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# MUSCULOSKELETAL TISSUE REGENERATION BY HUMAN NON-EMBRYONIC STEM CELLS

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ABSTRACT

The aim of the thesis is to investigate the regenerative potential of alternative sources of stem cells, derived from human dental pulp (hDPSCs) and amniotic fluid (hAFSCs) respectively, and, specifically to evaluate their capability to be committed towards the osteogenic and myogenic lineages, with the long-time objective to apply these stem cells to translational strategies in regenerative medicine for the repair of bone and skeletal muscle tissues. So far orthopaedic surgery, based on the use of conventional prosthesis and engineering biomaterials, does not represent a definitive solution in the treatment of some diseases, because the implanted material either does not offer the best histo-integration and does not replace the function of the original tissue.

The tissue engineering approach could be therefore a promising tool to restore bone defects and deficiencies that currently are surgically treated through the application of artificial permanent implants. In particular, the *in vitro* bone production by stem cells may represent a radical breakthrough in the treatment of congenital or acquired pathologies and secondary traumas characterized by critical bone mass defects, which yet do not have any medical or surgical solution. Therefore, hDPSCs and hAFSCs were seeded and pre-differentiated on different scaffolds to test their capability to subsequently reach the osteogenic differentiation *in vivo*, in order to recover critical size bone defects.

Equine collagen, silk fibroin and P(d,l)LA were utilized to determine the best scaffold for hDPSCs and hAFSCs to undergo the effective osteogenic differentiation *in vivo*, to repair surgically operated parietal bone defects in rats. Results obtained in a recent study on ectopic implants allowed to identify fibroin scaffolds as a promising tool for osteogenic differentiation of human stem cells.

This study demonstrated that fibroin scaffold promotes mature bone formation and defect correction when combined to both hDPSCs and hAFSCs, in particular, with a higher bone amount produced by hAFSC-seeded scaffolds.

Since the ultimate goal of cell-based therapy is to utilize new stem cell sources for clinical applications with minimal safety concerns, this study has investigated a culture condition that might allow human DPSCs to be used for human cell therapy in compliance with good manufacturing practices (GMPs). This study demonstrated that human serum (HS) is an appropriate supplement for the *in vitro* expansion of human DPSCs, since its addition to culture medium produced a good proliferation rate in comparison with the one evaluated after culturing the cells with foetal calf serum (FCS). As a matter of fact, human serum appeared to be an adequate additive to the osteogenic medium in order to achieve the expression of bone related proteins and induce the mineralization of the extracellular matrix by hDPSCs, *in vitro*. Also, hDPSCs pre-differentiated towards the osteogenic lineage with the addition of HS showed a huge contribution in regenerating of the critical size bone defects *in vivo*.

Similarly, cell-based therapy with hDPSCs and hAFSCs could represent an alternative therapeutic approach for patients with musculoskeletal traumas or diseases. This thesis investigated the ability of these stem cells to undergo the myogenic commitment *in vitro* by using different protocols, either when co-cultured with murine myoblasts (C2C12) and when differentiated alone after the demethylating treatment with 5-aza-2'-deoxycytidine. Human DPSCs and AFSCs, indeed, expressed under both these conditions regulatory factors and markers – such as myogenin and myosin - that are typical of the myogenesis process. Furthermore, after being pre-differentiated by means of demethylation, with or without the addition of conditioned media from differentiated C2C12, hDPSCs and

