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AMNIOTIC FLUID-DERIVED STEMS ISOLATION, CHARACTERIZATION AND DIFFERENTIATION

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1. INTRODUCTION

1.1 Stem cells

Stem cells have been defined as self-renewing cell's populations that through symmetric or asymmetric divisions replicate themselves or differentiate into specialized progeny. Symmetric division gives rise to two identical daughter cells, both endowed with stem cell properties. On the other hand, asymmetric division also called "bivalent mitosis" produces only one stem cell and a progenitor cell with limited self-renewal potential. Progenitors can go through several rounds of cell division before finally differentiating into a mature cell, phenotypically and functionally specialized (Cai et al. 2004). These two types of cell division ensure the maintenance of the stem cell pool of a tissue (Figure1). In developing embryos, stem cells can differentiate into all specialized cell types, while in adult organisms; they act as a repair system for the body, replenishing adult tissues. Therefore, two broad types of stem cells have been found in mammals: embryonic stem cells, which are isolated from the inner cell mass of blastocysts, and adult stem cells, which are found in various tissues. According to the potential to differentiate into different cell types stem cells can be classified as:

Totipotent stem cells: stem cells that can differentiate into embryonic and extraembryonic tissue. They are derived from embryos at the 4-8-cell stage, 1-3 days after fertilization.

Pluripotent stem cells: embryonic stem cells at the blastocyst stage (4-14 days after fertilization). They are able to differentiate into embryonic tissues (ectoderm, mesoderm, and endoderm);

Multipotent stem cells: they can differentiate into a number of cells, but only if derived from the same germ layer. They have the ability to replicate in vitro, but not unlimitedly. Adult stem cells belong to this category.

Unipotent stem cells: they are present in adult tissues, and are able to self-renew and to differentiate into the same cell type of the tissue of belonging, ensuring tissue repair, maintenance and growth throughout life.

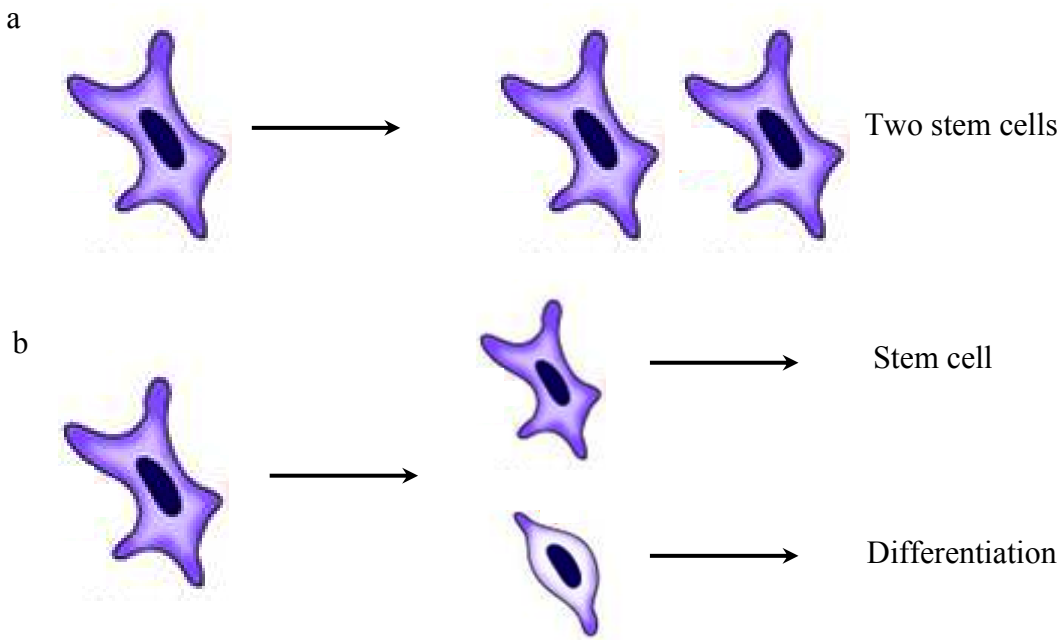

Figure 1: The two types of cellular division of a stem cell. The symmetric division produces two identical stem cells (a), while the asymmetric cell division produces two daughter cells with different cellular fates (b).

1.1.2 Adult stem cells

Adult stem cells are undifferentiated cells found throughout the body after development. They multiply by cell division to replenish dying cells and regenerate damaged tissues. Also known as somatic stem cells, they can be found in juvenile as well as adult animals and human bodies. A series of recent investigations have suggested that adult stem cells might have the ability to differentiate into cell types from different germ layers. According to this hypothesis, neural stem cells from the brain, which are derived from ectoderm, can be tricked to differentiate into ectoderm, mesoderm, and endoderm derived tissue (Clarke et al. 1999); bone marrow stem cells can be differentiated into cells of regenerating organs, such as the liver and the gastrointestinal tract, or even differentiated tissues such as the brain and the heart (Krause et al. 2001). This phenomenon is referred to as stem cell plasticity or transdifferentiation. Significant data supporting stem cell transdifferentiation have come from tissue regeneration studies in mice. It can be induced by the modification of the growth medium where stem cells are cultured in vitro or by transplanting them into an organ of the body different from the one they were originally isolated in vivo. These observations show the regenerative capacity of adult tissues and suggest a large amount of potential applications in regenerative medicine (Krampera et al. 2003) without the ethical problems associated with the use of embryonic stem cells.

1.1.3 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) are not hematopoietic precursors; they were initially isolated from bone marrow as adherent elements, highly proliferating, with self-renewal and multi-lineage differentiation capacity (Friedestein et al. 1976, Jiang et al. 2002) (Figure2). MSCs are adult stem cells that derive from the mesoderm, the middle germ layer, from which originate the connective tissues of the entire organism, and that differentiates at about the third month of gestation (Ambrosi et al. 2001). However, they are able to differentiate not only in tissues of mesenchymal origin, including stromal bone marrow, adipose tissue, bone, cartilage, tendon, skeletal muscle, visceral mesoderm and endothelial cells (Friedestein et al. 1976, Jiang et al. 2002), but also in organs and tissues derived from ectoderm and endoderm such as neuronal and epithelial tissues, digestive tract, liver and lung (Jiang et al. 2002, Reyes et al. 2001, Kotton et al. 2001). Several properties, such the ability of isolating and the high potential for in vitro culture and expansion, make MSCs an interesting resource of stem cells usable in a wide range of clinical applications, in cell and gene therapy and in regenerative medicine (Minguell et al. 2001). The first determined source of MSCs in adult individuals was bone marrow, where they are immersed in the stroma (Pittinger et al. 1999). In bone marrow, they are present at a low frequency and recent studies, employing the CFU-F assay, suggest that in humans there is one MSC every 34,000 nucleated cells (Wexler et al. 2003). Afterwards, MSCs have been found in several other tissues and in ontogeny (Table 1). They have been isolated from brain, thymus, liver, spleen, kidney, muscle and lungs.

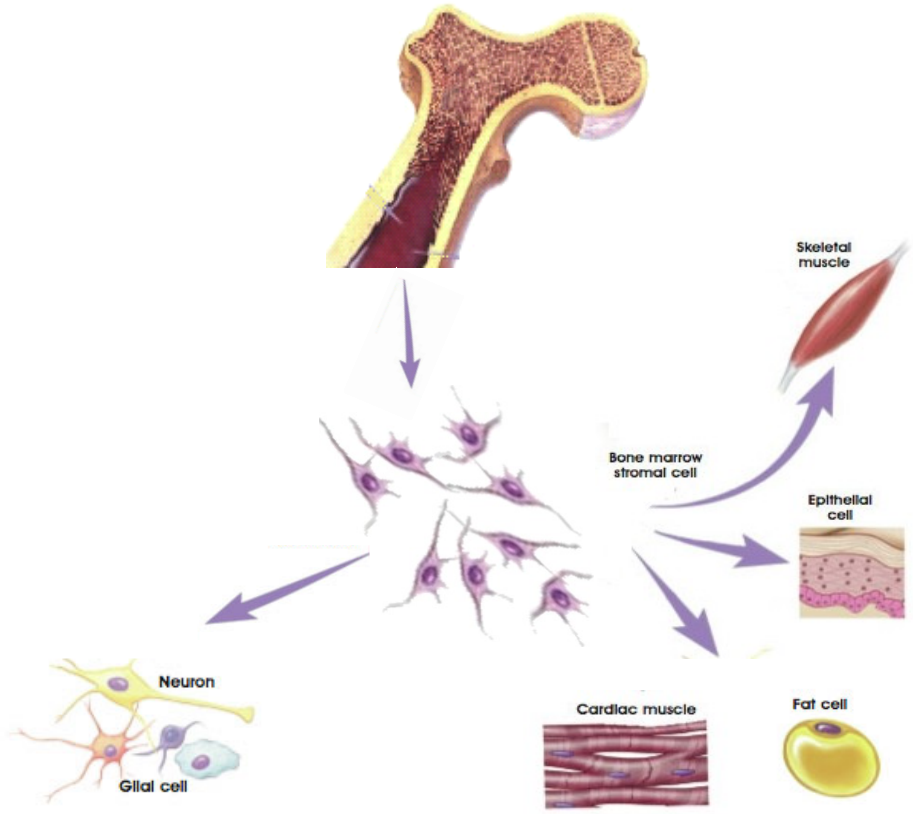

Figure 2: Multi-lineage differentiation capacity of MSCs. MSCs isolated from bone marrow are able to differentiate in tissues of mesenchymal origin such as adipose tissue, skeletal and cardiac muscle, but also in tissues derived from ectoderm and endoderm such as neuronal and epithelial tissues.

Site	Ontogenetic Stage	Reference
Adipose tissue	Postnatal	Zuk et al. 2001, 2002
Pancreas	Fetal	Hu et al. 2003
Bone marrow	Fetal	Campagnoli et al. 2001
Liver	Fetal	Campagnoli et al. 2001
Blood	Fetal	Campagnoli et al. 2001
Amniotic liquid	Fetal	In' t Anker et al. 2003
Peripheral blood	Postnatal	Zvaifler et al. 2000
Umbilical cord blood	Fetal/Postnatal	Alfonso et al. 2000

Table 1

Mesenchymal stem cells are morphologically characterized by a small cell body with a few cell processes that are long and thin. The cell body contains a large, round nucleus with a prominent nucleolus, which is surrounded by finely dispersed chromatin particles, giving the nucleus a clear appearance. The remainder of the cell body contains a small amount of Golgi apparatus, rough endoplasmic reticulum, mitochondria and polyribosomes. Phenotypic characterization of MSCs must still be investigated due the lack of a specific marker for the analysis and isolation of MSCs (Campioni et al. 2003). In fact, since MSCs have no unique distinctive markers, they are identified through the analysis of complex immunophenotypic patterns, which includes the lack of antigens, typical of hematopoietic stem cells, such as CD45, CD34 and CD14, and the expression of a series of surface molecules, such as CD90, also called Thy-1, CD105 or endoglin (Barry et al. 1999), CD29 or β subunit of the receptor for fibronectin, CD44 or receptor-III of the extracellular matrix and others. These markers result uniformly and strongly expressed on MSCs isolated from tissues of different origins (Majumdar et al. 2003, Vogel et al. 2003) and they are maintained after in vitro expansion. During the last few years, isolations of adult mesenchymal stem cells from different sources have been reported, including adult and birth-associated tissues.

