	5-TGACCTTCGAGGCAAGTTT-3
		Reverse	5-CAGGAAGGCTCAGCTCTCA-3
TNNI3K	221	Forward	5-CATTTTCATTCTTGCCGAAA-3
		Reverse	5-CACAAATCCAAAGCCTGCTA-3

3.6 Agarose gel electrophoresis

After PCR amplification, the amplified were loaded in 2% agarose gel and were run in a Borax-EDTA 1x buffer. Sybr Safe DNA Gel Stain (Invitrogen) was used to stain DNA. All samples were loaded with Loading Buffer 10X (TBE 1X, bromophenol blue, glycerol and SDS 10%). The size of DNA bands was estimated by Step Ladder, 50bp (SIGMA) , a marker with known molecular weight.

3.7 Clonogenicity assays

Clonogenic assay is an in vitro cell assay based on the ability of a single cell to undergo unlimited division and grow into a colony, composed at the least of 50 cells. We applied a limiting dilution as reported Weiss et al, 2006. Briefly, cells at different culture passages were plated as single cells into each well of a 96-well plate. The addition of a single cell per well was confirmed by phase contrast microscopy. After 2 weeks in culture, with medium change each second day, the presence of clones was assessed by phase-contrast microscope.

3.8 Osteogenic differentiation and histological stain

HSE-MSCs at different passages were cultured in osteogenic differentiation medium, as reported previously. Briefly, cells were cultured for 3 weeks in osteogenic medium, DMEM with 10%FCS(PAA) supplemented with 0.1μM dexamethasone, 50μM ascorbate-2 phosphate, 10 μM β-glycerophosphate (Sigma), 1% antibiotic/antimycotic (GIBCO). The formation of cell clusters resembling intra-membranous ossification was monitored by phase-contrast microscopy along culturing. Controls included HSE-MSC

cultured in normal growth medium for 3 weeks to monitor the eventual spontaneous formation of bone-like nodules.

To demonstrated the acquisition of osteogenic phenotype was perform: Alizarin Red Stain which highlights the presence of extra-cellular calcium storages and Alkaline phosphatase Stain

which measures indirectly enzyme activity. According to protocol of Alizarin Red Stain, cells cultured in 8-well chamber slides (BD Bioscience) ,deprived of the medium, were washed with PBS and then fixed with 4% paraformaldehyde for 20 minutes at room temperature. Eliminated the fixative, they were performed three washes with distilled water and subsequently was added for 30 minutes at room temperature, 1% water solution of Alizarin Red S (SIGMA) with range of pH between 4.6-6. After incubation with dye were carried out some washes with deionized water to remove excess stain, and then cells were photographed at the photomicroscope.

3.9 Adipogenic differentiation and histological stain

Differentiation of cells was performed by culturing HSE-MSCs at different passages in adipogenic differentiation medium, as reported previously .

Culture medium was supplemented with 0.5 mM isobutylmethylxanthine (Sigma), 1 µM dexamethasone (Sigma), 10 µM insulin (Sigma), 200 µM indomethacin (Sigma), 10% FCS (PAA), and 1% antibiotic-antimycotic (Gibco).

Cells were cultured in six-well tissue culture plates for 3 weeks, and medium was replaced every second day. The formation of cytoplasmic lipid vacuoles was monitored by phase-contrast microscopy along culturing. Controls included MSCs cultured in standard growth

medium for 3 weeks to monitor the spontaneous formation of lipid vacuoles.

To confirm acquisition of adipogenic phenotype was performed Oil Red staining which highlights the presence lipid vacuoles. According to protocol, after medium aspiration and a brief wash with PBS , cells were fixed with 10% formalin (Sigma) for 30 min at room temperature and then washed with distilled water and 60% isopropanol. After drying was added Oil Red O working solution for 5-10 min, and were carried out four washes (5 min each) with distilled water. To counterstain was added Meyer's hematoxylin for 1 minute and the images were viewed and photographed using an inverted phase-contrast microscope. The lipid vacuoles appeared red and nuclei blue.

3.10 Chondrogenic differentiation and histological stain

In order to perform a chondrogenic differentiative protocol for HSE-MSC, cells at different passages were prepared for culture in alginate beads, as reported previously (Chubinskaja et al., 2001; Petit et al., 1996). Culture medium was supplemented with1% FCS (PAA), 10ng/ml TGFβ1, 50nM ascorbate-2 phosphate, 6,25 µg/ ml insulin (Sigma), and 1% antibiotic-antimycotic (Gibco).

Cells were embedded in an alginate solution (2,4 % w/v) to form beads with a density of 4x 10^6 cells/ml and then HSE-MSC were cultured in 24-well tissue culture plates for 3 weeks, with medium replacement every second day.

The formation of characteristics lacunae around cells in alginate beads was monitored by phase-contrast microscopy along culturing.

Controls were HSE-MSC embedded in alginate and cultured in standard growth medium for 3 weeks.

Beads were fixed with 2% paraformaldeyde (Sigma) and paraffin embedded. Thin sections were prepared and it was removed paraffin through passages in xilene and in ethanol with decreased concentrations (100%, 96%, 80% and 50%).

To confirm acquisition of chondrogenic phenotype we performed Alcian Blue staining which allows the stain of acid mucosubstances by combination of PAS staining with Alcian Blue. According to protocol the sections were deparaffinized in the typical manner and rehydrated; after drying we added alcian blue solution for 5 min, and subsequently two washes were carried out (5 min each) with running tap water and distilled water. Specimens were counterstained by using Nuclear fast red solution for 10 minutes, rinsing with distilled water and dehydrating with ascending alcohol series before mounting slides. The acid mucosubstances (glycosaminoglycans) appeared dark blue and nuclei red.

IV- Results and discussion

4.1 Cellular isolation and culturing

The cellular isolation protocol applied, previously published by our group, permitted reproducibly the isolation of fibroblastoid cells, which adhered on gelatin-coated flasks and should undergo repeated passages in culture and were successfully subcultured giving cells at various culture passages. Cells were extracted both from the sub-endocardial layer and from the infarct scar of human hearts from CHF patients which underwent heart transplantation. The first ones were analyzed during this project.

Figure 4.1 shows a panel of representative phase-contrast micrographs of HSE-MSCs at different culture passages. Panels are representative of what were obtained with different cell lines. As visible, primary cultured cells at the first passages, show a typical mesenchymal morphology: with processes extending between adjacent cells and the presence of cell–cell contacts. Moreover, confluent monolayer cells are arranged in parallel arrays. Cells at higher culture passages (Fig. 4.1) retained the standard fibroblastoid morphology and steadily grew in the standard culture medium.

Freezing, storage in liquid nitrogen and defrost of cultured cells were routinely performed at different passages in order to demonstrate their ability to survive deep freezing and therefore their long-term storability.

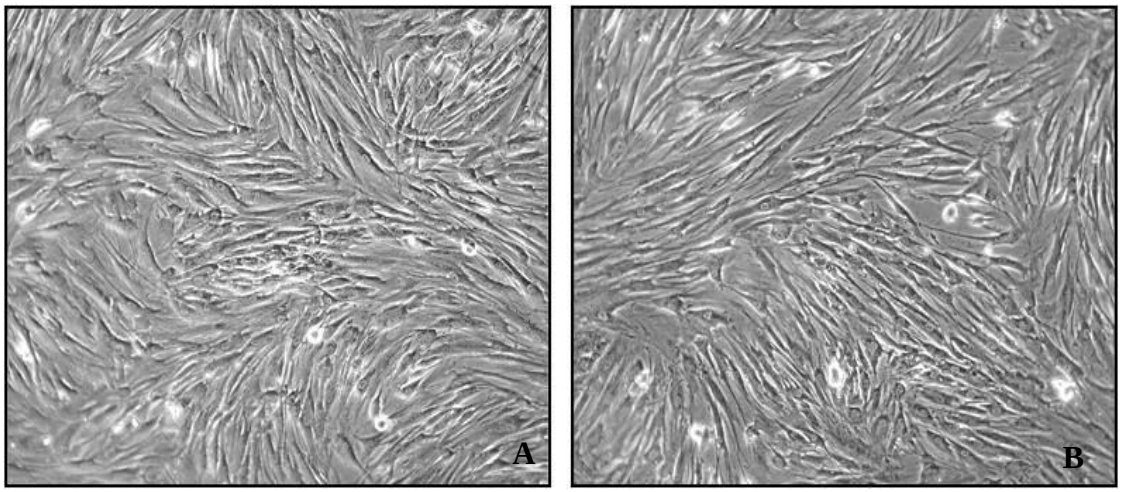


Fig. 4.1 Light microscopic Micrographs, monolayer culture at different culture passages. In monolayer culture, cells assumed a polymorphic, fibrobroblast-like morphology, which was maintained throughout the passaging process. Magnification 20x.

4.2 Characterization of MSC markers by immunocytochemistry

The characterization of markers expressed by HSE-MSCs was initially performed by immunocytochemical analysis. Experiments were repeated in triplicate on cell lines obtained from different subjects in order to assess the reproducibility of the results.

Figure 4.2 shows the representative results obtained by probing the cells for the expression at the protein level of a panel of key markers used to characterize cells. Present data show that, besides mesodermal markers as vimentin (Fig. 4.2a) and alphasmooth muscle actin (Fig. 4.2b), HSE-MSCs expressed c-Kit membrane antigen (CD117, Fig. 4.2c), which acts as receptor for stem cells factor (SCF) in hematopoietic stem cells (HSC). CD117 has been characterized as a key marker in HSC, but its expression has been reported in populations of adult- and fetal-derived mesenchymal stem cells. Noteworthy, this marker is not expressed in BM-MSC.

Nestin expression (Fig. 4.2d) in the vast majority of cells is a particularly significant finding. In fact, this molecule represents an intermediate filament, characterized in the neuroectodermic lineage as precursor of neurofilament proteins, and has also been demonstrate in endocrine pancreas progenitors.

Its expression in mesenchymal stem cells has been recognized by different authors, even if this molecule is not yet a key marker of these cells. Immunocytochemical analysis for expression of GATA-4 (Fig. 4.2e) showed an intense perinuclear positivity in HSE-MSCs. As stated previously, this transcription factor is involved in several differentiative pathways, as the cardiomyocyte one, where it is responsible of the transactivation of promoters specific for the

myocardial lineage, as reported for ANF (atrial natriuretic factor), BNP (brain natriuretic peptide), and the cardiac isoforms of troponin.
The perinuclear localization of this molecule is consistent with the undifferentiated phenotype of HSE-MSC, where, in the absence of a specific differentiation stimulus, the transcription factor is expressed, but is maintained in aninactive form outside the nucleus.
Finally, another marker expressed by the vast majority of cells is connexin-43 (Fig. 4.2f), a protein is known to be expressed at high levels in embryonic cells as well as in myocardial cells. Its expression is indicative of the capability of HSE-MSC to form gap junctions. The potential usefulness of some of these markers to predict differentiation along myocytes is an hypothesis which deserves much work to be demonstrated. Positivity to alpha-smooth muscle actin has been also assessed in HSE-MSCs (Fig. 4.2b). The expression of this molecule has previously been reported for various MSC populations, even if there is no general consensus to consider it as a MSC-specific marker.
Figure 4.3 shows another panel of key markers tested also at protein level used to characterize cells, we can see a positive signal for Keratin (Fig. 4.3 a), we used an antibody for Pan-cytokeratin, and the immunocytochemistry evidenced a consistent number of positive cells as firstly observed for MSC derived from Wharton's Jelly.
We observed also the expression of another mesenchymal marker prolyl-4-hydroxylase (Fig. 4.3b), this enzyme catalyzes the reaction that transforms prolin in hydroxiprolin. Its expression is indicative of the capability of these cells to form collagen. Further experiments have shown that alongside with passaging, cultured cells were morphologically and phenotypically similar to the parental cells, for the tested marker .