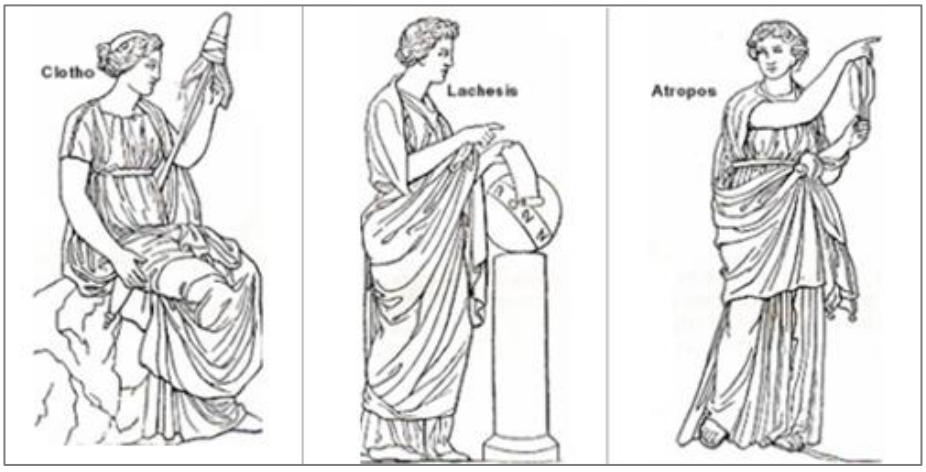
hAFSCs were tested for the capability to regenerate the damaged skeletal muscle of SCID/*mdx* mice, animal model of Duchenne Muscular Dystrophy (DMD). When injected into dystrophic muscles of SCID/*mdx* mice, pre-differentiated hDPSCs and hAFSCs were able to recover the skeletal muscle tissue, and more interestingly to restore dystrophin expression. These observations, altogether, suggest the eventual applicability of human DPSCs and AFSCs to translational strategies, in order to enhance the repair of injured skeletal muscle in DMD patients.

## *Introduction*

*In Greek mythology, the Moirai also known in English as the Fates, and in Latin as the Parcae - euphemistically the "sparing ones" - were white-robed figures, personifying the inescapable destiny. Daughters of Zeus, they were three: Clotho (the spinner), who spun the thread the life from her distaff onto her spindle, Lachesis (the allotter), who measured the thread of life allotted to each person, and Atropos (the inexorable or inevitable), who was the cutter of the thread of life. Thus, they controlled the metaphorical thread of life of every mortal from birth to death.*

*In medical research stem cells – whose definition derives from the Latin "stamen" – can be therefore metaphorically considered as the thread of life, namely the starting point from which the human being comes from.*

*Actually, they have been demonstrated to carry out a fundamental role in maintaining, healing, regenerating and aging processes, therefore representing an adequate tool to be applied in regenerative therapies.*



## *Tissue Engineering*

The primary goal of tissue engineering scientists is to optimize cell isolation, proliferation and differentiation of stem cells and to develop scaffolds and/or delivery systems that enhance the coordinated growth of three-dimensional tissues. The most used basic strategy consists in harvesting cells from a patient, expand them in cell culture, and seed them onto a scaffold that provides a biomechanical environment that leads the formation of the required tissue upon the production of a certain extracellular matrix by stem cells. The tissue can be grown on a scaffold that will completely resorb as the new tissue grows, so that only the new tissue will be implanted, or that will be gradually reabsorbed after implantation. Therefore, the tissue engineered complex must be able to survive, restore normal function, and integrate with the surrounding tissues (Polak and Bishop, 2006).

## *Cell sources*

As a primary factor to achieve successful results in tissue engineering it is desirable and necessary to obtain a sufficient number of cells that show constantly an adequate phenotype and the ability to perform their specific biological functions, i.e. the correct organization of the extracellular matrix, the secretion of signaling molecules, and the interaction with the surrounding cells and tissues. Taken altogether, these functions are meant to enhance the repair (Polak and Bishop, 2006).

### *Primary Cells*

These cells are tissue specific, are generally harvested from explant samples collected by surgical procedure. Primary cells are the most desirable with regard to immunological

compatibility, but they are differentiated and post-mitotic. When cultured *ex vivo* a de-differentiation of the primary cells themselves as well as the development of an inappropriate phenotype might be observed.