1.2 Different sources of MSCs

Although most of the published data refer to cell cultures obtained from bone marrow, there is an increasing number of publications on MSCs obtained from alternative sources such as adipose tissue, umbilical cord and fetal tissues. Cells derived from different tissues show phenotypic heterogeneity and different growth abilities, but they also show similarities, like the potential to differentiate into the classical mesenchymal lineages and the expression of common surface markers (Baksh et al. 2004). The amounts and characteristics of mesenchymal stem cells, which can be obtained by these isolations, vary enormously, as explained in the sub-paragraphs below.

1.2.1 MSCs from Bone Marrow

MSCs were first found in bone marrow. In 1970, Friedenstein and co-workers demonstrated that, after placing whole bone marrow cells in plastic culture dishes with medium supplemented with 10% fetal calf serum, bone marrow contained hematopoietic non-adherent cells along with a rare population of plastic-adherent cells. These cells were able to form colonies derived from a single cell. After a few days, these adherent cells were able to differentiate into mature cells of mesenchymal lineages, such as osteoblasts (Friedenstein et al. 1970, Friedenstein et al. 1976). The initial clones of adherent cells expanded into round-shaped colonies composed by fibroblastoid

cells, thus the term of Colony Forming Unit – fibroblasts (CFU-f). Friedenstein and collaborators also discovered that some of the colonies could differentiate into aggregates, resembling small areas of bone or cartilage. Then, other research groups extended these initial observations, studying CFU-f proliferative abilities and phenotypic characteristics (Castro-Malaspina et al. 1980, Prockop 1997, Caplan 1991), and it was established that these cells were multipotential and could differentiate, under appropriate conditions, into osteoblasts, chondrocytes, adipocytes, and even myoblasts. Each bone marrow donor shows a specific frequency of CFU-f, which is dependent on the age and health of the donor. The current categorization of these cells as mesenchymal stem cells (MSCs) was proposed by Caplan (1991), based on their ability to differentiate into cells of the mesenchymal lineages. In addition, several studies show their ability to differentiate into cells of endodermal origin (hepatocytes, pneumocytes) and ectodermal origin (nerve cells, glial cells) (Jiang et al. 2002, Koc et al. 2000). However, two main issues limit the use of MSCs from bone marrow: the invasive removal procedure from the bone and the small number of stem cells obtained from each sample (Harvey et al 2007). For this reason, the search for new sources of MSCs is an important goal of research.

1.2.2 MSCs from umbilical cord blood

The umbilical cord blood is an excellent source of hematopoietic stem cells for allogeneic transplantation. Compared to the bone marrow, it presents a greater percentage of CD34+ cells, suggesting that in neonatal blood may be present progenitors with proliferative and differentiative potential. Some researchers have hypothesized that in the umbilical cord could be also present mesenchymal progenitors. In 2004, Bieback and collaborators have demonstrated that it is possible to isolate elements similar to MSCs (MSC-like cells) from cord blood. These cells are present with a lower frequency in the blood of the umbilical cord than in bone marrow but show a higher proliferative capacity.

1.2.3 MSCs from adipose tissue

The adipose tissue is a rich and accessible source of adult stem cells, consisting of a pluripotent cell population able to differentiate into cells of various tissues of mesodermal line (cells of the bone tissue, adipose, cartilage, heart and muscle) and not of the mesodermal line (neuron-like cells, endothelial cells, hepatocytes, pancreatic cells). MSCs extracted from adipose tissue show plasticity similar to mesenchymal stem cells extracted from bone marrow. Generally, the MSCs from adipose tissue have a population doubling time of 2-4 days, depending on the age of the donor, the location

(omental fat or subcutaneous fat), the type of surgical procedure, the culture conditions, the seeding density and, the composition of the culture medium (Gronthos et al. 2001).

1.2.4 MSCs from Amniotic fluid

Amniotic fluid is known to contain multiple cell types derived from the developing fetus (Priest et al. 1978, Polgar et al. 1989). Thus, human amniotic fluid has been used in prenatal diagnosis for a number of years. Through its screening and the cells contained within it, a variety of genetic and developmental disorders of the fetus can be diagnosed. When cells derived from amniotic fluid are isolated, the resultant culture consists of a heterogeneous cell population. Most cells in the fluid are terminally differentiated (epithelial lineages) and have limited proliferation and differentiation capabilities (von Koskull et al. 1981, Medina-Gomez and Johnston 1982). Previous studies have noted an interesting composition of the fluid consisting of a heterogeneous population that expresses markers from all three germ cell layers (Cremer et al. 1981). For this reason, many researches have been conducted on the source of these cells and on the fluid itself. Current theories suggest that the fluid is largely derived from the fetus's urine and peritoneal fluid, as well as from some ultrafiltrate belonging to the plasma of the mother entering through the placenta. The cells in the fluid have been shown to be coming from the fetus, and are thought to be mostly cells sloughed off the epithelium, digestive and urinary tracts of the fetus, in addition to the amnion (Brace et al. 1989). Recent studies have shown that it is possible to isolate from amniotic fluid a novel type of stem cell, which could be maintained in an undifferentiated state in culture for long periods and can be induced to differentiate into many different cell types (De Coppi et al., 2007a). Although stem cells can be isolated from many sources, including bone marrow, fetal and embryonic tissues, amnion-derived stem (AFS) cells are an attractive source of stem cells because of many logistical and ethical advantages. Harvesting these cells poses minimal risk of harming the fetus; in fact, AFS cells can be isolated from amniocentesis around 15–20 weeks of gestation. AFS cells isolated during an earlier phase of pregnancy have a higher proliferative capacity (Antonucci et al. 2011, Manuelpillai et al. 2011, Yu et al. 2009) and their versatility and plasticity properties fall somewhere in between the pluripotent embryonic and the multipotent adult stem cells (De Coppi et al. 2007a, Mauro et al. 2010). Analysis of stem cell markers shows that the AFS cells express the human embryonic stage-specific surface marker SSEA4 and the embryonic stem cell marker OCT4, both of which are typical of the undifferentiated state of ES cells. They also express mesenchymal and neuronal stem cell markers (CD29, CD44, CD73, CD90, and CD105), but they do not express SSEA1, SSEA3, CD4, CD8, CD34, CD133, C-MET, ABCG2, NCAM, BMP4, TRA1- 60, or

TRA1-81, to name a few (De Coppi et al. 2007a). This expression profile is of interest as it demonstrates that AFS cells have some key markers in common with the ES cell phenotype. Therefore, it is important to emphasize that AFS cells, which are not as primitive as ES cells, have greater potential than most adult stem cells. Moreover, these cells do not form teratomas in vivo when implanted into immunodeficient mice (Thomson et al. 1996). Lastly, the AFS cells expanded from a single cell maintain similar properties in growth and potential as the original mixed population of progenitor cells (Atala, 2009; Table 2).

Site	ES cells	MSCs	AFS cells
Source	Early stage embryo	Bone marrow and other adult tissues	Amniotic fluid
Feeder cells	Required	Not required	Not required
Markers	SSEA3/4, OCT3/4 SOX2	CD44, CD90, CD105 and others	SSEA4, OCT4, c-kit, CD44, CD105 and others
Plasticity	Pluripotent	Multipotent	Broadly multipotent
Teratoma formation	Yes	No	No
Doubling time (h)	31-57	Variable	30-36
Lifespan in vitro	Long	Short	Long
Ethical issues	Yes	No	No
Clinical trials	No	Yes	No

Table 2

Additionally, AFS cells have a high renewal capacity and can be expanded for over 250 doublings without any detectable loss of chromosomal telomere length. Telomere length conservation in the undifferentiated state, as well as telomerase activity, even in late passages, has been demonstrated. AFS cells have also been shown to maintain a normal karyotype at late passages, and have normal G1 and G2 cell cycle checkpoints. The AFS cell's population doubling time is approximately 30–36 hours (De Coppi et al. 2007a) and can differentiate into multiple lineages (Fauza et al. 2004, In't Anker et al. 2003, McLaughlin et al. 2006, Prusa et al. 2002, Tsai et al. 2004, Tsai et al. 2006). All

these data provide support to the notion that the amniotic fluid is a rich and promising source of stem cells for clinical applications.

1.3 Potential clinical use of MSCs

Much of the excitement surrounding human stem cells is connected with the hope of clinicians and patients that these cells can be used for cell therapies for a wide spectrum of human diseases. The objective of tissue engineering is to reconstruct tissues or organs in order to replace damaged and injured parts of the body (Atala et al. 2007, Raya-Rivera et al. 2011). Its major approaches are tissue-specific cells, biomaterials (also called a scaffolding system) and an appropriate environment for promoting tissue maturation/formation. A well balanced combination of these components can allow the engineering of a tissue construct in vitro and further facilitate the development of functional tissue substitutes following in vivo implantation (Langer and Vacanti 1993). Recently, a broad and multidisciplinary field termed "regenerative medicine" emerged through the integration of tissue engineering, stem cell biology, material sciences, developmental biology and molecular biology. The goal of regenerative medicine is to create functional tissues or organs to be used in repair or replacement procedures in patients based on clinically relevant approaches (Atala et al. 2007). However, these cell-based approaches are often limited by the availability of an appropriate cell source and current technologies are based mostly on the use of tissue-specific primary cells taken from the patient's own body. Even though these autologous cells are nonimmunogenic, they tend to be poorly proliferative and to easily lose their phenotype in culture. Consequently, most researchers have recently focused on stem cells as an alternative cell source for these cell-based approaches, because stem cells capability of differentiating to multiple lineages would be valuable for a wide range of therapies. The generation of ES cell lines through the use of human embryo raises a variety of ethical issues, with different approaches from country to country, while the use of adult stem cells in research and therapy is not considered as controversial. From a tissue engineering perspective, the most extensive MSC research has been in the area of bone regeneration. Ectopic bone formation has been generated by subcutaneous implantation of MSCs-seeded scaffolds in vivo (De Coppi et al. 2007a). By 18 weeks after implantation, highly mineralized tissues and blocks of bone-like material were observed in the recipient mice using micro-CT. These blocks displayed a density somewhat greater than the one of the mouse femoral bone. This indicated that MSCs cells could be used to engineer bone grafts for the repair of bone defects. Human MSCs cells can be induced toward osteoblastic differentiation by bone morphogenic protein 7 (BMP-7; Sun et al. 2010). Nanofiber scaffolds, which mimic the