On the contrary we obtained negative results testing endothelial-specific markers as CD31 (Fig. 4.3 e) and vWF (Fig. 4.3 c), or hematopoietic specific ones as CD79 (Fig. 4.3 d), and also testing sarcomeric actin (Fig. 4.3f), a molecule found in muscle tissue as a major constituent of the contractile apparatus.

Furthermore, other immunocytochemical analyses showed the expression of CD68 (Fig. 4.4 b) but not expression of CD34 (Fig. 4.4 a).

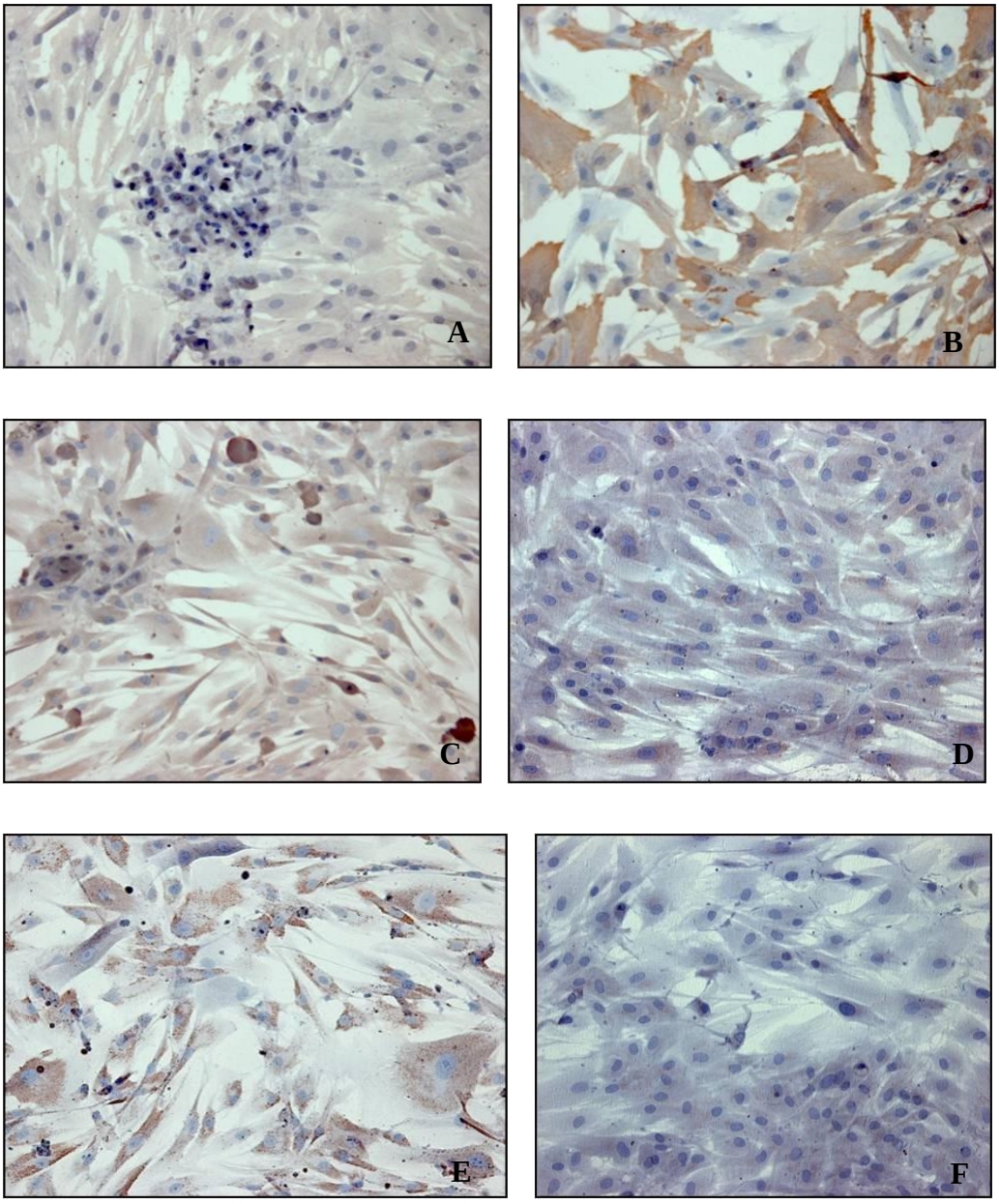


Fig. 4.2 Representative panels of immunocytochemical detection of mesenchymal markers on HSE-MSCs. The isolated cells showed a strong positive signal for vimentin (a), α-sma (b),CD117 (c-Kit, c), Nestin (d), Connexin-43 (e), GATA-4 (f). Magnification: 20x.

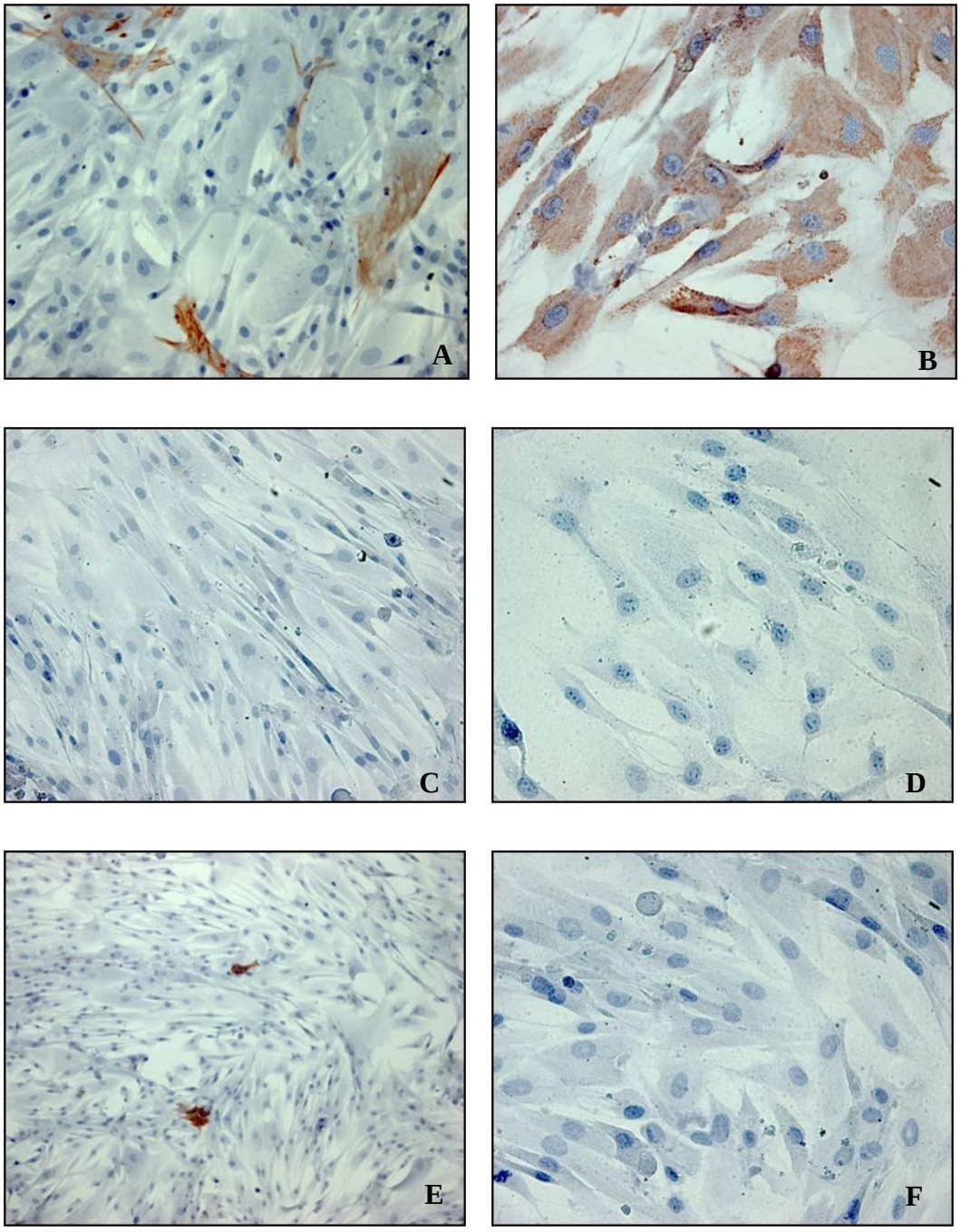


Fig. 4.3 Representative panels of immunocytochemical detection of mesenchymal markers on HSE-MSCs. The isolated cells showed a strong positive signal for Pan-cytokeratin (a), prol-4-hydroxilase (b), while don't show signal for vWF (c), CD79 (d), CD31 (e), sarcomeric actin (f). Magnification: 20x.

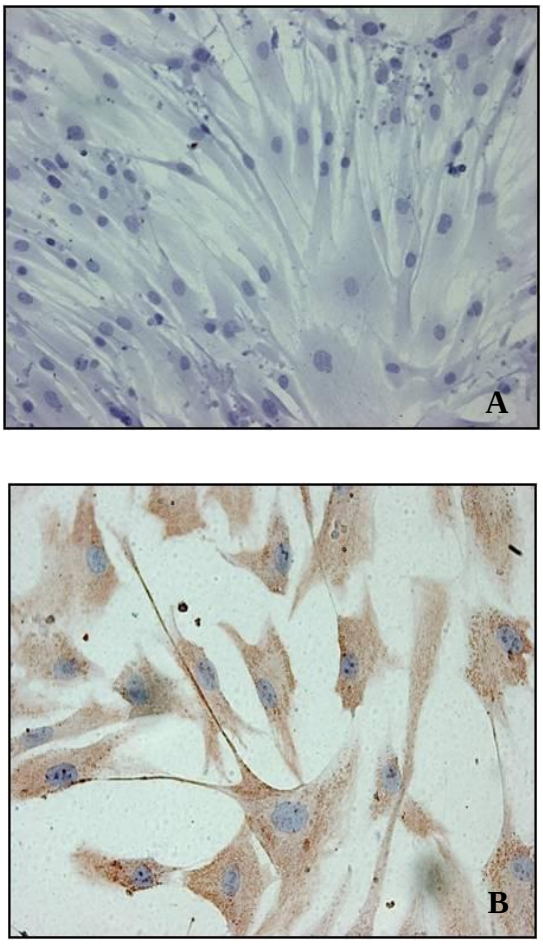


Fig. 4.4 Representative panels of immunocytochemical detection of markers on HSE-MSCs. The isolated cells showed no expression of CD34(a), while showed the expression CD68 (b). Magnification: 20x.

Table 4.1 shows the global results of the ICC analysis of HSE-MSCs. The cells did not show expression of endothelial- specific markers (CD31, vWF) or hematopoietic specific ones (CD34, CD45, CD79). These data are in good agreement with those reported in literature regarding the expression pattern of mesenchymal stem cells. On the contrary, HSE-MSC showed the expression of a MSC marker as CD13.

MSC Markers	Expression
α-SMA	++
CD117 (c-Kit)	+++
Vimentin	+++
Connexin 43	+++
Nestin	+++
Prol-4-hydroxilase	+++
vWF	-
GATA-4	+++
Pan-Keratin	+
CD13	+
CD31	-
CD34	-
CD45	-
CD79	-

Table 4.1 Markers expressed by heart-derived mesenchymal stem cells (ICC).

4.3 Gene expression by qualitative RT-PCR analysis

In order to confirm and extend the data obtained by immunocytochemical analysis, we performed total RNA extraction, followed by retrotranscription and specific amplification of gene fragments by qualitative RT-PCR.

Figure 4.5shows a representative electrophoretic gel of HSE-MSCs gene expression pattern. As visible, RT-PCR experiments allowed demonstrating the expression of other "core markers" of human MSCs as CD29, CD73, CD80 CD90 and CD166. As visible, we detected the expression of the B7-costimulator molecule CD80, which we also previously demonstrated in MSC from Wharton's Jelly.

The expression of GATA-4 at the RNA level confirms the datum obtained by immunocytochemistry.

Furthermore we have obtained the expression of the transcript of Desmin, an intermediate filament protein.