Moreover, functionality and proliferation rates tend to lower, and for the isolation of certain cell types, i.e. spinal cord neurons, the harvesting of primary cells is not recommended.

These aspects have triggered the pursuit of alternative cell sources for tissue engineering approaches and stem cells actually provide solutions to some problems related to the use of primary cells (Polak and Bishop, 2006).

***Stem Cells***

Stem cells can be described as undifferentiated cells that are characterized by three fundamental abilities: proliferation, self-renewal, and differentiation towards multiple cell lineages (Polak and Bishop, 2006).

They can be isolated from different sources, and with this regard are classified as embryonic stem cells (ESCs), foetal stem cells (FSCs), and adult stem cells (ASCs), whose range of differentiation potential may vary. Indeed, a more useful classification can be based on stem cells plasticity (Figure 1):

- **Totipotent** are the most plastic ones and differentiate into any cell type, including placenta (i.e. the zygote and morula at 8-cells stage)
- **Pluripotent** are the second most plastic stem cells after the totipotent ones, can differentiate into all cells, except for the totipotent and placental cells
- **Multipotent** are the precursors of cells differentiated towards specific tissues

- **Progenitor cells** may differentiate into only one or a limited number of cell types, therefore are the least plastic cells

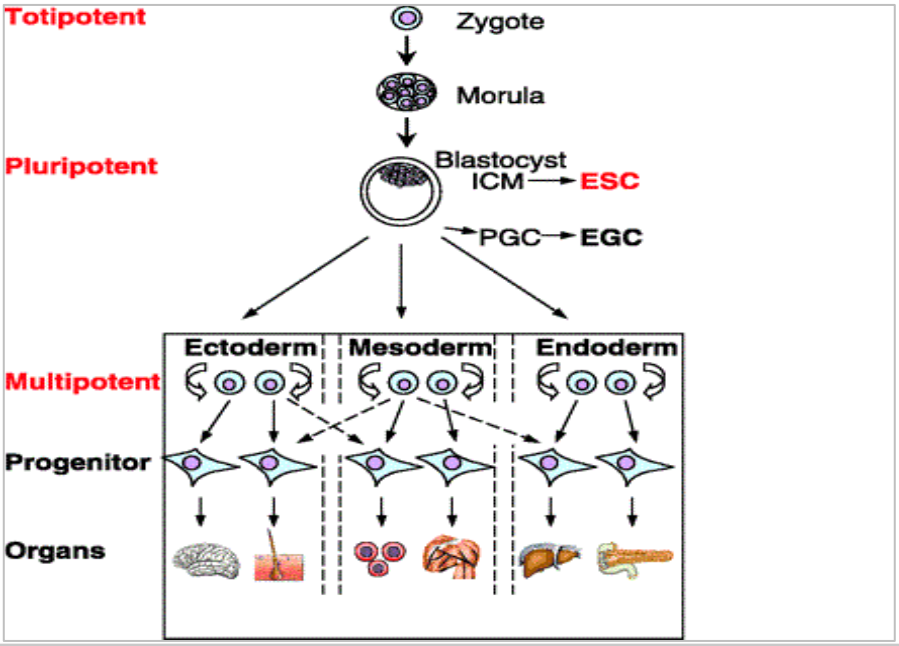



**Figure 1:** Stem cells classification by origin and plasticity

**Embryonic stem cells** (ESCs) are the most plastic source available in tissue engineering. They were first described when they were isolated from the inner cell mass of a mouse blastocyst and then expanded *in vitro* (Evans and Kaufman, 1981; 3 Martin GR, 1981). ESCs have actually been demonstrated to be pluripotent, namely able to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm (Nagy A *et al*, 1990; Bradley A *et al*, 1984; Xu RH *et al*, 2002). The murine ESCs were able to maintain an undifferentiated state while proliferating, and to differentiate towards all the mature somatic phenotypes, when stimulated with the appropriate signals. The initial isolation of murine ESC lines provided an easy model system to analyze the processes occurring during early development and cellular differentiation. These findings have also

led the way to tissue engineering applications with similar pluripotent cells obtained from human blastocyst. As a matter of fact, human ESCs, were first derived in 1998 for infertility purposes (Thomson JA *et al*, 1998; Reubinoff BE *et al*, 2000); they show various discrete differences from murine ESCs, in fact they proliferate more slowly and most of them form flat, instead of spherical, colonies. Also, they can be dissociated in single cells more easily than their murine counterparts (Laslett AL *et al*, 2003).