morphology of natural collagen fibers, facilitated osteogenic differentiation of human MSCs in vitro and bone formation in vivo (Peister et al. 2009). Moreover, these cells have the potential to differentiate along the chondrogenic lineage, thus establishing the feasibility of using these cells for cartilage tissue engineering applications. Transplantation of MSCs cells has been explored in neurological disorders (Antonucci et al. 2011, Manuelpillai et al. 2011, Yu et al. 2009). Under standard neuronal induction protocols for stem cells, MSCs posses preferential dopaminergic phenotypic commitment, making them a potentially valuable source of stem cells to treat Parkinson's disease. In a model of acute necrotizing injury of the urinary bladder, MSCs cells were transplanted for the treatment of the impaired detrusor muscle contractility resulting from the injury (De Coppi et al.2007b). MSCs transplanted into cryo-injured bladders formed a few small smooth muscle bundles in the detrusor muscle and gave rise to limited vasculogenesis. Some MSCs cells underwent cell fusion. However, it appears that the major effect of MSC transplantation in this model was preventing cryo-injury induced hypertrophy of the surviving smooth muscle cells via an as yet un- known paracrine mechanism. Human MSCs cells are able to integrate into murine lung and differentiate into lung-specific lineages after injury. After microinjection into cultured mouse embryonic lungs, MSCs can integrate into the epithelium and express the early human differentiation marker thyroid transcription factor 1 (TTF1). In adult nude mice exposed to hyperoxia, tail vein-injected MSCs localized in the distal lung and expressed both TTF1 and type II pneumocyte marker surfactant protein C. These results suggest a certain level of plasticity of MSCs cells which allows them to respond in different ways to different types of lung damage by expressing specific alveolar versus bronchiolar epithelial cell lineage markers, depending on the type of injury of the recipient lung (Carraro et al. 2008). There are several papers that describe the importance of indirect support of tissue regeneration through the secretion of biological factors by MSCs (Lee et al. 2011). A recent study (Mirabella et al. 2011) showed that conditioned medium (CM) from AFS cells cultures contained pro-angiogenic soluble factors, such as monocyte chemotactic protein (MCP)-1, interleukin (IL)-8, stromal- derived factor (SDF)-1 and vascular endothelial growth factor (VEGF). When injected into mice of a hind-limb ischemic model, CM prevented capillary loss and muscle tissue necrosis, and later induced neo-arteriogenesis and remodeling of pre-existing collateral arteries. This study asserts that stem cell-secreted factors can recruit endogenous stem and progenitor cells to induce efficient tissue repairs.

1.4 MSCs in heart diseases

Cardiomyopathies are the leading cause of death in the Occident. Loss of cardiomyocytes due to myocardial infarction or hereditary cardiomyopathies may represent causative factors in the progression toward heart failure. Several research groups are exploring the possibility of using stem cells to restore myocardial function in damaged hearts. There is growing enthusiasm for the application of MSCs-based therapies to repair or regenerate damaged myocardium. In mice, bone marrow-derived stem cells have been found to enhance functional recovery in infarcted hearts (Orlic et al. 2001), suggesting that the use of these cells may contribute to the regeneration of myocardial tissue. In fact, MSCs engraft, has the potential for myocyte differentiation (Pittenger et al. 1999, Makino et al. 1999), and releases cytokines and growth factors stimulating endogenous repair mechanisms (Tang et al 2001, Kucia et al. 2004). Furthermore, MSCs have several properties that support their ability to evade rejection (Bartholomew et al. 2002, Tse et al. 2003). In this regard, MSCs lack surface expression of key costimulatory molecules such as B7-1 and B7-2 (Majundar et al. 2003; Le Blanc et al. 2004) and they may also directly inhibit inflammatory responses. Additionally, human MSCs have been found capable to express vascular endothelial growth factor (Makino et al. 1999, Tang et al. 2004) and a broad spectrum of arteriogenic cytokines promoting in vitro and in vivo vasculogenesis through paracrine mechanisms (Kucia et al. 2004, Bartholomew et al. 2002). These effects, which are referred to as trophic effects, have been recently proposed as a major contribution to the therapeutic potential of MSCs in experimental models of myocardial infarction and dilated cardiomyopathy (Majundar et al. 2003). Therefore, MSCs may serve as an allogeneic graft, an extraordinary therapeutic advantage for this cell type. In these studies, human MSCs of bone marrow (BMhMSCs) were used. The use of bone marrow-derived cells, despite the advantages, is potentially associated with high degree of viral infection and significant decline in cell viability and differentiation with age. However, although human MSCs may encompass cells committable to cardiovascular lineages, it is well established that cardiac differentiation is an extremely low yield process even in ES cells. So MSCs pre-differentiated into cardiomyocytes transplanted into infarcted rat hearts result in enhanced cardiovascular repair (Ventura et al. 2006). Hence, affording high-throughput of cardiogenesis in human MSCs isolated from different sources would have obvious therapeutic potential.

1.5.1 Molecules with differentiating properties: hyaluronic, butyric, and retinoic acids

Numerous types of molecules are mainly essential during the biological processes, driving an organism to grow and develop. These molecules are able to diffuse through all cell membranes and

to carry signals regulating cellular differentiation programs. Molecules with differentiating properties are developed by the researchers in order to highlight their potential in the modification of gene expression program induced by chemical signals. The use of molecules with direct or synergistic action offers a new approach in tissue engineering and regenerative cell therapy thus avoiding possible nonspecific actions related to traditional methods such as "gene transfer". On the basis of the mechanisms of cell signaling knowledge, a mixture of hyaluronic (HA), butyric (BU), and retinoic (RA) acids has been recently investigated. This mixture demonstrated to act as a specific agent to promote cardiogenic differentiation (Ventura et al. 2007, Cavallari et al. 2012).

1.5.2 Hyaluronic acid

Hyaluronic acid (also called Hyaluronan or hyaluronate or HA) is an anionic, nonsulfated glucosaminoglycan consisting of repeating disaccharide units of D-glucuronic acid and D-N-acetylglucosamine with alternately glycosidic bonds β1,4 and β1,3 (Figure3). It is distributed widely throughout connective, epithelial, and neural tissues. In the solid state it has a helical configuration, while in aqueous solution assumes a conformation "random coil" that exposes along the chain, hydrophilic groups with a sequences of relatively hydrophobic residues (axial hydrogen atoms) that probably mediate interactions with cell membranes and hydrophobic proteins (Hascall, 1997). HA is the main constituent of the extracellular matrix of differentiated tissues and in the early stages of development forms a matrix similar to a highly hydrated gel that provides the space and the plasticity required for the rapid proliferation of embryonic cells. It is therefore extremely important not only for the maintenance of the biophysical properties and tissue homeostasis, but also for the participation in various morphogenetic events. Hyaluronan is synthesized by a class of integral membrane proteins called hyaluronan synthases, of which vertebrates have three types: HAS1, HAS2, and HAS3. These enzymes lengthen hyaluronan by repeatedly adding glucuronic acid and N-acetylglucosamine to the nascent polysaccharide as it is extruded via ABC-transporter through the cell membrane into the extracellular space (Shulz et al. 2007). The number of disaccharide units that constitute the polysaccharide can reach 10,000 units and 4 millions Daltons of molecular weight. It was shown that chains of hyaluronic acid with different molecular weights have different roles, and act differently in the processes of cellular signal transduction. The discovery that different HAS isoforms are able to synthesize hyaluronic acid with different average lengths, establishes an additional level of regulating cell physiology.

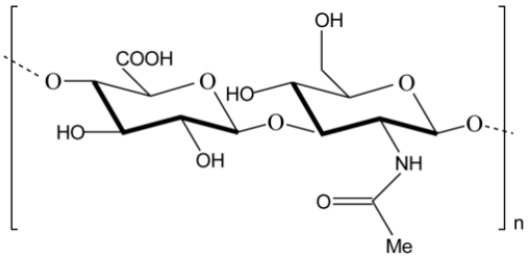

Figure 3: Hyaluronic Acid chemical structure. Hyaluronic Acid is a polymer of a disaccharide, composed by D-glucuronic acid and D-N-acetylglucosamine, linked via alternating β-1,4 and β-1,3 glycosidic bonds. Hyaluronan can be 25,000 disaccharide repeats in length. Polymers of hyaluronan can range in size from 5,000 to 20,000,000 Dalton in vivo.

The membrane receptor of hyaluronic acid is CD44, a monomeric transmembrane protein, highly glycosylated, encoded by a single gene but expressed as several isoforms due to alternative splicing. The simplest form widely distributed throughout the body is called standard form (CD44s) and comprises an extracellular region, a transmembrane single-elix region and a cytoplasmic region (Figure 4). The extracellular region of CD44s consists of two domains: the domain responsible for the binding of the hyaluronate (link module) and the domain closest to the membrane, which may have different isoforms. The cytoplasmic region may undergo phosphorylation of serine residues and contains the reasons of protein interaction with the cytoskeleton proteins (such as the ERM and the ankirine families) and for potential interactions with the intracellular signaling. The interaction of hyaluronic acid with the CD44 leads to internalization of the complex, through invagination of the membrane with a molecular mechanism (not yet identified) probably similar to endocytosis, which leads to the formation of clathrin coated vesicles; the formed endosome is routed to the lysosomal compartment due to molecular motors associated with the cytoskeleton leading to the subsequent degradation of the vesicular content (Tammi et al. 2001). Hyaluronate is able to interact with cellular processes by interacting with a specific protein called ialaderine, which translocate into the nucleus intervening in the MAP kinase pathway (Zhang et al. 1998). Therefore, hyaluronic acid is able to interact with cellular processes both through a CD44 dependent pathway, either through the interaction with other transmembrane or intracellular receptors; these characteristics have also led researchers to investigate the potential of HA as carrier to vehicular other molecules within the cell.

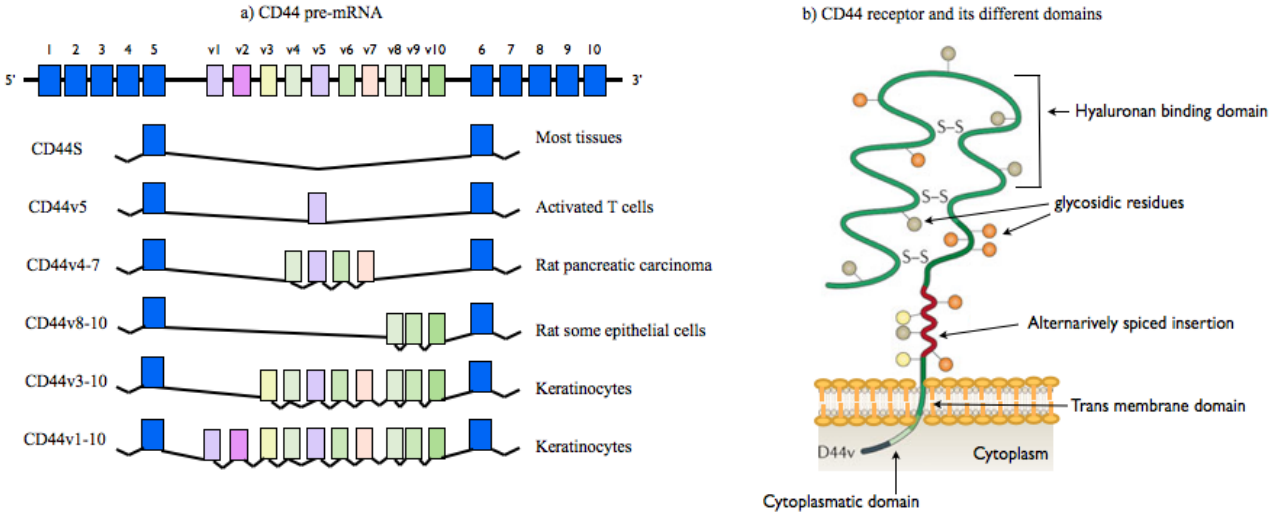

Figure 4: CD44 receptor. (a) A representation of the alternatively spliced CD44 isoforms, which range in size. Blue segments numbered from 1 to 5 and from 6 to 10 represent exons that encode for the constant region of the protein, while the colored segments numbered from v1 to v10 can be included or not in the mature mRNA due to alternative splicing and encode the variable region of the protein; b) CD44 is a monomeric transmembrane protein. Its structure comprises an extracellular region, highly glycosylated, with the domain responsible for the binding of the hyaluronate and the domain near the membrane that may have different isoforms. The cytoplasmic region may be phosphorylated and can interact with the cytoskeleton.