Moreover figure 4.6 shows the expression of CK18 and CK19, confirming data we obtained with ICC. These epithelial markers neither have been characterized previously in mesenchymal stem cells, nor are normally expressed in mesenchymal-derived cytotypes.

Also we observed the expression of CD105 and CD133, marker of MSC, and GATA-6 molecule present in differentiation pathways of diverse organs.

We investigated the presence of molecules involved in the immune recognition of MSCs by the immune system of the potential host. In fact, several authors reported that in "in vivo" settings the host response towards differentiated stem cells was related to the expression of B7 costimulatory molecules as CD80 (B7-1) and CD86 (B7-2). We determined by RT-PCR as shown in Figure 4.6 that HSE-

MSC did not express CD86, which induce lymphocyte response, while expressing CD80 as still described, this data can be correlate to reinforce the idea that these cells can have an immunosuppressive capability, a characteristic should be of key importance in the instauration of a response of the host to avoid transplant rejection.

Table 4.2 resume RT-PCR data obtained in mesenchymal stem cells derived from the sub-endocardial layer of hearts of CHF patients. These cells feature the presence of the embryonic markers Oct3/4A and Nanog. In particular OCT transcription factor, isoform OCT3/4 A, is a marker of embryonic stem cells involved in self-renewal and NANOG is a marker involved in maintenance of the undifferentiated state and pluripotency.

Moreover, RT-PCR experiments allowed confirming the expression of Nestin, GATA-4 and GATA-6 at the mRNA level, as showed by immunocytochemical analysis and also evidence the absence of GATA-5, a molecule involved pathways mainly in endodermal differentiation pathways. We must note in addition the absence of CD40, molecule implicated in immune response as co-stimulator, and the absence of CK8.

The transcription factor OCT-1 expressed in ESC and fetal liver cells was expressed by HSEMSCs, we obtained also the expression of OCT-2 mRNAwhich expression of this factor is normally restricted to the lymphoid lineage, and the expression of ABCG2, an ATP-binding cassette (ABC) transporter.

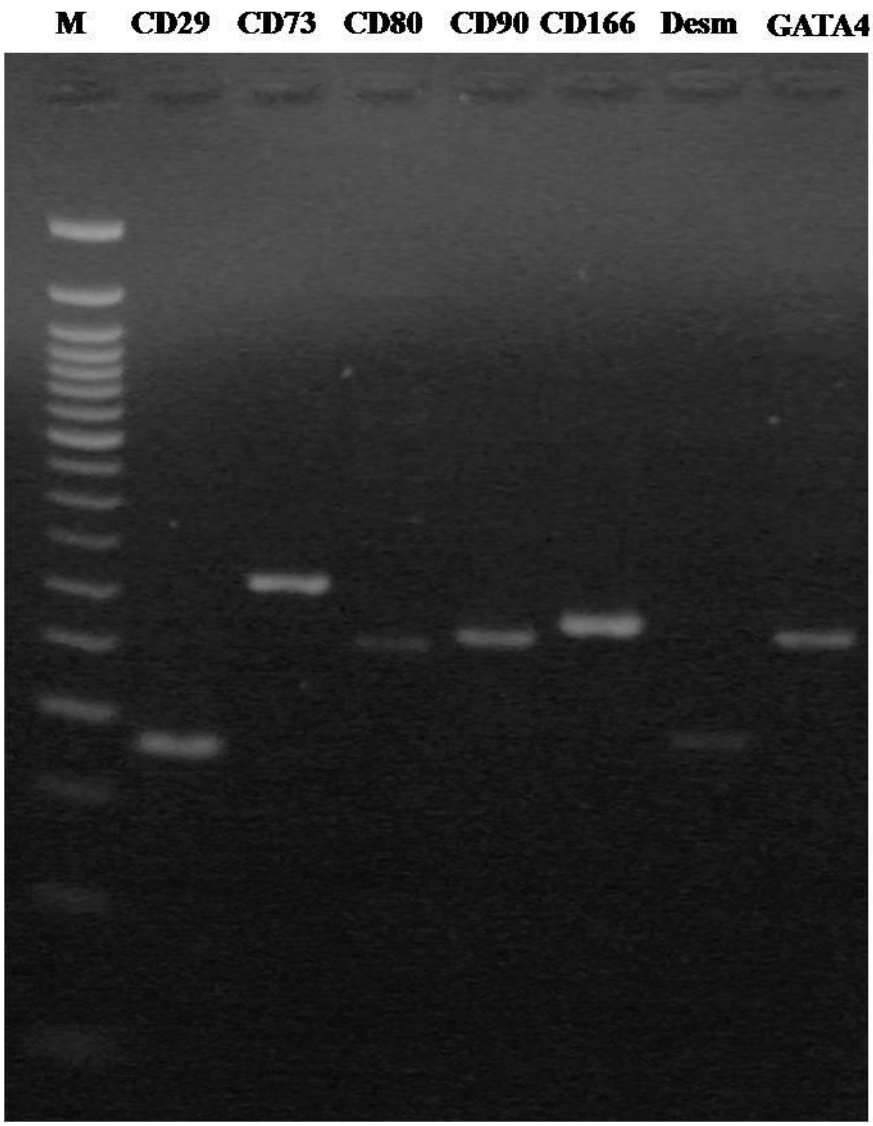


Fig. 4.5 Representative panel of RT-PCR analysis of HSE-MSC. **M** 50 bp ladder.

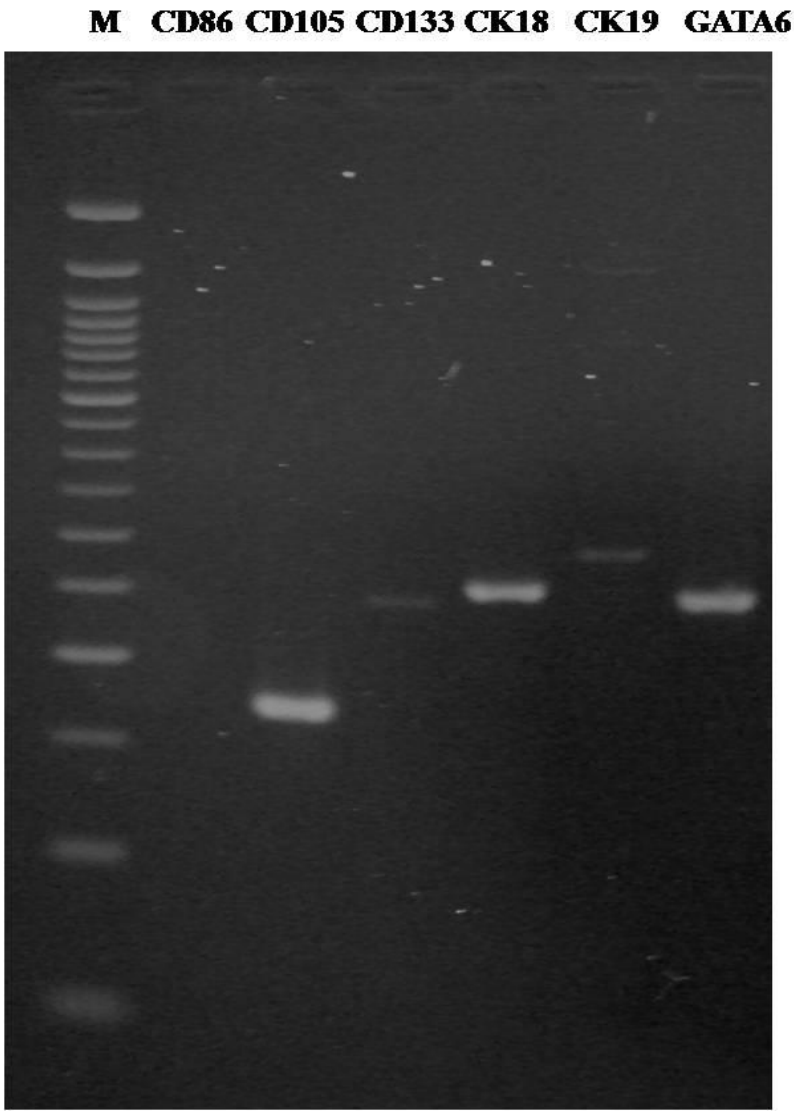


Fig. 4.6 Representative panel of RT-PCR analysis of HSE-MSCs. **M** 50 bp ladder.

MSC Markers	Expression	MSC Markers	Expression
CD29	+	CK8	-
CD73	+	CK18	+
CD80	+	CK19	+
CD90	+	Desmin	+
CD105	+	GATA-4	+
CD133	+	GATA-5	-
CD166	+	GATA-6	+
CD40	-	Nanog	+
CD86	-	Nestin	+
Actin beta	+	Oct 3/4A	+

Table 4.2 Expression of key markers of different lineages by HSE-MSCs assessed by qualitative RT-PCR analyses.

4.4 Extended characterization - cardiac transcription factors

In order to further extend the characterization of the basic features of such cells, we checked the expression of heart-specific TFs by RT-PCR.

These cells showed the expression of heart-specific TFs, which, as demonstrated for GATA-4 by ICC, are probably in an inactive state, so that they do not undergo nuclear translocation and therefore, in undifferentiated cells, cannot transactivate cardiac-specific genes.

RT-PCR analysis (results showed in table 4.3) demonstrated the expression of GATA-6, MEF2c, Myocardin and Nkx2.5 all of which are heart-specific transcription factor expressed in different stage of cardiogenesis.

MEF2C	+
Myocardin	+
Nkx2.5	+
GATA-4	+
GATA-6	+

Table 4.3 Results of RT-PCR analyses: cardiac TFs. For these analyses, NO-RT reactions were used as negative control, while beta-actin amplification was used as positive control.

4.5 Clonogenicity assays

The ability to generate clones, a cellular colony derived from a single cell, is a formal demonstration of the self-renewal ability, a characteristic of stem cell populations. Our gene expression data, suggesting expression of key molecules involved in self-renewal were thus confirmed by performing a clonogenicity assay. Briefly, cells at different culture passages were seeded in 96-well plates with an appropriate dilution to deliver single cell per well, as stated in Materials and methods. After 15 days of culture, colonies were counted, resulting in a cloning efficiency of 10–12% a result which is in good agreement with previous observations made by other groups on stem cells. Clonal lines were not further characterized.

4.6 Osteogenic differentiation of HSE-MSC

The differentiative potential of mesenchymal stem cells, has analyzed by several authors since the definitive demonstration of "stemness" for a cell line is the ability to differentiate towards more than one mature cell type.

In order to demonstrate the capability to differentiate towards osteoblasts, HSE-MSCs were cultured for 3 weeks in osteogenic medium. Following culture, fixed cells were observed at the microscope.

As visible in Fig. 4.7, untreated cells, cultured in standard medium for 3 weeks (Fig. 4.7 A, B), retained the normal fibroblastoid morphology, and were negative for alkaline phosphatase activity. On the contrary osteogenic-induced HSE-MSCs formed bone nodules (Fig. 4.7 C, D).

The formation of extracellular calcium deposits has been assessed by alizarin red S staining: differentiated HSE-MSCs were extensively

stained red (Fig. 4.8 C, D), both in nodules and sparse cells, while control cells were not stained (Fig. 4.8 A, B) and retained a fibroblastoid morphology.

Moreover, the acquisition of an osteoblast-like phenotype was also confirmed by RT-PCR. Figure 4.9 shows the results of semi-quantitative RT-PCR experiments. We demonstrated that the differentiation protocol used resulted in a significant upregulation of periostin and osteocalcin, with respect to the levels observed in untreated cells.