Because of their plasticity and potentially unlimited capacity for self-renewal, ESCs cell therapies have been proposed for regenerative medicine and tissue replacement in case of injury or disease. Diseases that might eventually be treated by ESCs cells include several blood and immune-system related genetic diseases, cancers, and disorders, type I diabetes, Parkinson's, blindness and spinal cord injuries. Nevertheless - beside the existing ethical controversy related to the use of embryonic stem cells - there is a huge concern with the possible transplantation of ESC into patients as therapies, because of the related possible formation of tumors, including teratoma (Knoepfler PS, 2009). Therefore, investigations have been carried out in order to find alternative solutions to ES cells. Takahashi and Yamanaka published a milestone manuscript in 2006 that defined a specific set of transcription factors capable of reverting differentiated cells back to a pluripotent status, thus creating **induced pluripotent stem cells (iPS cells)** (Takahashi and Yamanaka, 2006): four key transcription factors - Oct4, Sox2, Klf4, and c-Myc - identified by screening 24 preselected mouse embryonic stem cell–specific factors were sufficient to reprogram adult mouse fibroblasts into embryonic stem cell–like iPS cells (Takahashi and Yamanaka, 2006). The same combination of transcription factors was demonstrated to be sufficient for the pluripotent induction of human cells as well (Takahashi K *et al*, 2007). Considering the convenience and reproducibility of generating iPS cells, experts have

raised the hope that iPS cells might accomplish much of the promise of human ESCs in regenerative medicine (Pera MF, 2008). Nonetheless, findings from different research groups have demonstrated that iPS cells injected into immunodeficient mice give rise to teratomas comprising all three embryonic germ layers, as similarly observed with ESCs. A further potential complication related to the use of iPS cells is the use of retroviral and lentiviral vectors to activate the necessary reprogramming transcription factors, in fact, even though much progress has been made in generating integration-free murine iPS cells, the safety of iPS cells needs to be rigorously tested, since all essential reprogramming factors are oncogenes, and their overexpression has been linked with cancers (Liu SV, 2008).

**Foetal stem cells** (FSCs) are the mesenchymal stem cells derived from the fetus. Recently, they have been isolated from foetal blood and raised the possibility of using autologous cell to treat fetuses *in utero* (Campagnoli C *et al*, 2001).

The mesenchymal population obtained from foetal blood is characterized by adherent cells that divide upon 40 passages in culture and are able to differentiate towards the chondrogenic and osteogenic lineages, but also can form oligodendrocytes and hematopoietic cells. Furthermore, they are a unique source of stem cells since they can engraft into multiple organs and differentiate in a tissue-specific manner (Polak and Bishop, 2006).

**Adult stem cells** (or postnatal somatic cells) are undifferentiated cells found among differentiated cells in a tissue or organ that can renew itself. These stem cells are located in a variety of tissues, including bone marrow, brain, liver, skin, and blood (Presnell SC *et al*,

2002). Their principal role is to maintain and repair the tissue they derive from. Although in a first moment they were considered to own a very limited differentiation potential, there has been much evidence that they show a considerable degree of plasticity, not without some debate about it (Hawley and Sobieski, 2002; Holden and Vogel, 2002; Verfaillie CM *et al*, 2002; Poulsom R *et al*, 2002; Raff M, 2003). Unlike ESCs, the application of adult stem cells in research and therapy is not considered to be controversial, since they do not require the destruction of embryos and can be accessibly obtained from adult tissue samples.