1.5.3 Butyric acid

Butyric acid (BA), also known under the systematic name of butanoic acid, is a carboxylic acid with the structural formula $CH_3CH_2CH_2COOH$ capable of modulating a variety of fundamental cellular processes, it belongs to the class of histone deacetylase inhibitors (HDACi), potent inducers of the arrest of cell growth, differentiation and apoptosis in transformed cells (Coradini et al. 1997). The mechanism of action of these molecules is based on the ability to inhibit the activity of histone deacetylase (HDAC), which plays an antagonistic role compared to the histone acetyltransferase (HAT) and together are involved in gene expression regulation by modulating the degree of acetylation of histones. Histones are a family of basic proteins (H1, H2A, H2B, H3, H4) present in eukaryotic nuclei, which are one of the primary elements of structural organization of chromatin. The five types of histone proteins are rich in basic amino acids charged positively, that interact with the negatively charged phosphate groups of DNA. In fact, histones, which constitute the core protein of a nucleosome (a higher-order DNA structure organized by histones), contain a flexible amino-terminal sequence, consisting of 20-40 amino acid residues, which extend from their globular domains with several lysine residues. These positively charged lysine residues are exposed to reversible reactions of acetylation and deacetylation; in acetylated form the positive charge of the amino group of a lysine residue is neutralized and therefore the interaction of the N termini of

histones with the negatively charged phosphate groups of DNA is decreased. Consequently, high level of acetylation of the histones amino terminations transform the condensed chromatin into a more relaxed structure that is associated with greater levels of gene transcription. This relaxed structure of chromatin can be reversed by HDAC activity. The transcriptionally inactive genes are typically located in areas of more compact chromatin (heterochromatin); in this context the structural organization of chromatin constitutes an important level of endogenous regulation of gene expression that can be modulated, for example, through the use of substances that inhibit the HDAC activity (Figure 5).

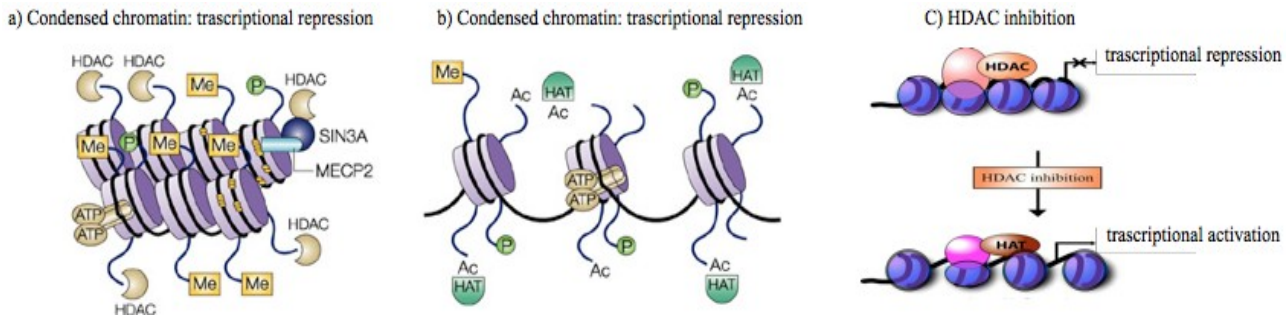

Figure 5: HDAC inhibitors general action. The histones are acetylated and deacetylated on lysine residues in the N-terminal tail as part of gene regulation. These reactions are typically catalyzed by enzymes with "histone acetyltransferase" (HAT) or "histone deacetylase" (HDAC) activity (a and b). Histone deacetylase inhibitors (HDAC inhibitors, HDI) are a class of compounds that interfere with the function of histone deacetylase (c).

The sodium butyrate (NaB) is an HDAC inhibitor approved for clinical use and in particular as a antineoplastic agent; the inhibition of HDAC results in the accumulation of hyperacetylated nucleosome core histones leading to transcriptional activation of some genes including those responsible for the control of growth and cell differentiation (Marks et al. 2001). However, due to its rapid cell metabolism (short half-life) the sodium butyrate must be used at high concentrations and does not constitute an ideal pharmacological agent. Coradini and coworkers demonstrated the efficacy as an antitumor agent of an ester of hyaluronic acid with butyric acid (HB) in the treatment of hepatocellular carcinoma. In this study, it was showed that HB has efficacy 10 times greater than NaB in vitro, suggesting that the use of hyaluronic acid as a carrier can significantly increase the biological activity of butyrate. The same study has confirmed that HB determines an inhibition of liver metastases formation in mouse models (Coradini et al. 2004). Further studies on the effect of sodium butyrate on murine embryonic carcinoma cells showed that the hypoacetylation of histones

induces the activation of the heterodimer RAR/RXR, promoting the processes of cell differentiation (McCue et al. 2005).

1.5.4 Retinoic acid

Retinoic acid (RA; Figure 6) is one of the most important biologically active derivatives of vitamin A. Retinoids function as important signal molecules for the regulation of cell growth and differentiation, during embryogenesis and adult organisms (Summerbell et al. 1990). Retinoic acid acts (Figure 7) by interacting with the retinoic acid receptor (RAR), binding to DNA as a heterodimer with the retinoid X receptor (RXR) in regions called retinoic acid response elements (RAREs). The binding of the retinoic acid ligand to RAR alters the conformation of the RAR, which affects the binding of other proteins that either induce or repress transcription of a nearby gene (including Hox genes and several other target genes). Retinoic acid receptors mediate transcription of different sets of genes by controlling the differentiation of a variety of cell types, thus the target genes regulation depends upon the target cells. In some cells, one of the target genes is the gene for the retinoic acid receptor itself (RAR-beta in mammals), which amplifies the response (Wingender, 1994). The retinoic acid levels are controlled by a suite of proteins, responsible for its synthesis and degradation (Duester 2008, Holland et al. 2007).

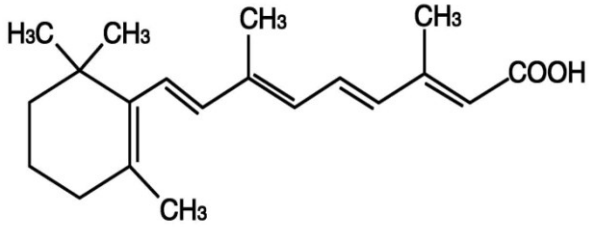

Figure 6: Retinoic Acid chemical structure. Retinoic acid is a metabolite of vitamin A (retinol) that mediates its functions required for growth and development.

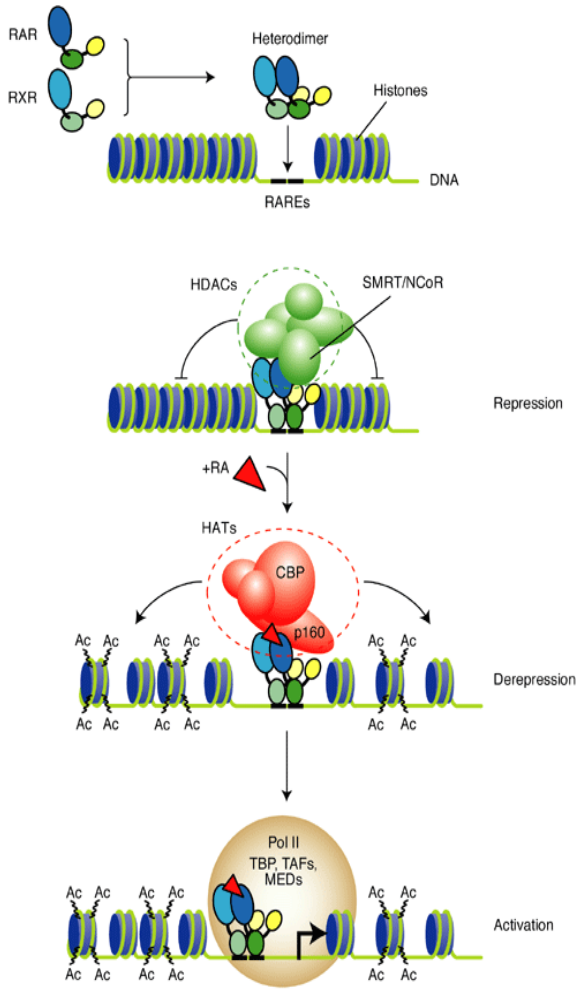

Figure 7: Retinoic acid mechanism of action. Retinoic acid exerts its molecular actions through RAR and RXR nuclear receptors. These receptors belong to the superfamily of nuclear hormone receptors and act as ligand-activated transcription factors of several genes by binding to specific retinoic acid response elements (RAREs).

1.5.5 Mixture of Hyaluronic, Butyric, and Retinoic acids

The integrated action of a mixture of Hyaluronic, Butyric, and retinoic acids (HA+BU+RA) suggests the differentiative logic which drives these molecules: Retinoic acid exerts its differentiating action at the nuclear level, facilitated by the decondensing action on the chromatin of Butyrate; in this context Hyaluronic acid acts as a carrier for the internalization of the molecule and for its conveyance at nuclear level.

2. AIM OF THE PROJECT

The existence of stem cells in human amniotic fluid was reported for the first time almost ten years ago (De Coppi et al., 2007a). Since this discovery, the knowledge concerning these cells has increased considerably. Today, amniotic fluid stem cells are widely accepted as a new powerful tool for basic research as well as for the establishment of new stem-cell-based therapy concepts. It is possible to generate genomically stable AFS cells lines harboring high proliferative potential without raising ethical issues and many different groups have demonstrated that AFS cells can be differentiated into all three germ layer lineages (In ’t Anker et al. 2003, Karlmark et al. 2005, Bossolasco et al. 2006, De Coppi et al. 2007a), which is of primary relevance for their scientific and therapeutic uses. Of special importance for the latter is the fact that AFS cells are less tumorigenic than other pluripotent stem cell types (Rosner et al. 2011, Kim et al. 2010, De Sacco et al. 2010). The use of MSCs pre-differentiated into cardiomyocytes transplanted in infarcted rat hearts results in successful cardiovascular repair (Ventura et al. 2007). Hence, affording high-throughput of cardiogenesis in human MSCs should have obvious therapeutic potential. Moreover, new molecules with differentiating properties are now being investigated by researchers in order to highlight their potential in the modification of gene expression program induced by chemical signals.