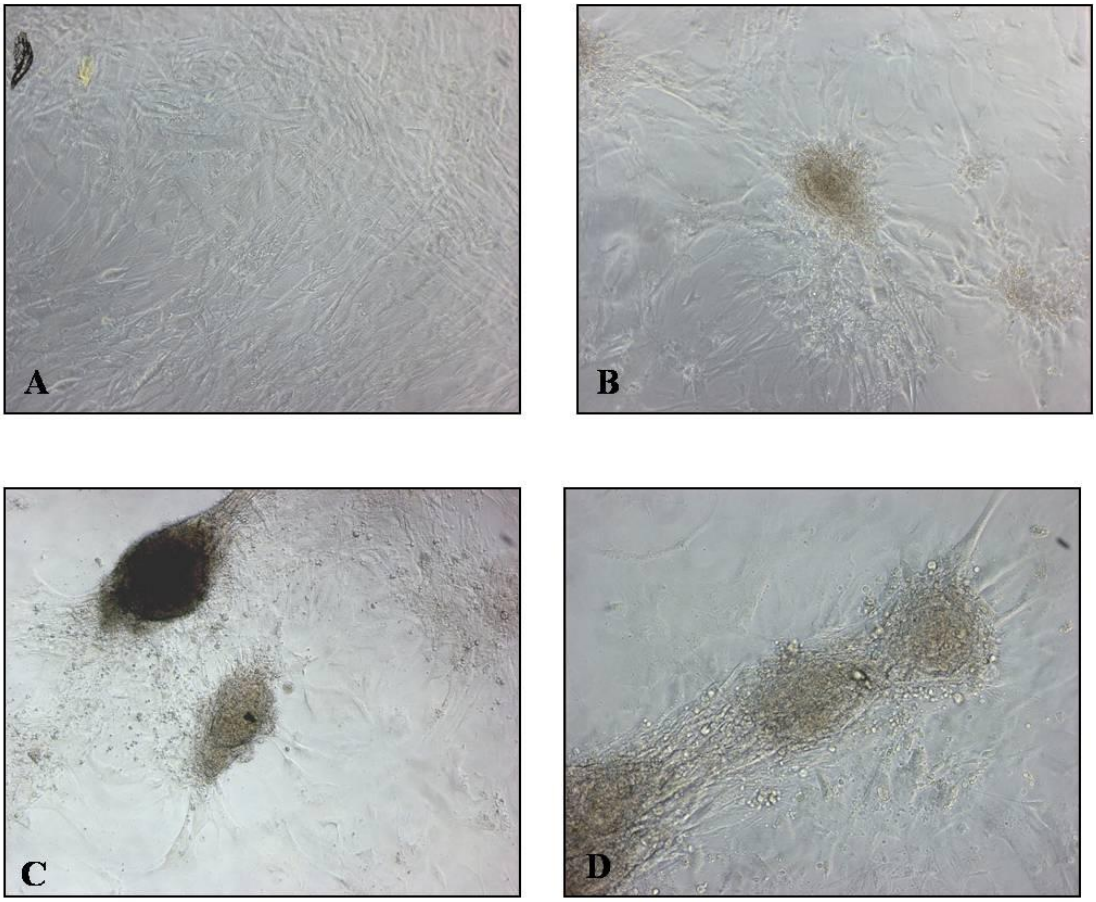


Fig.4.7 Phase-contrast micrographs after a three-weeks osteogenic differentiation period. A, B: control cells were cultured for three weeks in standard culture medium. C, D: treated cells were cultured for three weeks in supplemented medium. Magnification: 10x.

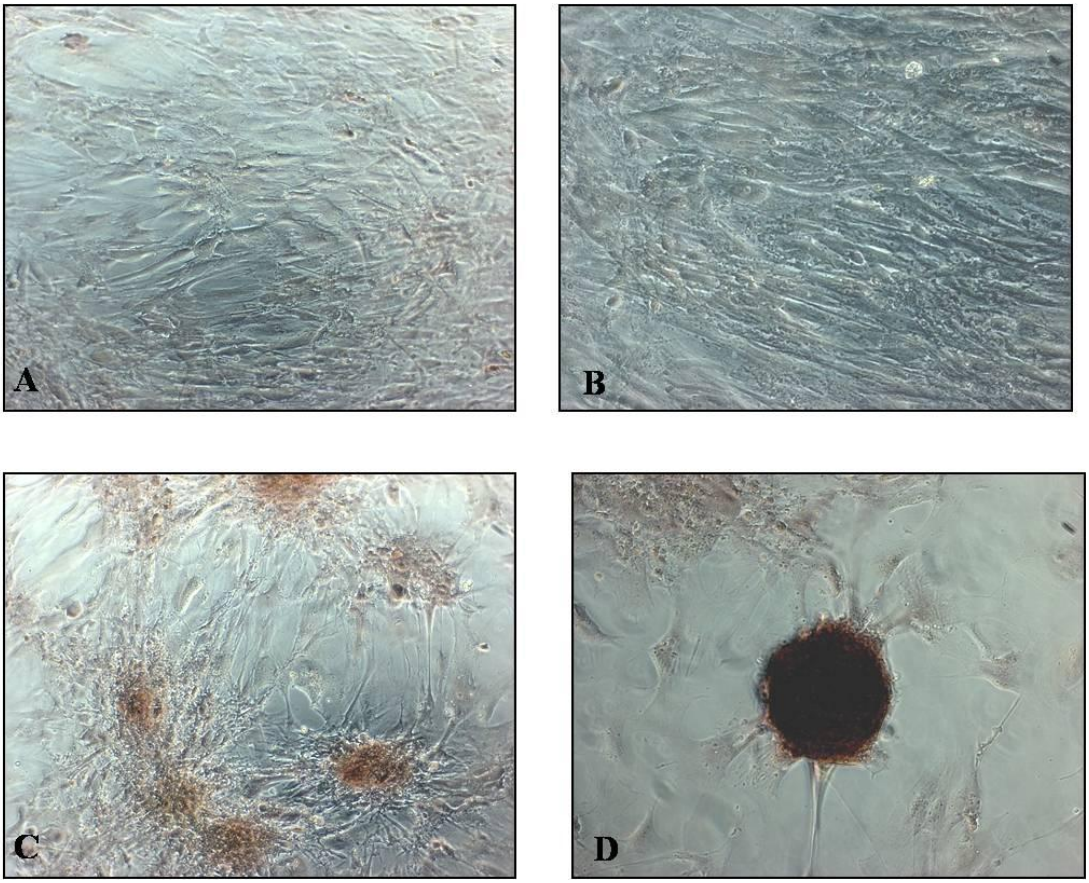


Fig. 4.8 Histochemical evaluation by Alizarin Red S stainings after a three-weeks osteogenic differentiation period. Control cells (A, B) were cultured for three weeks in standard culture medium, treated cells (C, D) were cultured for three weeks in opportunely supplemented medium. Magnification: 10x.

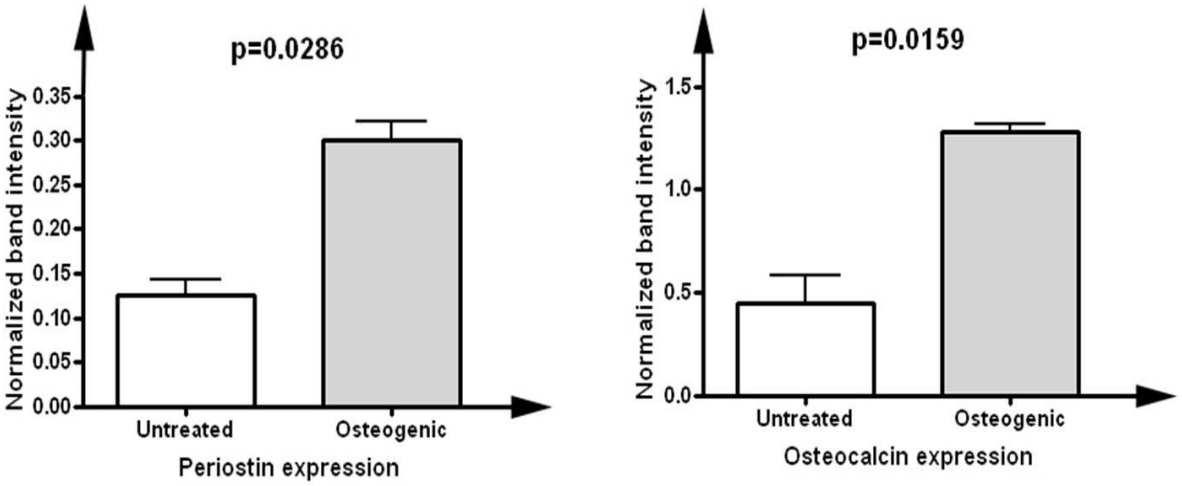


Fig. 4.9 RT-PCR analysis of the expression of specific markers following osteogenic differentiation of HEMSCs. Following the osteogenic differentiation protocol, cells significantly increased the expression of osteoblast-specific markers osteonectin and periostin.

4.7 Adipogenic differentiation of HSE-MSC

HSE-MSCs were cultured for 3 weeks either in adipogenic medium or in standard culture medium (control cells) prior to Oil Red O staining, a histochemical staining specific for lipids. As visible in Fig. 4.10, control cells, indicate as C, do not show any staining for lipid vacuoles, on the contrary, cells cultured for 3 weeks in adipogenic medium show the presence of multiple red vacuoles, resembling multivacuolar adipocytes (Fig. 4.12 ADI). Figure 4.11 also shows the characteristic red vacuoles, in higher-magnification images.

Moreover, adipogenic differentiation resulted in the expression of molecules, characteristic of the adipocyte lineage. Results obtained with adipogenic differentiation were confirmed by semi-quantitative RT-PCR. Figure 4.12 shows the results of a semi-quantitative multiplex RT-PCR analysis (SM-RT-PCR) which was used to determine the variations of FABP4 expression in untreated versus adipocyte-differentiated cells. FABP4 is the fatty acid binding protein-4, a specific marker for adipocytes. The graph shows that the levels of FABP4 are consistently and significantly higher in differentiated cells with respect to control ones, independently from the cycles of amplification. Therefore, this differentiation protocol resulted in the acquisition of a morphologic and functional phenotype adherents to that of adipocytes in vivo.

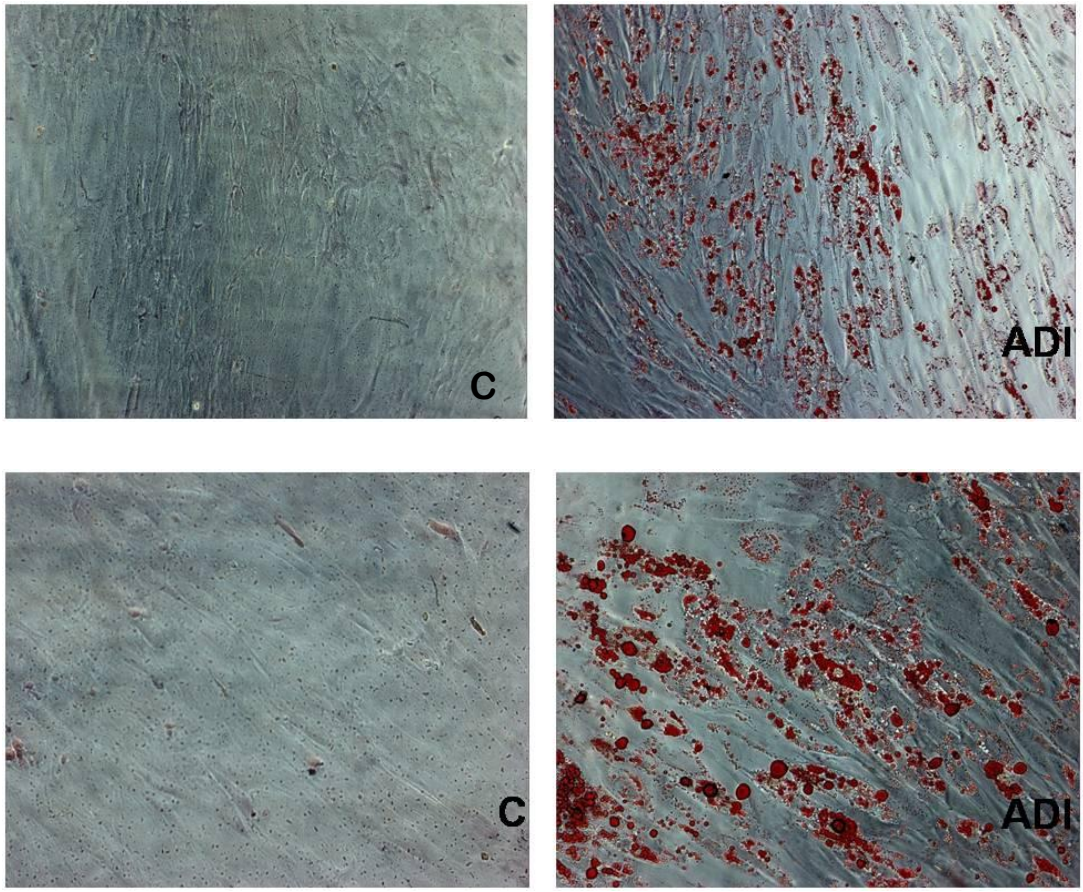


Fig. 4.10 Histochemical evaluation by Oil Red O staining after a three-weeks adipogenic differentiation protocol. Control cells were cultured for three weeks in standard culture medium. Magnification 10x.