In comparison with ESCs and FSCs, adult stem cells generally exhibit a lower differentiation potential and yield a more limited number of distinct progenitor cells. However, adult stem cells can significantly undergo proliferation and differentiation into more mature and tissue-specific cell types, according to changes in the specialized microenvironment where they reside - the *niche* - and the stem cell behavior is regulated through direct contact and paracrine signaling (Fuchs E *et al*, 2004; Niemann C, 2006; Wilson and Trumpp, 2006; Morrison and Spradling, 2008; Jones and Wagers, 2008).

As mentioned above, stem cells are defined by their ability to produce more stem cells and also cells that differentiate. Stem cells can accomplish these two tasks by means of asymmetric cell division (Figure 2b), whereby each stem cell divides to generate one daughter with a stem-cell fate (self-renewal) and one daughter that differentiates (Betschinger and Knoblich, 2004; Clevers H, 2005; Doe and Bowerman, 2001; Yamashita YM *et al*, 2005). However, a limit of this division type is that stem cells would be unable to expand in number. Thus, asymmetric cell divisions cannot be the only explanation. Stem cells must have further self-renewal strategies that allow a dynamic control of their numbers: in fact, with symmetric divisions they can self-renew and produce differentiated

progeny (Figure 2c). Symmetric divisions are defined as the generation of daughter cells that are intended to acquire the same fate. Although the idea that stem cells can divide symmetrically may sound like a contradiction, stem cells are defined by their "potential" to generate more stem cells and differentiated daughters, rather than by their production of a stem cell and a differentiated daughter at each division. Therefore, when thought as a population, a pool of stem cells with identical developmental potential may generate only stem-cell daughters in some divisions and only differentiated daughters in others (Figure 2d). Broadly, stem cells can rely either completely on symmetric divisions or on a combination of symmetric and asymmetric divisions (Morrison and Kimble, 2006).