The aim of this project is to evaluate the capacity of AFS cells to differentiate into cardiac and vascular lineages. For this purpose the AFS cells were extracted from amniotic fluid, derived from amniocentesis, through immunoselection techniques, cultured in vitro, and characterized by flow cytometry. Subsequently, AFS cells were inducted to differentiate through the stimulation with a mixture of hyaluronic, butyric, and retinoic acids. Differentiation will be evaluated by the analysis of the cells phenotype and the correlation of specific mRNA and protein levels.

3. MATERIALS AND METHODS

3.1 Isolation of AFS cells

Amniotic fluid samples from women at 15-18 weeks of gestation were provided by the Operative Unit of Medical Genetics at the University of Sassari. For each sample, 2–3 ml of amniotic fluid, were centrifuged for 10 minutes at 1800 rpm. Pellets were resuspended in Alpha-MEM supplemented with 15% FBS, 1% L-glutamine, 20% Chang Medium (Irvine Scientific) and antibiotics, then incubated at 37°C in a humidified 5% CO_2 incubator. After 3 days, non-adherent cells were removed and the adherent cells allowed to growth in the same medium, which was changed every 3 days. When culture reached confluence, cells were immunoselected using an anti-c-kit antibody conjugated with magnetic beads and MACS Cell Separation kit (Mltenyi Biotec) according to the manufacturer's instruction.

3.2 Cell culture and differentiation

AFS cells were grown in tissue culture flasks in presence of Alpha-MEM, supplemented with 15% FBS, 1% L-glutamine, 20% Chang Medium (Irvine Scientific) and antibiotics (Growth Medium, GM). To induce cardiac differentiation, cells were plated into special plates (Costar ultra low attachment clusters) containing the culture medium added with HA 2 mg/ml, BU 5mM and RA 0,1 mM. After 3 days of culture, the embryoid bodies (EBs) were plated into tissue culture multiwell plates.

3.3 Immunoblotting

Cell were lysated in lysis buffer (20 mM Tris HCl pH 8; 137 mM NaCl; 10% glycerol 1% Nonidet P-40; 2 mM EDTA; Protease Inhibitor Cocktails), and the protein concentration was determined with Nanodrop (Thermo Scientific). The protein extracted (60μg) was resolved with Novex® NuPAGE® SDS-PAGE Gel System (Invitrogen) using NuPAGE® 4–12% Bis-Tris Gel (Invitrogen) and transferred to a nitrocellulose membrane using iBlot® 7-Minute Blotting System (Invitrogen). The blots were blocked with TBS-T containing 5% non-fat dry milk. The protein levels were detected with the following antibody: anti-α-sarcomeric actinin (Sigma-Aldricht), anti-GATA4 (Santa Cruz), anti-Nkx2.5 (Santa Cruz) anti-dynorphin B (Abcam), anti-MYH (Santa Cruz) and anti-von Willebrand factor (-vWF) (Abcam). Equal loading was controlled with anti-GAPDH (Santa Cruz). Antibodies were used in TBS-T containing 3% non-fat dry milk. Anti-mouse

(1:10,000) and anti-rabbit (1:10,000) peroxidase conjugated (Santa Cruz) and ECL Western blotting detection reagents (Amersham Biosciences) were used for detection.

3.4 Gene Expression

Total RNA was isolated using Trizol reagent according to the manufacturer's instruction (Invitrogen). RNA was dissolved in RNAase-free water and, for RT-PCR cDNA was synthesized in a 50-µl-reaction volume with 1 µg of total RNA and MuMLV reverse transcriptase (RT) according to the manufacturer's instruction (Invitrogen). Quantitative real-time PCR was performed using the iCycler Thermal Cycler (Bio-Rad). Two µl of cDNA were amplified in 50-µl reactions using Platinum Supermix UDG (Invitrogen), 200 nM of each primer, 10 nM of fluorescein (BioRad) and Sybr Green. After an initial denaturation step at 94°C for 10 min, temperature cycling was initiated. Each cycle consisted in 94 °C for 15 s, 55–59 °C for 30 s and, 60 °C for 30 s, the fluorescence being read at the end of this step. All primers used in this study were from Invitrogen. To evaluate the quality of the product resulting from real-time PCR assays, melting curve analysis was performed after each assay. Relative expression was determined using the ''delta-delta-CT method'' with GAPDH as reference gene. Each data point was obtained from at least three independent experiments.

3.5 Immunostaining

For immunofluorescence analysis, cells were washed with PBS and fixed with 4% formaldehyde at room temperature for 1 h. After fixation, cells were washed three times with PBS and permeabilized with 0.2% Triton X-100 at room temperature for 15 min. Non-specific antibody binding sites were blocked by incubating with 1% bovine serum albumin for 1 h at 37°C. Then, cells were labeled at 4°C O/N with anti-α-sarcomeric actinin antibody (Sigma-Aldrich), anti-MYH antibody (Santa Cruz) and with anti-vWF antibody (Abcam). Cells were then stained with FITC-conjugated anti-rabbit or anti mouse IgG. All microscopy analysis was performed with a Leica confocal microscope (Leica TCS SP5). DNA was visualized with Propidium Iodide (1 ug/ml) or DAPI (1 mg/ml).

3.6 Flow Cytometry Analysis

Flow Cytometry Analysis was used for AFS cells characterization. Cells were harvested by treatment with 0.08% trypsin EDTA, after centrifugation and the supernatant was discarded. AFS cells were stained with 1μ/10^6 cells of FITC or PE-conjugated antibodies for 40 min at 4°C in the dark. The used antibodies were anti-CD44 (BD Biosciences), anti-CD29 (BD Biosciences), anti-CD73 (BD Biosciences), anti-CD105 (BD Biosciences), anti-CD90 (BD Biosciences) anti-CD31 (BD Biosciences), anti-CD34 (BD Biosciences) and, anti-CD45 (BD Biosciences). After washing, cells were analyzed on a flow cytometer (BD Acuri C6 Flow Cytometer) by collecting 10,000 events, and the data were examined using the BD Accuri C6 software (BD Biosciences). Flow cytometry analysis was also exploited to assess the percentage of AFS cells, expressing α-sarcomeric actinin and von Willebrand factor. Cell pellets were collected and, after a fixation/permeabilization step using the FIX & PERM® Fixation and Permeabilization Kit (Invitrogen), AFS cells were incubated with a primary antibody directed against α-sarcomeric actinin (Sigma-Aldrich) or vWF (Abcam) for 1 h at 4°C. Then the pellets were resuspended in 100 μL of permeabilization buffer and 10 μL of secondary antibody (50 μg/mL FITC-conjugated goat anti-rabbit or anti-mouse IgG antibody, SANTA CRUZ) was added. The samples were kept in the dark, at room temperature for 30 min and, after washing; cells were analyzed on a flow cytometer (BD Acuri C6 Flow Cytometer) by collecting 10,000 events. Data were analyzed using the BD Accuri C6 software (BD Biosciences).

4. RESULTS

4.1 Isolation and characterization of AFS cells

Amniotic fluid samples were obtained from pregnant women. 2–3 ml of amniotic fluid were centrifuged and the cell pellets were re-suspended in Growth medium and placed at 37°C in a humidified 5% CO_2 incubator. After 1 day, non-adherent cells were removed allowing the adherent cells to grow with fresh medium. When culture reached confluent cells were immunoselected using an anti-c-kit antibody, conjugated with magnetic beads and the MACS® Cell Separation kit (Mltenyi Biotec). MACS technology is based on MACS MicroBeads, MACS Separators, and MACS Columns. MACS MicroBeads are superparamagnetic particles of approximately 50 nanometers in diameter coupled to a specific antibody. They are composed by a biodegradable matrix, and it is therefore not necessary to remove them from cells after the separation process. Immunoselection takes place within MACS Columns. When a MACS Column is placed in a MACS Separator, a strong magnetic field is induced on the column matrix, strong enough to retain cells labeled with minimal amounts of MACS MicroBeads. Unlabeled cells pass through while labeled cells are released after removal of the column from the magnet. Thus both labeled and unlabeled cell fractions could be easily isolated with high purity. For the positive selection an anti-c-kit antibody coupled to magnetic MicroBeads was used. Therefore, immunoselected c-kit positive cells were cultured and expanded in vitro (Figure 8).

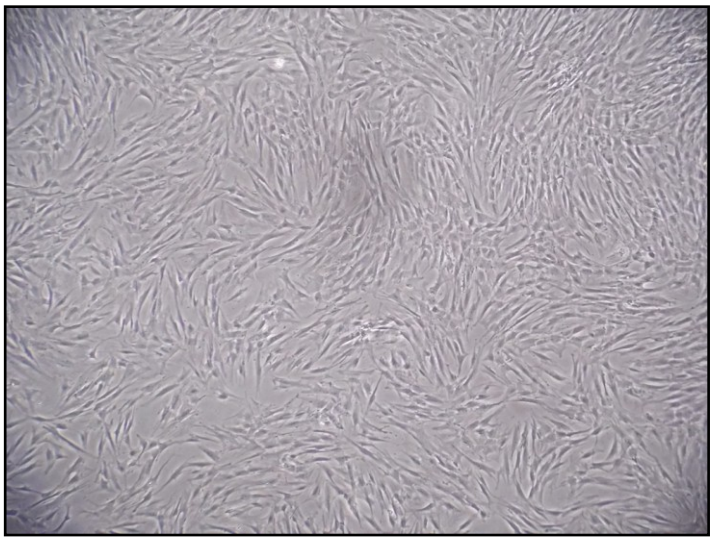

Figure 8: Morphology of AFS cells after immunoselection and expansion in vitro. AFS cells morphology was imaged with an optical microscope (10X enlargement). A small cell body with a few cellular processes indicates that AFS cells are similar to MSCs.

Phenotypic characterization of AFS cells was assessed trough flow cytometry. We observed the expression of a complex immunophenotype, which includes the expression of a series of surface hMSC markers, such as CD29 or CD44 and the lack of antigens typical of hematopoietic stem cells, such as CD45 and CD34 (Figure 9) MSCs surface markers resulted uniformly and strongly expressed on AFS cells and maintained after in vitro expansion, while the hematopoietic markers were absent. The table below (Table 3) summarizes the data from three independent experiments and shows the percentage of positive and negative cells for each marker. A percentage close to 100% of AFS cells expresses MSCs markers (CD44, CD29, CD105, CD73 and CD90), whereas hematopoietic markers are not expressed (CD45, CD31 and CD34). All these data indicated that we succeeded in isolating from amniotic fluid a highly pure population of stem cells with a mesenchymal profile.