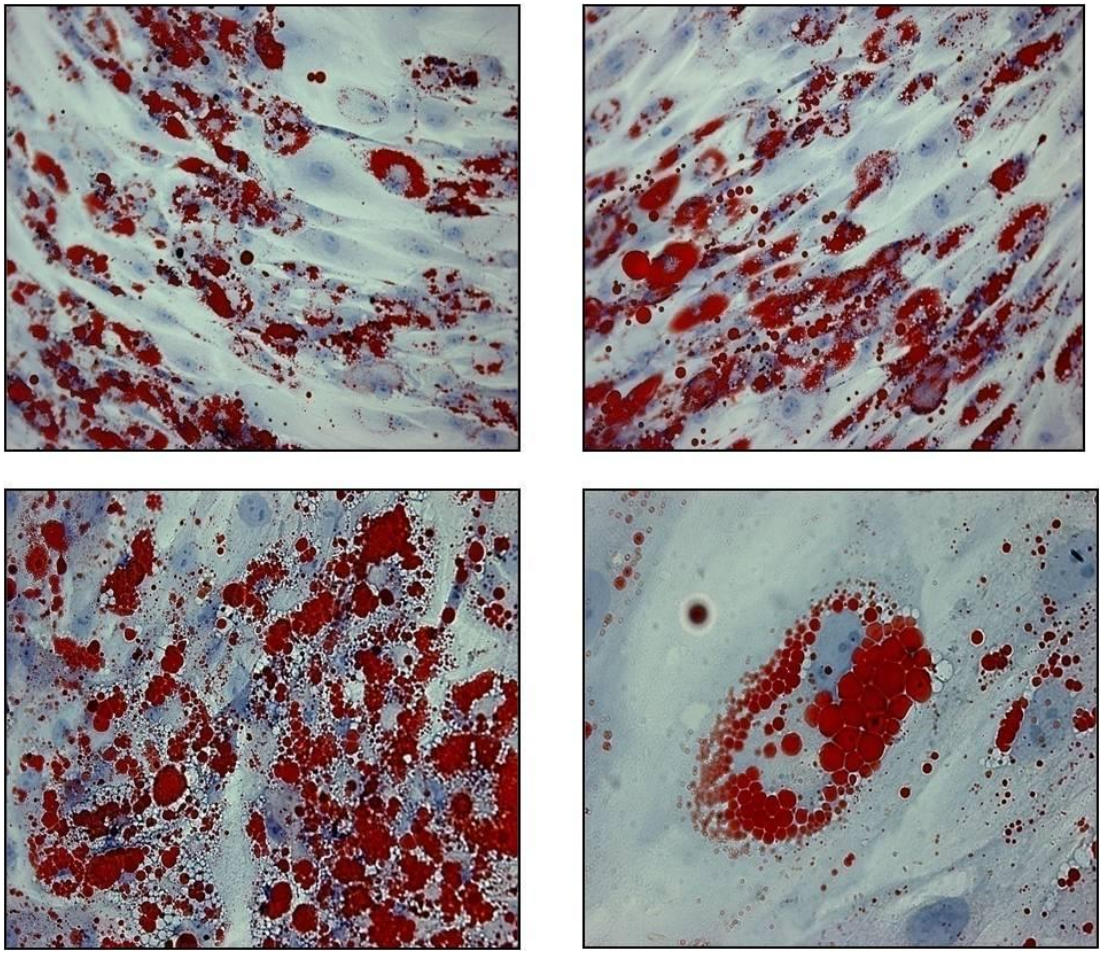


Fig. 4.11 Histochemical evaluation by Oil Red O staining after a three-weeks differentiation period. Magnification: 20x (A-B); 40x (C); 100x (D).

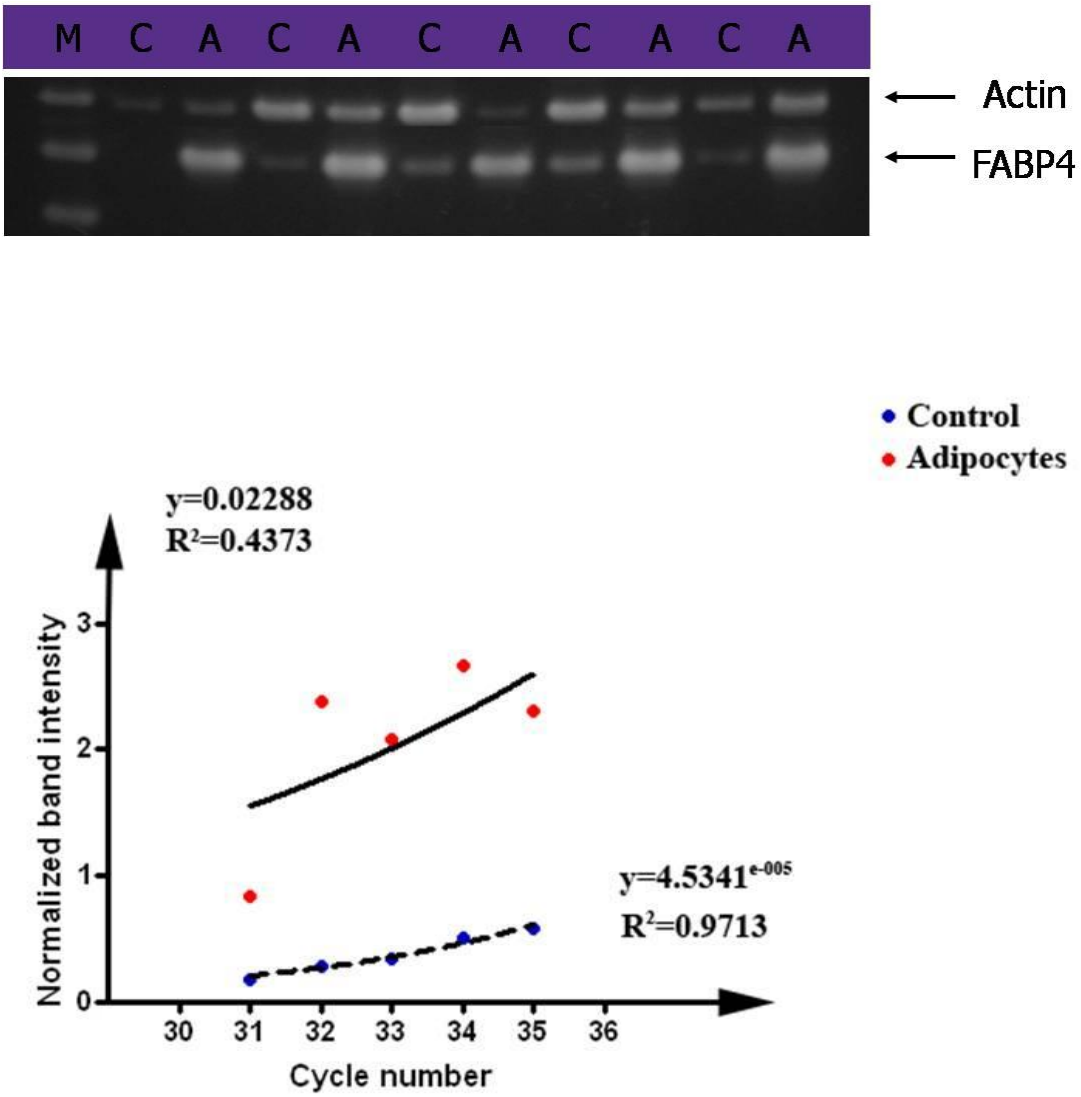


Fig.4.12 Results of the SM-RT-PCR experiments showing the increase of expression of FABP4 in adipocyte-differentiated cells compared to control ones.

4.8 Chondrogenic differentiation of HSE-MSC

In order to demonstrate the capability to differentiate towards chondrocytes, HSE-MSC were cultured in alginate beads for 3 weeks in chondrogenic medium. During culture, cells were observed at the microscope. Control cells were encapsulated in alginate beads and cultured with standard medium.

As visible in Fig. 4.13, untreated cells, cultured in standard medium for 3 weeks (Fig. 4.13 A, C), retained a spheroidal morphology in alginate beads, which did not change over the entire culture period, maintaining a close contact with the embedding matrix. On the contrary chondrogenic-induced HSE-MSC formed enlarged spaces around cells, thus resembling the formation of lacunae which are characteristic of the cartilage tissue (Fig. 4.13 B, D).

The formation of extracellular glycosaminoglycans deposits has been assessed by Alcian Blue staining: control beads contained cells tightly embedded in the matrix, without evident deposits of glycosamminoglycans, as visible from the pale blue color (Fig. 4.14 A-C). Differentiated HSE-MSC showed, as visible in Fig. 4.15, the formation of lacunae around cells and the embredding matrix developed a strong blue staining, which evidences the presence of deposits of glycosaminoglycans.

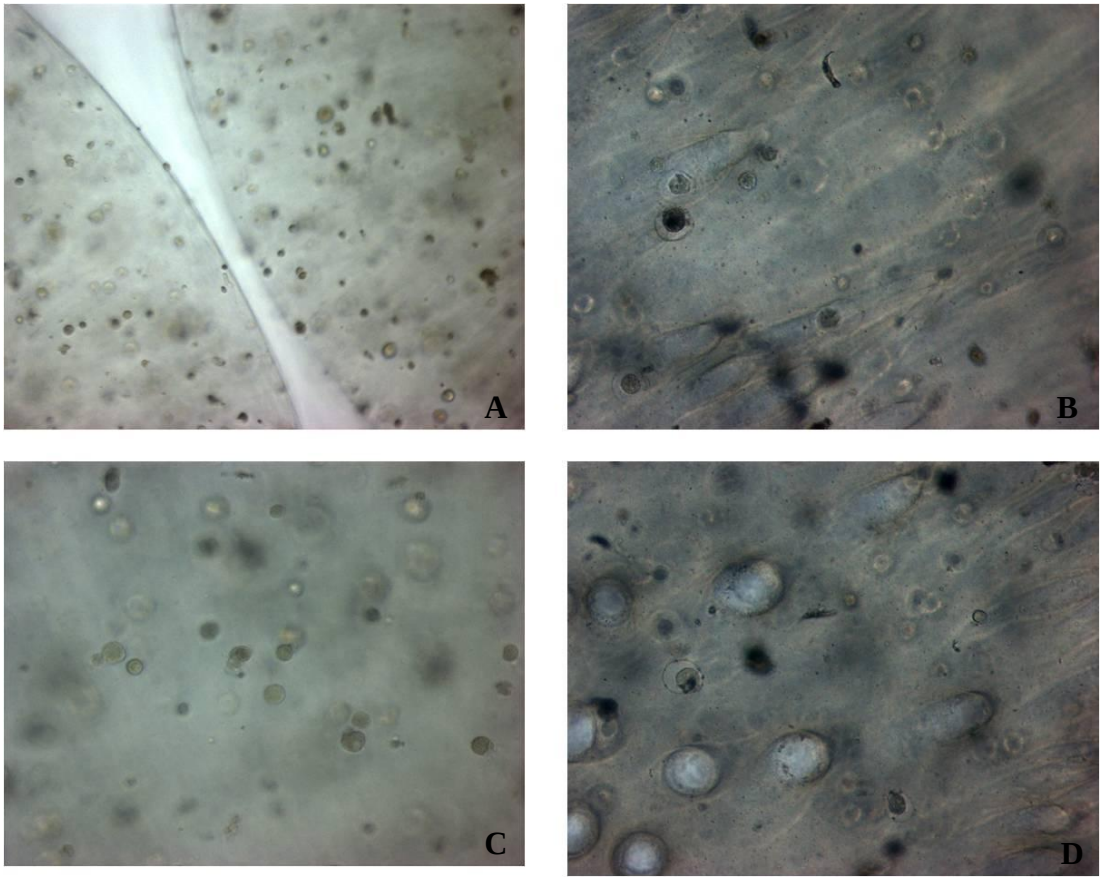


Fig 4.13 Phase-contrast micrographs after a three-weeks differentiation period. A, C: control cells cultured for three weeks in standard culture medium. B, D: cells cultured for three weeks in differentiation medium. Magnification: 10x (A), 20x (B, C, D).

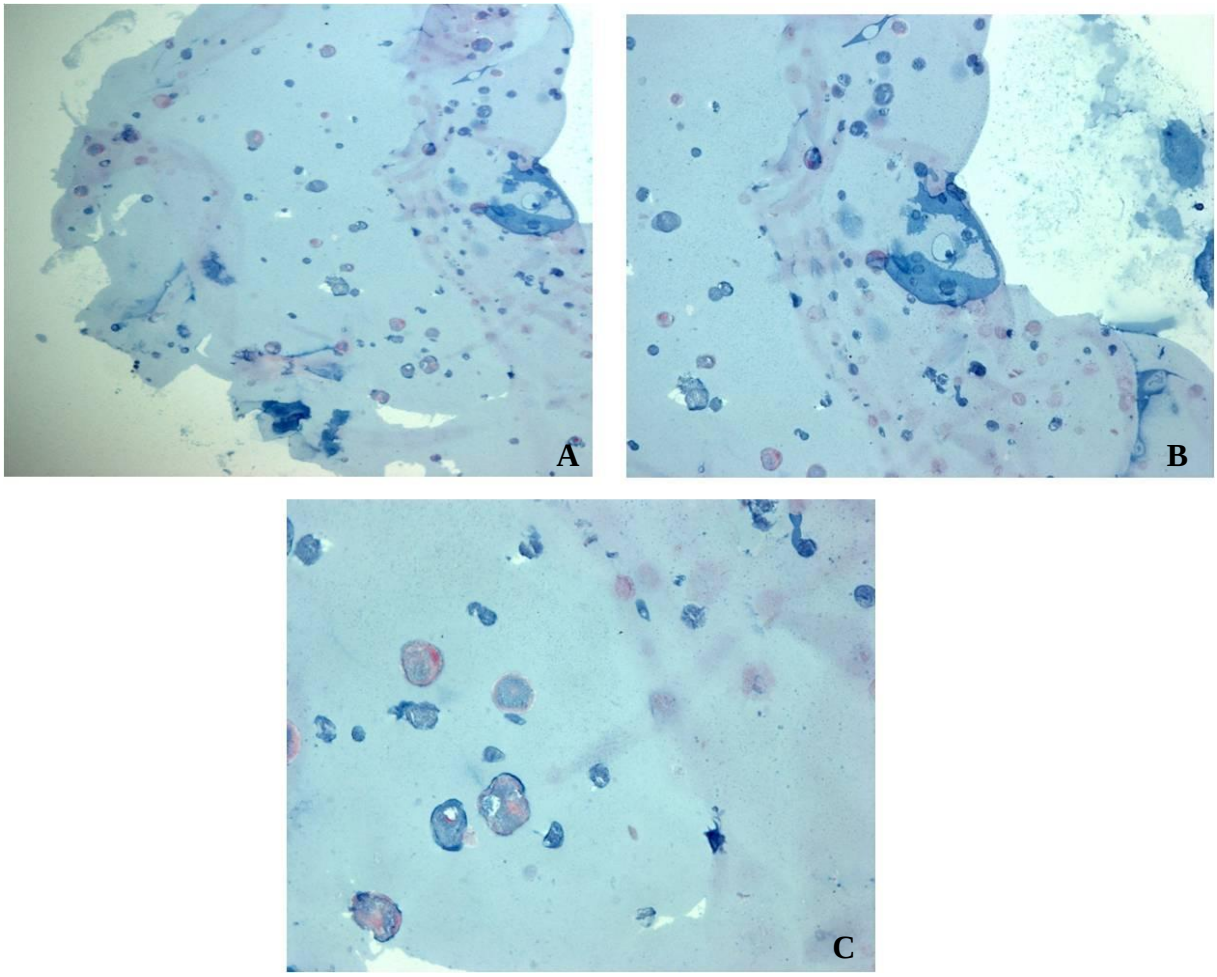


Fig. 4.14 Histochemical evaluation of control cells grown in an alginate hydrogel and standard medium for three weeks and subjected to Alcian Blue staining. Magnification: 20x(A, B), 40x (C).

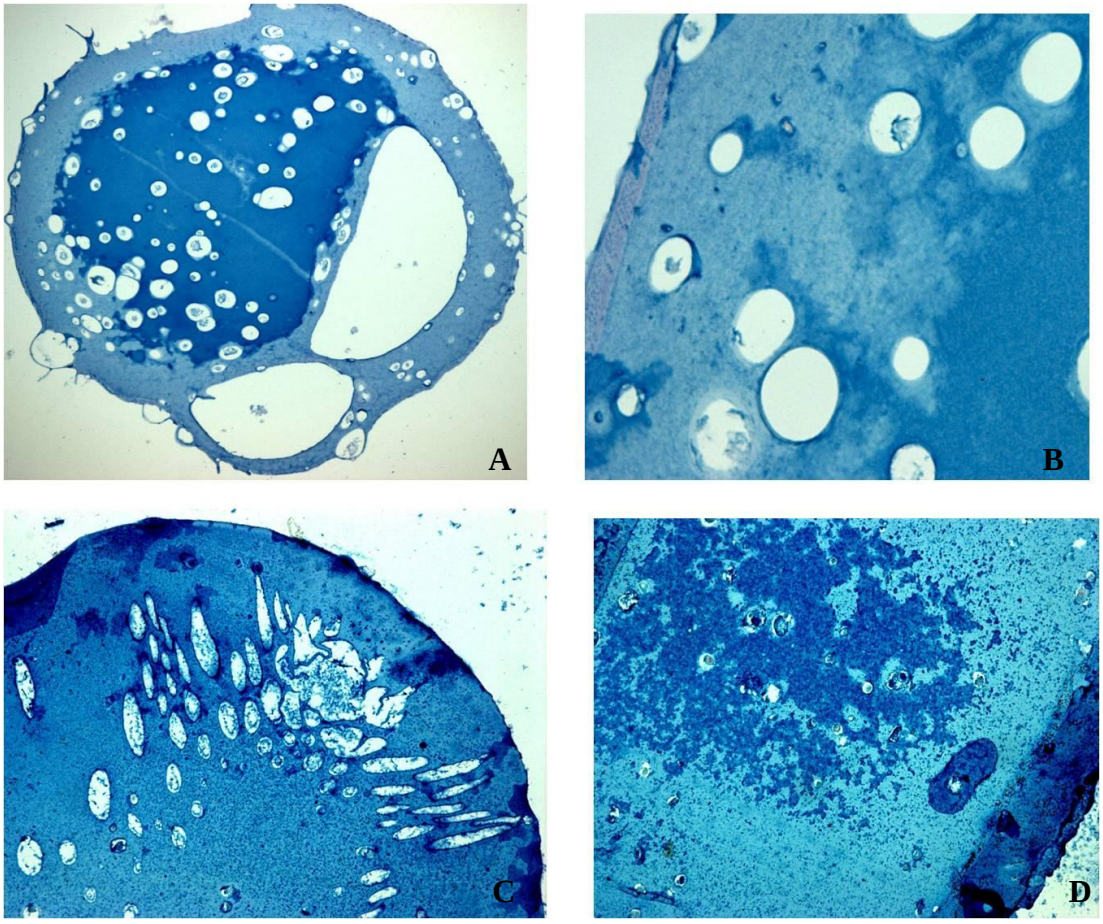


Fig. 4.15 Histochemical evaluation of HSE-MSC grown in an alginate hydrogel and differentiated for three weeks by Alcian Blue staining. Magnification: 10x (A), 20x (C, D) 40x (B).

4.9 Expression of Immunomodulatory molecules by HSE-MSC

Immune modulation is emerging as a key feature of MSC isolated from different sources. These cells in fact, normally do not express type II HLA MHC (major histocompatibility complex) molecules as HLA-DR, while expressing, albeit at low levels, type I HLA MHC molecules (as HLA A,B,C). Data present in literature further extended the range of molecules expressed by MSC, with a potential to modulate the immune response in the host. Between these molecules, a new class of type I HLA (described as non-classical) have been investigated. Therefore, to extend the characterization of HSE-MSC with the analysis of molecules which should have a potential impact in cell therapy approaches, we performed ICC analyses to determine the expression of type I and type II HLA. As visible in figure 4.16, HSE-MSC express type I HLA molecules, albeit at low levels, and do not express HLA-DR (Fig. 4.16 A-B). More interestingly, and we demonstrated this for the first time in heart-derived MSC, our cells did express HLA-E (Fig. 4.16 C), a non-classical type I HLA molecule which has been implicated in immune modulation by these cells for its potential to bind inhibitory receptors on NK cells. This first observation should disclose further potentials of HSE-MSC which, in parallel to their frank multipotency, present a promising HLA setting for in vivo applications.

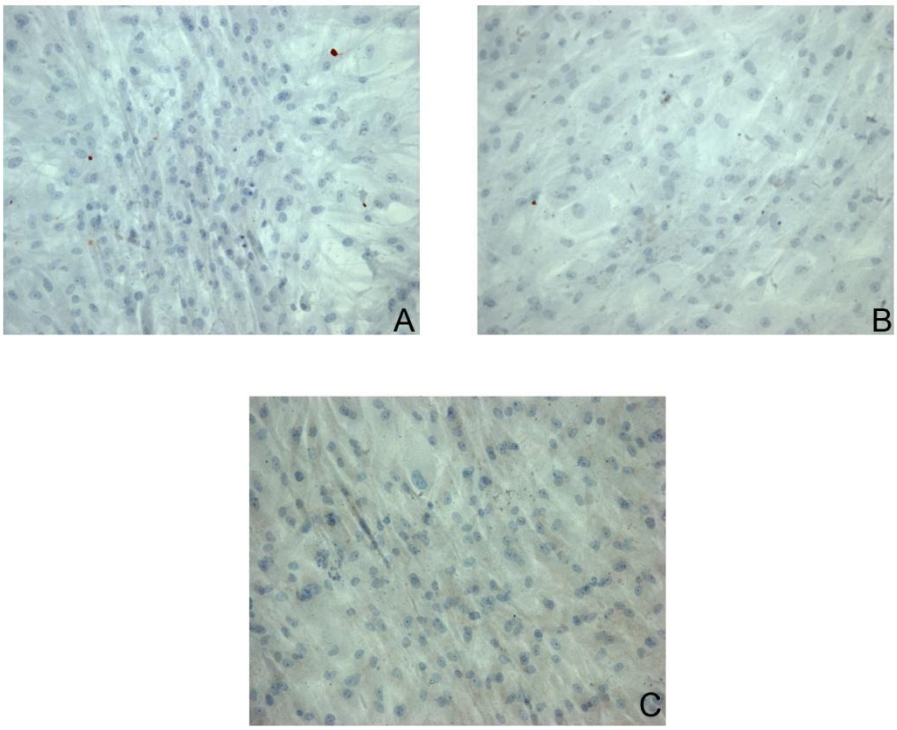


Fig. 4.16 Analysis of the expression of HLA molecules by HSE-MSC at the protein level by ICC. A: HSE-MSC expressed low levels of type I HLA (anti HLA A,B,C). B: HSE-MSC resulted negative for the expression of type II HLA (anti HLA-DR). C: HSE-MSC expressed in nearly 100% of cells the non-classical type I HLA-E. Magnification: 20x.