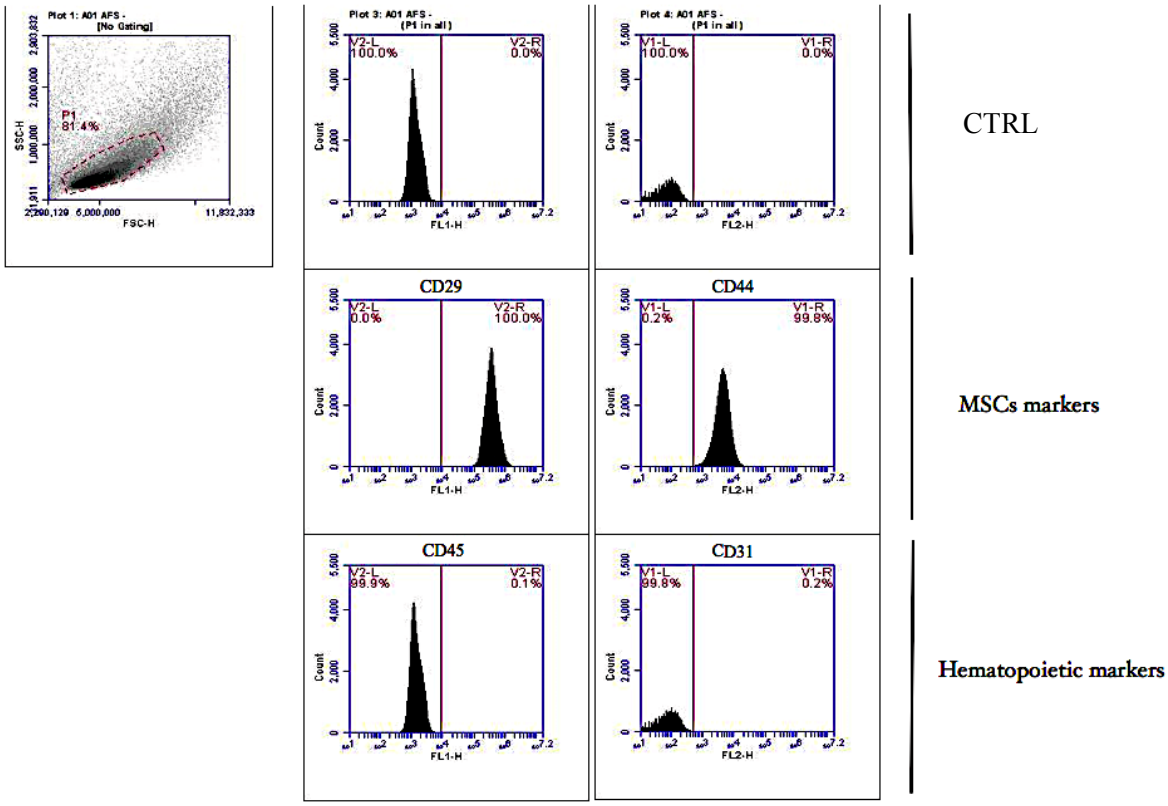

Figure 9: Flow cytometry analysis of CD29, CD44, CD45 and CD31 markers in AFS cells. Cells were stained with fluorescently tagged antibodies specific for the marker of interest. The samples labeled with anti-MSCs markers (CD29 and CD44) show an increase of fluorescence compared to the sample stained with anti-hematopoietic markers (CD45 and CD31). Unstained samples were used as a negative control.

Antigen	Percentage of positive AFS cells
CD44	96,8% ± 3,1%
CD29	97,8% ± 1,8%
CD105	96% ± 3,2%
CD73	95,2% ±4%
CD90	94% ± 3%
CD45	0%
CD31	0%
CD34	0%

Table 3

4.2 The synergistic action of hyaluronic, butyric and retinoic acids drives the gene expression of Nkx2.5, GATA4 and dynorphin B in AFS cells

A hyaluronic, butyric and retinoic acid mixture (HA+BU+RA) was administered to AFS cells for 10 days then the cardiomyogenesis was verified by the evaluation of cells phenotype (figure 10) and the determination of specific mRNA and proteins expression. After 1 day from the induction of cardiomyogenic, differentiation cells formed embryoid bodies that become more numerous after 2 and 3 days. At the 7^{th} day the cells showed an elongated morphology with numerous small protrusions of the cytoplasmic membrane and cellular connection more clearly visible at the 10th day of differentiation.

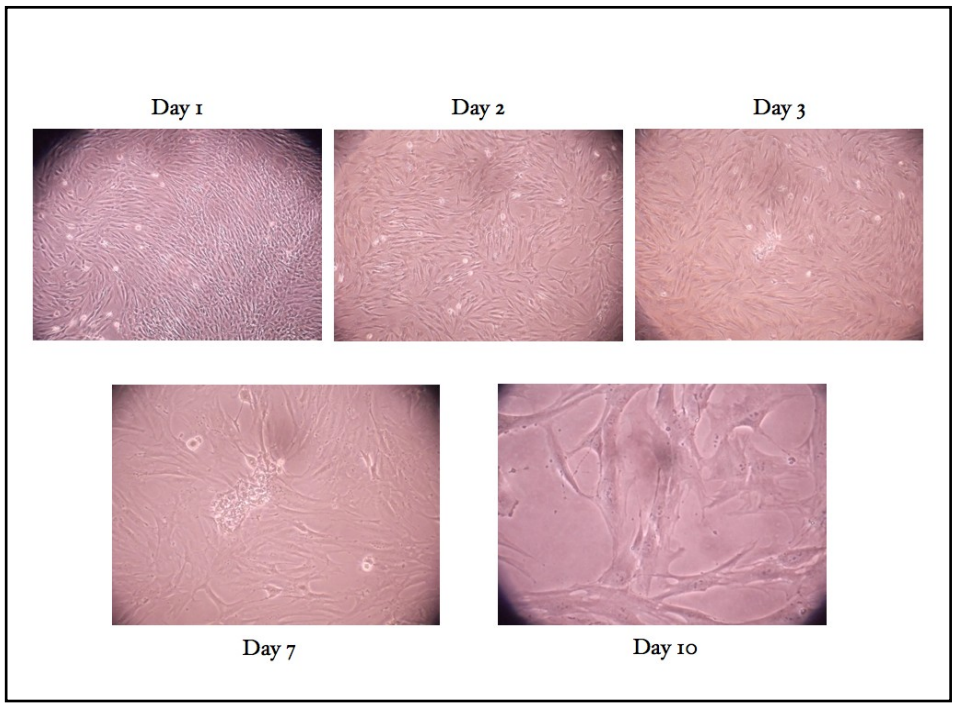

Figure 10: Differentiation of AFS cells toward cardiomyogenic lineages. AFS cells grown to 95% confluence and induced to differentiate by HA+BU+RA administration for 10 days were imaged with an optical microscope (10X enlargement). In the first 3 days from the induction of cardiomyogenic differentiation, cells formed embryoid bodies. At the 7th day, the AFS cells showed an elongated morphology. Finally at the 10th day, cellular connections are clearly visible.

Gene and protein expressions were investigated by real-time PCR and western blotting at different times of AFS cells culture in the absence or presence of HA+BU+RA. RT-PCR analysis revealed that cell treatment with HA+BU+RA, used at a concentration previously shown to manage a maximal cardiogenic response (HA 2 mg/ml, BA 5mM and RA 0,1 mM; Ventura et al. 2004; Ventura et al. 2007; Cavallari 2012), remarkably increased GATA-4, Nkx-2.5 and prodynorphin expression (Figure 11). In fact, after 24 hours of culture, following HA+BU+RA treatment, AFS cells consistently exhibited extremely higher gene and protein expression levels of GATA4, Nkx2,5 and prodynorphin compared to untreated cells. Also, after 48 and 72 hours, in HA+BU+RA-treated cells the expression of GATA4, Nkx2,5 and prodynorphin further increased. The expression of Nkx2.5 and prodynorphin declined after 72 hours of culture, while GATA4 expression was retained high even after 10 days in culture. GATA4 and Nkx2.5 are respectively a zinc finger-containing transcription factor and a homeodomain shown to be essential for cardiogenesis in different animal species (Biben et al 1997; Lints et al. 1993), including humans (Benson et al. 1999). HA+BU+RA also enhanced prodynorphin mRNA expression and the levels of dynorphin B, a natural κ opioid receptor agonist. This finding is particularly important because the prodynorphin gene and dynorphin B primed GATA-4 and Nkx-2.5 transcription (Ventura et al. 2003) and triggered protein

kinase C (PKC) signaling through complex subcellular redistribution patterning of targeted PKC isozymes, one of the major requirement for stem cells commitment to the cardiac lineage (Ventura et al. 2003). Additionally, dynorphin B acted as an agonist of nuclear opioid receptors coupling nuclear PKC activation to the transcription of cardiogenic genes, indicating that intracrine signals for cardiac differentiation may also be fashioned by the prodynorphin gene and its related peptides (Ventura et al. 2003). These results confirm that HA+BU+RA activates the cardiomyogenic program in AFS cells driving the expression of cardiac lineage-promoting genes.

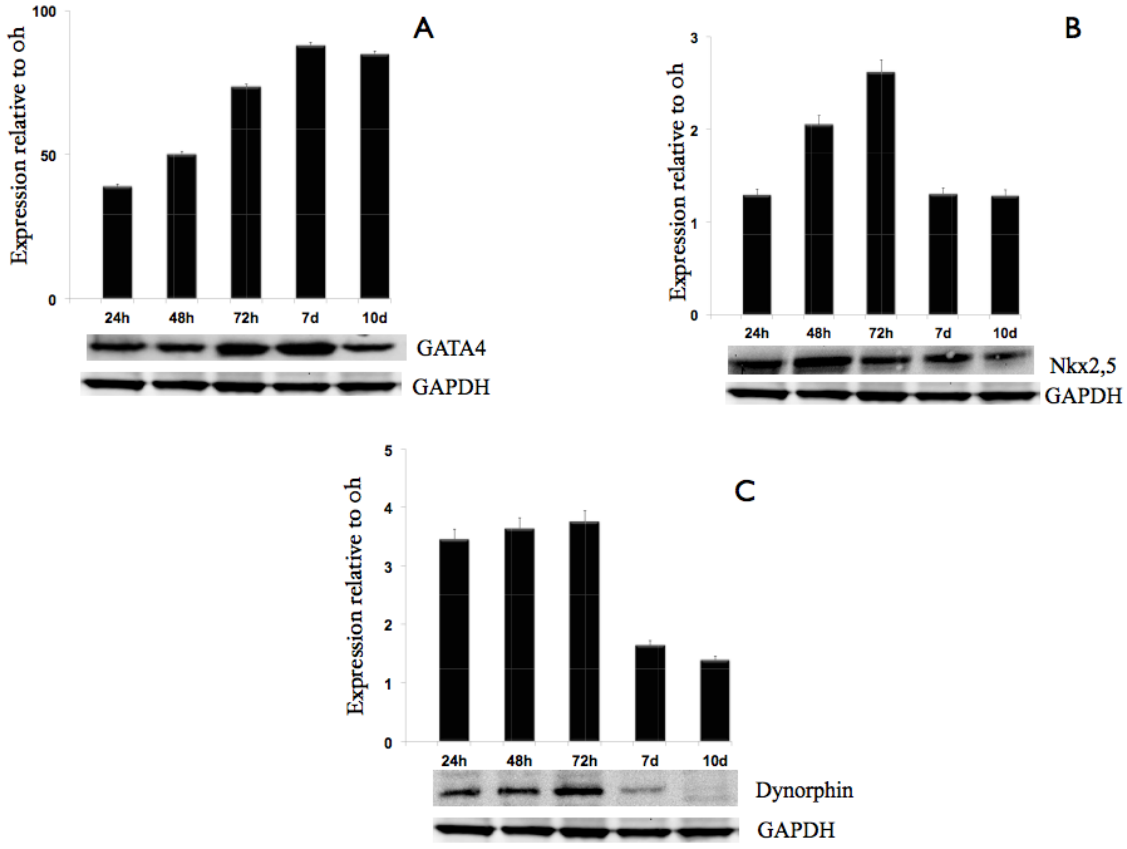

Figure 11: A mixture of HA+BU+RA triggers the expression of cardiac lineage-promoting transcripts. AFS cells were treated for 10 days with HA+BU+RA. GATA-4 (A), Nkx-2.5 (B) and prodynorphin (C) mRNA expression levels were measured by real-time PCR. The reported data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. For the immunoblotting analysis the protein levels were detected with the followed antibody: anti-GATA4, anti-Nkx2.5 and anti-dynorphin. Equal loading was controlled with anti-GAPDH.

4.3 A mixture of Hyaluronic, butyric and retinoic acids affords a high throughput of cardiogenesis in AFS cells

In our study, we assessed the ability of HA+BU+RA to regulate the intracellular patterning of cardiac marker proteins at the intact cell level. To this end, immunofluorescence analysis was performed in AFS cells committed toward cardiac lineage in the absence or presence of the mixed compounds. As shown in figure 12, HA+BU+RA elicited a remarkable increase in the fluorescent staining for sarcomeric actin and MYH, when compared to unexposed cells. Cardiac lineage commitment was further inferred from the observation that HA+BU+RA exposure was associated with a sarcomeric-like organization of sarcomeric actinin staining. These observations further validate that, following the activation of the cardiomyogenic program; AFS cells cells exposed to HA+BU+RA are able to differentiate toward the cardiac phenotype.

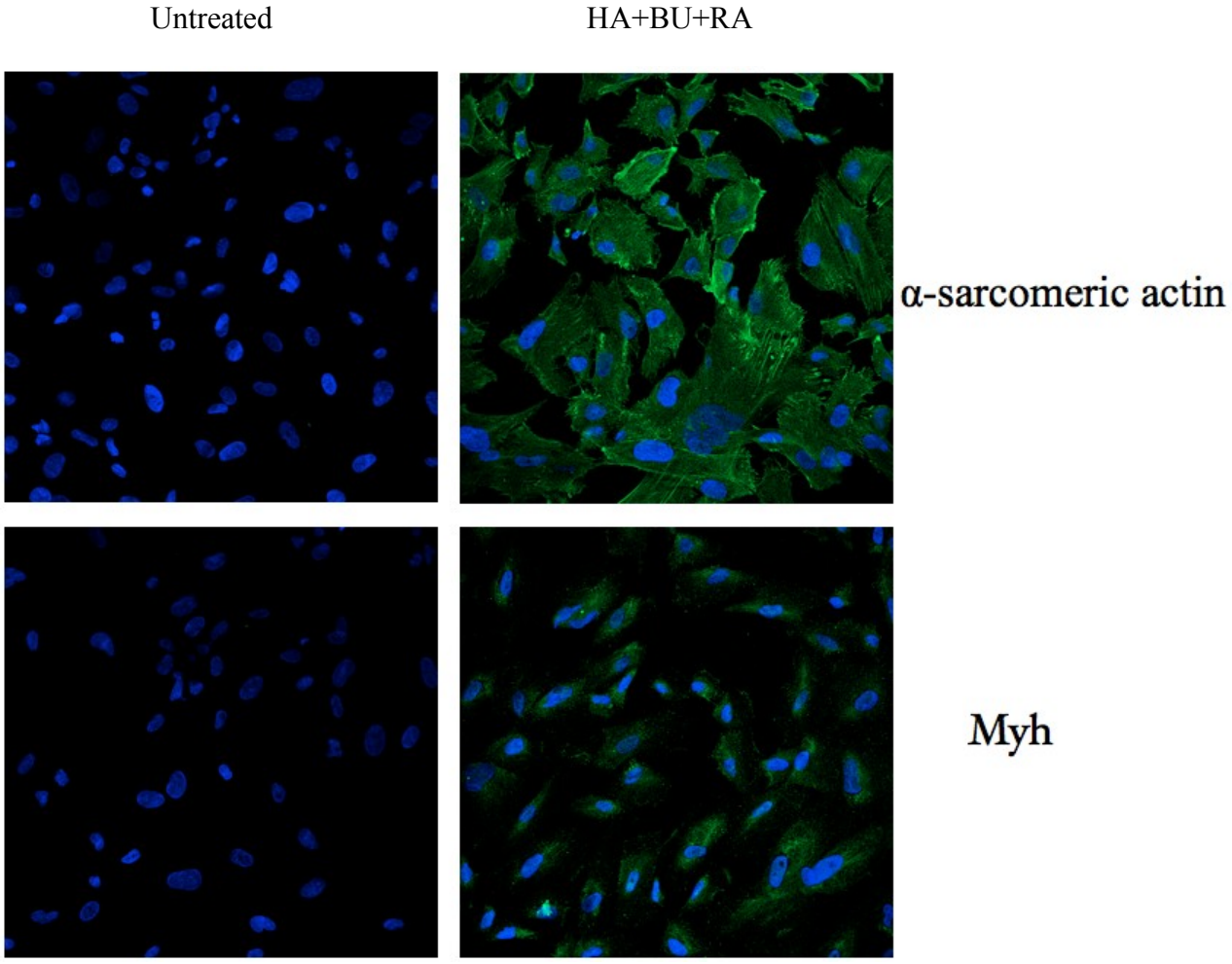

Figure 12: Immunofluorescence analysis of the expression of α-sarcomeric actinin and MYH in AFS cells. Expression of α-sarcomeric actinin and MYH (green fluorescence) was assessed in AFS cells cultured for 15 days in absence or presence of Ha 2 mg/ml, Bu 5mM and Ra 0,1 mM. Nuclei are labeled with DAPI (blue).

Moreover, immunofluorescence analysis demonstrated that AFS cells, after 15 days of HA+BU+RA treatment, showed a clearly evident von Willebrand factor (vWF) expression, a known marker for vasculogenesis (Figure 13).

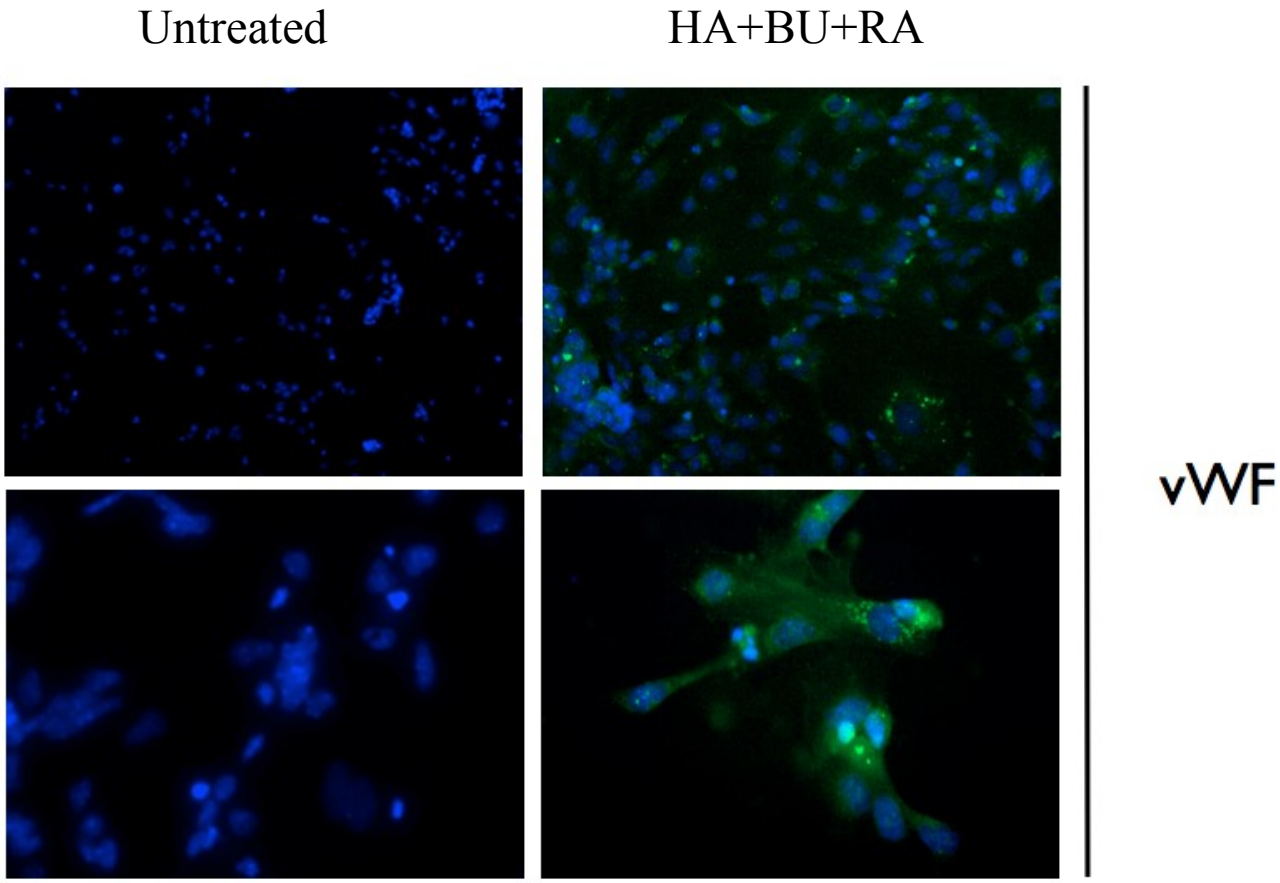

Figure 13: immunofluorescence analysis of the expression of vWF in AFS cells. Expression of vWF (green fluorescence) was assessed in AFS cells cultured for 15 days in presence of HA+BU+RA. Nuclei are labeled with DAPI (blue).

To assess the percentage of AFS cells expressing α-sarcomeric actinin, MYH and vWF cytofluorimetric analysis were performed. The percentage of cells expressing sarcomeric actinin MYH or vWF is considerably greater in treated compared to untreated cells (Figure 14). In conclusion, HA+BU+RA increased the yield of cardiac and endothelial differentiated elements (Table 4).