4.10 Evaluation of myocardiocyte-specific markers in undifferentiated HSE-MSC

Moreover by means of RT-PCR, we evaluated the expression of key marker of myocardiocyte lineage, as **Myl 2 (**Myosin regulatory light chain 2, ventricular/cardiac muscle isoform), **ANF** (atrial natriuretic factor), **Ryr2 (**cardiac ryanodine receptor), which governs the release of Ca2+ from the sarcoplasmic reticulum and initiates muscle contraction, **MYBPC3** that encodes the cardiac isoform of myosin-binding protein C which is a myosin-associated protein found in the cross-bridge-bearing zone (C region) of A bands in striated muscle, **TNNI3,** Troponin I cardiac muscle that is one of 3 subunits that form the troponin complex of the thin filaments of striated muscle, **TNNI3K** that is a new cardiac-specific MAP kinase, it has a role in the cardiac myogenesis process and in the repair of ischemic injury. As visible in table 4.4, undifferentiated HSE-MSC do not show the presence of these cardiac-specific markers. This, in conjunction with the expression of cardiac-specific transcription factors, outlined above, suggests that the expressed transcription factors are maintained in an inactive state in these cells, since no reliable markers of the myocardiocyte phenotype can be detected in undifferentiated cells.

MEF2C	+
Myocardin	+
Nkx2.5	+
GATA-4	+
GATA-6	+

Table 4.4 Evaluation of the expression of key markers of myocardiocyte lineage by HSE-MSCs, assessed by qualitative RT-PCR analyses.

V- Conclusions

The results of the present thesis work showed that HSE-MSC can be successfully isolated from human hearts explanted from CHF patients undergoing transplantation. These cells express the typical markers of other MSC populations and further ones (Oct3/4A, Nanog) which indicate their ability to maintain an undifferentiated state and self-renewal. Moreover, heart-derived MSC (HSE-MSC) express several markers characterized in BM-derived and UC-derived MSC. Interestingly, and differently from BM-MSC, these cells feature a high expression of c-Kit protein.

Another novel datum for mesenchymal stem cells, HSE-MSC showed the expression of heart-specific TFs, which, as demonstrated for GATA-4 by ICC, are probably in an inactive state (i.e. do not undergo nuclear translocation), and therefore in cells kept undifferentiated, transactivation of cardiac-specific genes is prevented.

As multipotent cells, HSE-MSC showed differentiative ability towards both the oasteoblastic, adipocyte and chondrogenic lineages, thus confirming their "mesenchymal" phenotype. The acquisition of the differentiated phenotypes has been demonstrated both by upregulation of lineage-specific genes and tissue-specific histochemical stainings.

In addition, we demonstrated for the first time in such cells the expression of HLA-E, which, together with the absence of HLA-DR and the presence of type I HLA, should suggest a possible immunomodulatory effect for such cells, which should be important when in vivo applications will be performed.

Further work is ongoing to determine their immune features in comparison to other MSC populations. However the presence of such

cells in vivo in a diseased setting as that of post-infarct chronic heart failure shows that also diseased hearts bear stem cellular populations.

Bibliography

Anversa P, Leri A, Rota M, Hosoda T, Bearzi C, Urbanek K, Kajstura J, Bolli R. Concise Review: Stem Cells, Myocardial Regeneration, and Methodological Artifacts. Stem Cells 25: 589-601, 2007.

Anversa P, Torella D, Kajstura J, Nadal-Ginard B, Leri A. Myocardial regeneration. European Heart Journal Supplements 4 (Supplement G): G67-G71, 2002.

Anzalone R, La Rocca G, Di Stefano A, et al. Role of endothelial cell stress in the pathogenesis of chronic heart failure. Frontiers in Bioscience 14: 2238–2247, 2009.

Anzalone R, Lo Iacono M, Corrao S, Magno F, Loria T, Cappello F, Zummo C, Farina F, La Rocca G. New Emerging Potentials for Human Wharton's Jelly Mesenchymal Stem Cells: Immunological Features and Hepatocyte-Like Differentiative Capacity. Stem Cells and Development vol. 19, n. 4: 423-438, 2010.

Anzalone Z, Lo Iacono M, Loria T, Di Stefano A, Giannuzzi P, Farina F, La Rocca G. Wharton's Jelly Mesenchymal Stem Cells as Candidates for Beta Cells Regeneration: Extending the Differentiative and Immunomodulatory Benefits of Adult Mesenchymal. Stem Cells for the Treatment of Type 1 Diabetes. Stem Cell Rev and Rep vol. ND, p. ND, ISSN: 1550-8943, DOI 10.1007/s12015-010-9196-4, 2010.

Banerjef I, Yekkala K, Borg TK, Baudino TA. Dynamic Interaction between Myocytes, Fibroblasts, and Extracellular Matrix. Ann.

N.Y. Acad. Sci, 1080: 76-84, 2006.

Barile L, Messina E, Giacomello A, Marbán E. Endogenous Cardiac Stem Cells. Progress in Cardiovascular Diseases vol. 50, n. 1: 31-48, 2007.

Bartholomew A, Sturgeon C, Siatskas M, Ferrer K, McIntosh K, Patil S, Hardy W, Devine S, Ucker D, Deans R, Moseley A, Hoffmann R. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. Experimental Hematology 30, 42-48, 2002.

Bearzi C, Rota M, Hosoda T, Tillmanns J, Nascimbene A, De Angelis A, Yasuzawa-Amano S, Trofimova I, Siggins RW, LeCapitaine N, Cascapera S, Beltrami AP, D'Alessandro DA, Zias E, Quaini F, Urbanek K, Michler RE, Bolli R, Kajstura J, Leri A, Anversa P. Human cardiac stem cells. PNAS vol. 104, n. 35: 14068-14073, 2007.

Beltrami AP, Barlucchi L, Torella D, Baker M, Limana F, Chimenti S, Kasahara H, Rota M, Musso E, Urbanek K, Leri A, Kajstura J, Nadal-Ginard B, Anversa P. Adult Cardiac Stem Cells Are Multipotent and Support Myocardial Regeneration. Cell vol. 114: 763-776, 2003.

Beltrami AP, Cesselli D, Bergamin N, Marcon P, Rigo S, Puppato E, D'Aurizio F, Verardo R, Piazza S, Pignatelli A, Poz A, Baccarani U, Damiani D, Fanin R, Mariuzzi L, Finato N, Masolini P, Burelli S, Belluzzi O, Schneider C, Beltrami CA. Multipotent cells can be generated in vitro from several adult human organs (heart, liver, and bone marrow). Blood 110: 3438-3446, 2007.

Beltrami AP, Urbanek K, Kajstura J, Yan S, Finato N, Bussani R, Nada-Ginard B, Silvestri F, Leri A, Beltrami A, Anversa P. Evidence that Human Cardiac Myocytes Divide After Myocardial Infarction. N Engl J Med 344: 1750-1757, 2001.

Bonab MM, Alimoghaddam K, Talebian F, Ghaffaru SH, Ghavamzadeh A, Nikbin B. Aging of Mesenchymal stem cell in vitro. BMC Cell Biology 7: 1-7, 2006.

Borg TK, Thompson R, Fitzharris TP, Terracio L, Carver W. Overview: extracellular matrix: Nature vs Nurture in cardiac development. In Clark EB, Nakazawa M, Takao A. Etiology and morphogenesis of congenital heart disease. Armonk, NY, Futura Publishing Co, 2000: 153-162.

Brand T. Heart development: molecular insights into cardiac specification and early morphogenesis. Developmental Biology 258: 1-19, 2003.

Callis TE, Dongsun C, Wang DZ. Bone Morphogenetic Protein Signaling Modulates Myocardin Transactivation of Cardiac Genes. Circulation Research 97: 992-1000, 2005.

Can A, Karahuseyinoglu S. Concise Review: Human Umbilical Cord Stroma with Regard to the Source of Fetus-Derived Stem Cells. Stem Cells 11: 2886-95, 2007.

Chamberlain G, Fox J, Ashton B, Middleton J. Mesenchymal Stem Cells: their Phenotype, Differentiation Capacity, Immunological Features and Potential for Homing. Stem Cells Express July 26 2007: 1-19.

Chien KR, Domian IJ, Parker KK. Cardiogenesis and the Complex

Biology of Regenerative Cardiovascular Medicine. Science vol 322: 1494-1497, 2008.

Chubinskaya S, Huch K, Schulze M, Otten L, Aydelotte MB, Cole AA. Gene Expression by Human Articular Chondrocytes Cultured in Alginate Beads. J Histochem Cytocem 49: 1211-1219, 2011.

Chubinskaya S, Huch K, Schulze M, Otten L, Aydelotte MB, Cole AA. Human Articular Chondrocyrtes Cultured in Alginate Beads Maintain their Gene Expression. Cell and Materials vol. 8: 151-160, 1998.

Corda S, Samuel JL, Rappaport L. Extracellular Matrix and Growth Factors During Heart Growth. Heart Failure Reviews 5: 119.130, 2000.

Cripps RM, Olson EN. Control of Cardiac Development by an Evolutionarily Conserved Transcriptional Network. Developmental Biology 246: 12-28, 2002.

Davani S, Deschaseaux F, Chalmers D, Tiberghien P, Kantelip JP. Can stem cells mend a broken heart?. Cardiovascular Research 65: 305-316, 2005.

Dhein S, Jongsma HJ. Forming the network – gap junction in the cardiovascular system. Cardiovascular Research 62: 225-227, 2004.

Di Nicola M, Carlo-Stella C, Magni M, Milanesi M, Longoni PD, Matteucci P, Grisanti S, Gianni AM. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. Blood 99: 3838-3842, 2002.

Eric N. Gene Regulatory Networks in the Evolution and

Development of the Heart. Science vol 313: 1922-1927, 206.

Frangogiannis NG. The immune system and cardiac repair. Pharmacological Research 58: 88-111, 2008.

Gojo S, Gojo N, Takeda Y, Mori T, Abe H, Kyo S, Hata J, Umezawa A. In vivo cardiovascullogenesis by direct injection of isolated adult mesenchymal stem cells. Experimental Cell Research 288: 51-59, 2003.

Goldsmith EC, Borg TK. The Dynamic Interaction of the Extracellular Matrix in Cardiac Remodeling. Journal of Cardiac Failure vol. 8 n. 6 suppl.: S314-S318, 2002.

Goldsmith EC, Carver W, Borg TK. The role of the extracellular matrix and its receptors modulating cardiac development. In Tomacek R, Runyan RB. Formation of the heart and its regulation. New York, NY, Birkhauser Press, 2001: 147-169.

Goldsmith EC, Hoffman A, Morales MO, Potts JD, Price RL, McFadden A, Rice M, Borg TK. Organization of Fibroblasts in the Heart. Developmental Dynamics 230: 787-794, 2004.

Gray's Anatomy, Elsevier Churchill Livingstone, 2005.

Grépin C, Nemer G, Nemer M. Enhanced cardiogenesis in embryonic stem cells overexpressing the GATA-4 transcription factor. Development 124: 2387-2395, 1997.

Grogan SP, Miyaki S, Asahara H, D'Lima DD, Lotz MK. Mesenchymal progenitor cell markers in human articular cartilage: normal distribution and changes in osteoarthritis. Arthritis Research & Therapy 11: R85, DOI 10.1186/ar2719, 2009.

Hagège AA, Menasché P. Cellular cardiomyoplasty: a new hope in heart failure?. Heart 84: 465-466, 2000.