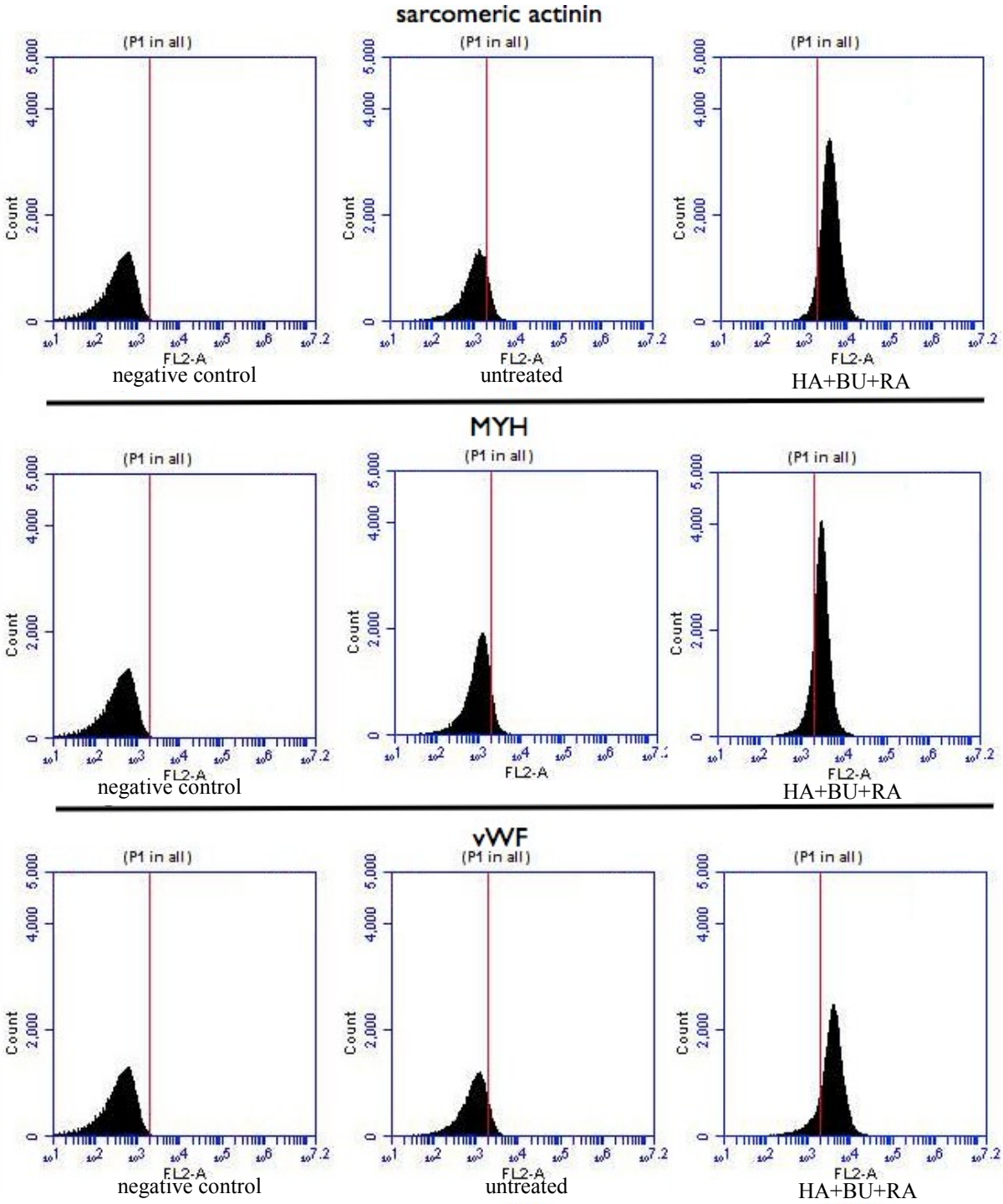

Figure 14: Flow cytometry analysis of sarcomeric actin, MYH and vWF in AFS cells treated with a mixture of HA+BU+RA. Cells were stained with primary antibodies specific for the marker of interest and FITC-conjugated secondary antibodies. The graphics show an increase of fluorescence and percentage of differentiated cells in the treated samples (HA+BU+RA) compared to the untreated samples. Unstained cells were used as a negative control.

	Sarcomeric Actin	MYH	vWF
HA+BU+RA	56,7% +/- 1,8%	57,2% +/- 1,6%	41,2% +/-3,6%
UNTREATED	4,7% +/- 1,3%	4,8% +/- 1,4%	4,2% +/- 0,9%

Table 4

5. DISCUSSION

Cardiomyopathies are a various group of myocardial diseases correlated with cardiac dysfunction. Enormous importance has been given to the possibility that cardiac metabolism deregulation may play a role in the mechanisms leading to the deterioration of the heart functions and to its maladaptive remodeling. These assorted myocardial diseases were once considered as specific diagnoses but there is now considerable evidence that they are caused by a common genetic origin. Although research has made great efforts in this direction by identifying many different gene mutations, at the moment, the best therapeutic solution is tissues or organ transplantation. One of the most important aspects of clinical practice of tissue grafts and organ transplants concerns the immune-known phenomenon of so-called "rejection". The rejection is caused by the recipient's body not recognizing the transplanted organs or tissues as "its" because of the cytological heterogeneity that has its origin in a genomic difference between the donor and the recipient cells. Another significant concern regarding this clinical approach is the lack of available organs. In this context, the possibility to have a source of large quantities of every cell types of tissues, represented by stem cells, opens new scenarios for the therapeutic approach to chronic and degenerative diseases. Stem cells, cultured in vitro and directed to differentiate into specific cell types would constitute a renewable source of cells, eliminating the problem of organ's availability. Cell therapy is now an interesting target for the repair and regeneration of damaged myocardium (Orlic et al. 2001). In particular, the use of stem cells pre-differentiated into cardiomyocytes and transplanted into infarcted hearts has been demonstrated improving cardiovascular repair in rats (Ventura et al. 2007). The possibility to have stem cells capable of regenerating the heart damage would represent a very important goal for the prevention and therapy of cardiomyopathies. In this scenario, originally the attention has been placed on adult stem cells of the bone marrow. In fact, numerous studies have shown the ability of mesenchymal stem cells derived from bone marrow (hBM-MSCs) to differentiate into cardiomyocytes. Although this mesenchymal cell population is characterized by a good proliferative potential, high genetic stability and ability to improve the regenerative process of many tissues (Pittenger et al. 1999), there are a number of problems limiting the possible clinical applications. In fact, they are obtained from conventional bone marrow via a highly invasive procedure, moreover, in each sample are available only a small number of stem cells, and finally they have the tendency to age and to get infections (Rao et al. 2001). This has prompted the research to the characterization of alternatives sources of mesenchymal stem cells in adult tissues able to overcome these limitations. Furthermore, in relation to the phenomenon of the rejection of

transplants and immune tolerance described previously, the interest of some scientists working on stem cells has focused on the possibility of obtaining cultures of autologous stem cells. To date, despite the considerable efforts of many researchers, protocols based on the use of autologous cells appear to be difficult to be put in place, for the need to obtain patient's biopsies and culturing cells in vitro, to perform tests and to store them properly before the administration of the therapy. The use of allogeneic stem cells, on the contrary, allows selecting the donor in advance in order to examine and analyze the MSC populations and to exclude malfunctions. Crucial is the fact that stem cells can be immediately available for the administration in case of necessity.

Amniotic stem cells are stem cells of mesenchymal origin isolated from amniotic fluid. The use of amniotic fluid-derived stem cells provides very interesting perspectives. AFS cells have a high renewal capacity, can be expanded for over 250 doublings without any detectable loss of chromosomal telomere length (De Coppi et al. 2007a) and can differentiate into multiple lineages (Fauza et al. 2004, In't Anker et al. 2003, McLaughlin et al. 2006, Prusa et al. 2002, Tsai et al. 2004, Tsai et al. 2006). Tolerogenic properties of AFS cells also make them ideal instruments in cell therapy and compatible with efficient therapeutic protocols (Atala, 2009).

In this study, the amniotic fluid has been used as a source of mesenchimal stem cells. The isolated AFS cells were induced to differentiate into cardiomyocytes through the administration of a mixture of hyaluronic, butyric, and retinoic acids. Its mechanism of action is based on the ability of hyaluronan to be internalized through the specific receptor CD44. Hyaluronate acts, therefore, as a "carrier" for the internalization of the other chemical entities (butyric and retinoic acids); at the intracellular level, this results in a sequence of concerted epigenetic modifications, mediated by butyrate (relaxation of chromatin) and transcriptional events induced by retinoic acid and its specific receptors. The cells isolated from amniotic fluid have been expanded and characterized by flow cytometry to confirm a MSCs profile (CD29 +, CD73 +, CD90 +, CD105 +, CD44 +, CD34-, CD45-, CD31-). The data obtained show that the antigenic profile of AFS cells is analogous to the one belonging to hBM-MSCs. In particular, the presence of the receptor for hyaluronic acid CD44 in more than 96% of the cells enhances the rational of using HA+BU+RA. In our study, AFS cells were treated with HA+BU+RA to induce cardiac differentiation. The mixture has demonstrated to be able to induce the cardiomyogenic differentiation in these cells, activating the gene expression pattern typical of the cardiac lineage commitment. mRNA and protein levels were analyzed at several stages of the differentiation process. Even after one day of treatment with HA+BU+RA, the transcription factors GATA4 and Nkx2.5, which are required for cardiogenic commitment, were remarkably expressed (Biben et al 1997; Lints et al. 1993; Benson et al. 1999). HA+BU+RA also

enhanced prodynorphin mRNA expression and the levels of dynorphin B, another important protein for stem cells commitment to the cardiac lineage (Ventura et al. 2003). Moreover, immunofluorescence and citofluorimetry analysis showed the expression of the two cardiac specific proteins α-sarcomeric actinin and MYH. Interestingly, the exposure to HA+BU+RA induced the transcription of vWF, a gene tightly involved in vasculogenesis and angiogenesis, leading to the development of vWF-expressing endothelial cells.

In conclusion, in this study, we have illustrated the possibility of using amniotic fluid as source of stem cells with considerable capability to differentiate into cardiomyocytes and endothelial cells through the use of HA+BU+RA. AFS cells own many benefits such as being readily isolated, easy to grow; highly expansive including clonal growth and, able to differentiate into all germ layers. In addition, they lack immunogenicity due to a low expression of the major histocompatibility complex (MHC) class II antigen and have not shown tumorigenic property in vitro and in vivo (Cipriani et al., 2007; Kunisaki et al., Tissue Eng. 2007; Tsai et al., 2006; In 't Anker et al., 2003; Magatti et al., 2008, Joo et al., 2012; Chemg et al., 2011). In conclusion, based on all the above reasons AFS cells represent an extremely suitable source of cells that might be used for therapeutic cell transplantation.

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