Hernández-Torres F, Martínez-Fernández S, Zuluaga S, Nebreda Á, Porras A, Aránega, Navarro F. A role for p38α mitogen-activated protein kinase in embryonic cardiac differentiation. FEBS Letters 582: 1025-1031, 2008.

Ivanova NB, Dimos JT, Schaniel C, Hackney JA, Moore KA, Lemischka IR. A Stem Cell Molecular Signature. Science vol. 298: 601-604, 2002.

Javazon E, Beggs KJ, Flake AW. Mesenchymal stem cells: Paradoxes of passing. Experimental Hematology 32: 414-425, 2004.

Kattman S, Adler ED, Keller GM. Specification of Multipotential Cardiovascular Progenitor Cells During Embryonic Stem Cell Differentiation and Embryonic Development. Trends Cardiovascular Med 17: 240-246, 2007.

Kenyon J, Gerson SL. The role of DNA damage repair in aging of adult stem cells. Oxford University Press: ND, 2007.

Klein L, O'Connor CM, Gattis WA, Zampino M, de Luca L, Vitarelli A, Fedele F, Gheorghiade M. Pharmacologic Therapy for Patients with Chronic Heart Failure and Reduced Systolic Function: Review of Trial and Practical Considerations. Am J Cardiol 91 (suppl): 18F-40F, 2003.

Koutsourakis M, Langeveld A, Patient R, Beddington R, Grosveld F. The transcription factor GATA6 in essential for early

extraembryonic development. Development 126: 723-732, 1999.

La Rocca G, Anzalone R, Farina F. The expression of CD68 in human umbilical cord mesenchymal stem cells: new evidences of presence in non-myeloid cell types. Scandinavian Journal of Immunology 70: 161–162, 2009.

La Rocca G, Anzalone R, Corrao S, et al. Isolation and characterization of Oct-4+/HLA-G+ mesenchymal stem cells from human umbilical cord matrix: differentiation potential and detection of new markers. Histochemistry and Cell Biology 131: 267–282, 2009.

La Rocca G, Di Stefano A, Eleuteri E, et al. Oxidative stress induces myeloperoxidase expression in endocardial endothelial cells from patients with chronic heart failure. Basic Research in Cardiology 104: 307–320, 2009.

Laflamme MA, Myerson D, Saffitz JE, Murry CE. Evidence for Cardiomyocyte Repopulation by Extracardiac Progenitor in Transplanted Human Hearts. Circulation Research 90: 634-640, 2002.

Li X, Yu X, Lin Q, Deng C, Shan Z, Yang M, Lin S. Bone marrow mesenchymal stem cells differentiate into functional cardiac phenotypes by cardiac microenvironment. Journal of Molecular and Cellular Cardiology 42(2): 295-303, 2007.

Linke A, Müller P, Nurzynska D, Casarsa C, Torella D, Nascimbene A, Castaldo C, Cascapera S, Böhm M, Quaini F, Urbanek K, Leri A, Hintze TH, Anversa P. Stem cells in the dog heart are self-renewing, clonogenic, and multipotent and regenerate

infarcted myocardium, improving cardiac function. PNAS vol. 102, n. 25: 8966-8971, 2005.

Lips DJ, deWindt LJ, van Kraaij DJW, Doevendans PA. Molecular determinants of myocardial hypertrophy and failure: alternative pathways for beneficial and maladaptive hypertrophy. European Heart Journal 24: 883-896, 2003.

Marcus AJ, Woodbury D. Fetal stem cells from extra-embryonic tissue: Do not discard. J Cell Mol Med 12(3):730-42, 2008.

Martini FH, Timmons MJ, Tallisch RB. Anatomia Umana. Edises 2004.

Martin-Puig S, Zhong W, Chien KR. Lives of a Heart Cell: Tracing the Origins of Cardiac Progenitors. Cell Stem Cell 2: 320-331, 2008.

Murry CE, Reinecke H, Pabon LM. Regeneration Gaps. Observation on Stem Cells and Cardiac Repair. J Am Coll Cardiol 47: 1777-85, 2006.

Nadal-Ginard B, Kajstura J, Leri A, Anversa P. Myocyte Death, Growth, and Regeneration in Cardiac Hypertrophy and Failure. Circ Res 92: 139-150, 2003.

Nah DY, Rhee MY. The Inflammatory Response and Cardiac Repair After Myocardial Infarction. Korean Circ J 39: 393-398, 2009.

Nemer N, Nemer M. Cooperative interaction between GATA5 and NF-ATc regulates endothelial-endocardial differentiation of cardiogenic cells. Development 129: 4045-4055, 2002.

Oh J, Wang Z, Wang DZ, Lien CL, Xing W, Olson EN. Target Gene-Specific Modulation of Myocardin Activity by GATA Transcription Factors. Molecular and Cellular Biology vol. 24, n. 19: 8519-8528, 2004.

Ohnishi S, Nagaya N. Prepare Cells to Repair the Heart: Mesenchymal Stem Cells for the Treatment of Heart Failure. Am J Nephrol 27: 301-307, 2007.

Oka T, Xu J, Molkentin JD. Re-employment of developmental transcription factors in adult heart disease. Seminars in Cell § Developmental Biology 18: 117-131, 2007.

Olson EN, Perry M, Schulz RA. Regulation of Muscle Differentiation by the MEF2 Family of MADS Box Transcription Factors. Developmental Biology 172: 2-14, 1995.

Olson EN. Gene regulatory networks in the evolution and development of the heart. Science vol. 313: 1922-1927, 2006.

Park M, Yaich LE, Bodmer R. Mesodermal cell decisions in *Drosophila* are under the control of the lineage genes *numb*, *Notch*, and *sampodo*. Mechanisms of Development 75: 117-126, 1998.

Parmaceck MS. Myocardin – Not Quite MyoD. Arterioscler Thromb Vasc Biol 24: 1535-1537, 2003.

Păunescu V, Deak E, Herman D, Raluca Siska I, Tănasie G, Bunu C, Anghel S, Tatu CA, Oprea TI, Henschler R, Rüster B, Bistrian R, Seifried E. In vitro differentiation of human Mesenchymal stem cells to epithelial lineage. J. Cell. Mol. Med. Vol. 11, n. 3: 502-508, 2007.

Peterkin T, Gibson A, Loose M, Patient R. The roles of GATA-4, -5 and -6 in vertebrate heart development. Seminars in Cell § Developmental Biology 16: 83-94, 2005.

Peterkin T, Gibson A, Patient R. GATA-6 maitains BMP-4 and Nkx2 expression during cardiomyocyte precursor maturation. The EMBO Journal vol. 22 n. 16: 4260-4273, 2003.

Petit B, Masuda K, D'Souza AL, Otten L, Pietryla D, Hartmann DJ, Morris NP, Uebelhart D, Schmid TM, Thonar EJMA. Characterization of Crosslinked Collagens Synthesized by Mature Articular Chondrocytes Cultured in Alginate Beads: Comparison of Two Distinct Matrix Compartments. Experimental Cell Research 225, 151-161, 1996.

Pikkarainen S, Tokola H, Kerkelä R, Ruskoaho H. GATA transcription factors in the developing and adult heart. Cardiovascular Research 63: 196-207, 2004.

Puymirat E, Geha R, Tomescot A, Bellamy V, Larghero J, Trinquart L, Bruneval P, Desnos M, Hagège, Pucéat M, Menasché P. Can Mesenchymal Stem Cells Induce Tolerance to Cotransplanted Human Embryonic Stem Cells?. Molecular Therapy vol. 17, n. 1: 176-182, 2009.

Quiao C, Xu W, Zhu W, Hu J, Qian H, Yin Q, Jiang R, Yan Y, Mao F, Yang H, Wang X, Chen Y. Human mesenchymal stem cells isolated from the umbilical cord. Cell Biology International 32(1): 8-15, 2008.

Reffelmann T, Kloner RA. Cellular cardiomyplasty–cardioyocytes, skeletal myoblasts, or stem cells for regenerating myocardium and

treatment of heart failure?. Cardiovascular Research 58: 358-368, 2003.

Reiter JF, Kikuchi Y, Stainier YR. Multiple roles for GATA5 in zebrafish endoderm formation. Development 128: 125-135, 2001.

Rizzino A. A Challenge for Regenerative Medicine: Proper Genetic Programming, Not Cellular Mimicry. Developmental Dynamics 236: 3199-3207, 2007.

Rubio D, Garcia S, Paz MF, De la Cueva T, Lopez-Fernandez LA, Lloyd AC, Garcia-Castro J, Bernad A. Molecular Characterization of Spontaneous Mesenchymal Stem Cell Transformation. PLoS ONE 3(1) e1398: 1-15, 2008.

Sarugaser R, Lockorish D, Baksh D, Hosseini MM, Davies JE. Human Umbilical Cord Perivascular (HUCPV) Cells: A Sources of Mesenchymal Progenitors. Stem Cells 23: 220-229, 2005.

Schellings MWM, Pinto YM, Heymans S. Matricellular proteins in the heart: possible role during stress and remodeling. Cardiovascular Research 64: 24-31, 2004.

Schlüter KD, Piper HM. Regulation of growth in the adult cardiomyocytes. The FASEB Journal Supplement vol. 13: S17-22, 1999.

Sotiropoulou PA, Perez SA, Gritzapis AD, Baxevanis CN, Papamichail M. Interaction Between Human Mesenchymal Stem Cells and Natural Killer Cells. Stem Cells 24: 74-85, 2006.

Thowfeequ S, Myatt EJ, Tosh D. Transdifferentiation in Developmental Biology, Disease, and in Therapy. Developmental

Dynamics 236: 3208-3217, 2007.

Tremain N, Korkko J, Ibberson D, Kopen GC, DiGirolamo C, Phinney DG. MicroSAGE Analysis of 2,353 Expressed Genes in a Single Cell-Derived Colony of Undifferentiated Human Mesenchymal Stem Cells Reveals mRNAs of Multiple Cell Lineages. Stem Cells 19: 408-418, 2001.

Troyer DL, Weiss ML. Concise Review: Wharton's Jelly-derived Cells are a Primitive Stromal Cell Population. Stem Cells 26 (3): 591-9, 2008.

Urbanek K, Torella D, Sheikh F, De Angelis A, Nurzynska D, Silvestri F, Beltrami CA, Bussani R, Beltrami AP, Quaini , Bolli R, Leri A, Kajstura J, Anversa P. Myocardial regeneration by activation of multipotent cardiac stem cells in ischemic heart failure. PNAS vol. 102, n. 24: 8692-8697, 2005.

Wang TT, Tio M, Lee W, Beerheide W, Udolph G. Neural differentiation of mesenchymal-like stem cells from cord blood is mediated by PKA. Biochem Biophys Res Commun 357: 1021-1027, 2007.

Warejcka DJ, Harvey R, Jackson Taylor B, Young HE, Lucas PA. A population of Cells Isolated from Rat Heart Capable of Differentiating into Several Mesodermal Phenotypes. Journal of Surgical Research 62: 233-242, 1996.

Wolbert KC, Drexler H. Clinical Applications of Stem Cells for the Heart. Circulation Research 96: 151-163, 2005.

Yokoyama M, Miwa H, Maeda S, Wakitani S, Takagi M. Influence of Fetal Calf Serum on Differentiation of Mesenchymal Stem to

Chondrocytes during Expansion. Journal of Bioscience and Bioengineering vol. 106, n. 1: 46-50, 2008.

Zaffran S, Frasch M, Early Signals in Cardiac Development. Circulation Research 91: 457-469, 2002.

Zeng L, Rahrmann E, Hu Q, Lund T, Sandquist L, Felten M, O'Brien TD, Zhang J, Verfaillie C. Multipotent Adult Progenitor Cells from Swine Bone Marrow. Stem Cells 24: 2355-2366, 2006.

Zuba-Surma EK, Kucia M, Ratajczak J, Ratajczak MZ. "Small Stem Cells" in Adult Tissue: Very Small Embryonic-Like Stem Cells Stand Up!. Cytometry Part A 75A: 4-13, 2009.