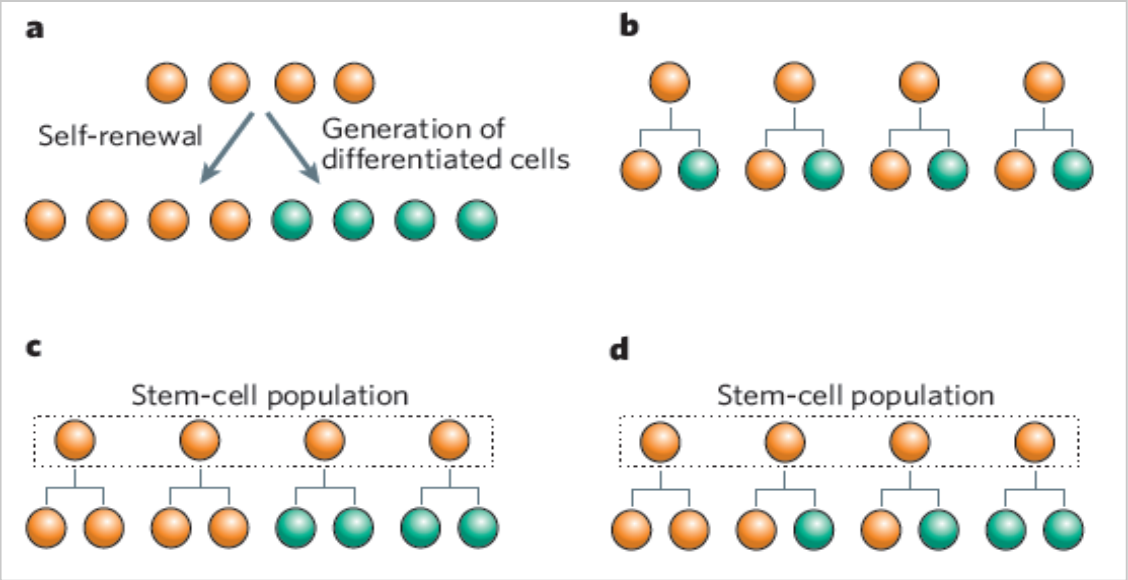



**Figure 2:** Stem cells (orange) must accomplish the dual task of self-renewal and generation of differentiated cells (green); b–d: possible stem-cell strategies that maintain a balance of stem cells and differentiated progeny. b: asymmetric cell division; c: symmetric cell division; d: combination of cell divisions: each stem cell can divide either symmetrically or asymmetrically (Morrison and Kimble, 2006).

***Mesenchymal Stem Cells***

Mesenchymal stem cells (MSCs) can be found in bone marrow and in many other tissues. Bone marrow is a mesoderm derived tissue, basically constituted by elements from the stroma and the hematopoietic system, and is present in the interior of bones (Arai F *et al*, 2004).

For long time, it has been known that the bone marrow contains two types of stem cells, the hematopoietic stem cells (HSCs) – committed to differentiate towards mature blood cells – and the more undifferentiated stromal mesenchymal cells (MCs).

The identification of the mesenchymal stem cell has so far depended on *in vitro* culture systems, which have provided very heterogeneous information on MSCs.

They were identified through a combination of poorly defined physical, phenotypic, and functional properties. The first direct evidence that non-hematopoietic, mesenchymal precursor cells were present in the bone marrow derived from the research performed during the late 1960s by Friedenstein and colleagues: bone marrow samples incubated in tissue culture flasks gave rise to a fraction of adherent cells within a few days, and soon after individual aggregates of 2 to 4 fibroblasts were observed, which could differentiate into cells able to form small deposits of bone or cartilage (Friedenstein AJ *et al*, 1976). These cells were called "colony forming unit-fibroblasts" or CFU-F. During the 1980s, several studies showed that cells isolated by the Friedenstein method were multipotent and able to differentiate towards osteoblasts, chondroblasts, adipocytes, and also myoblasts (Prockop DJ, 1997). The formation of CFU-F has been considered peculiar of mesenchymal stem cells, although a direct relationship between the two has not been clearly established, likely because of the high heterogeneity in morphology, cell size and differentiation potential observed among species and between colonies (Javazon EH *et al*,

2004). Caplan demonstrated that bone and cartilage turnover was mediated by MSCs, and the surrounding conditions were critical to inducing MSC differentiation (Caplan AI, 1991). Later, the multilineage differentiation capability of MSCs was definitively demonstrated by Pittenger (Pittenger MF *et al*, 1999).

The defining features of mesenchymal stem cells are inconsistent among scientists; to address this issue, the Mesenchymal and Tissue Stem Cell Committee of International Society for Cellular Therapy (ISCT) has recently proposed a set of standards to define human MSCs either for laboratory-based scientific investigations and for preclinical studies. First, MSCs must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks. Second, ≥ 95% of the MSCs population must express CD105 (endoglin), CD73 (ecto-5'-nucleotidase) and CD90 (Thy-1), as determined by flow cytometry. Further, no more than 2% of these cells may express CD45 (pan-leucocyte marker), CD34 (hematopoietic progenitor and endothelial cell marker), CD14 or CD11b (monocyte and macrophage markers), CD79α or CD19 (B cell markers) and HLA-DR (marker of stimulated MSCs). Third, the cells must be able to differentiate towards osteoblasts, adipocytes and chondroblasts, under the standard differentiating conditions *in vitro* (Dominici M *et al*, 2006).

MSCs embryologically derive from two major sources, neural crest and mesoderm, however these cells have been identified in not only mesoderm-derived but also endoderm- and ectoderm-derived tissues. Examples of tissues where MSCs have been characterized include, but are not limited to: *mesodermal* - bone marrow, trabecular bone, synovium, cartilage, fat, muscle, and tonsil; *endodermal* – thymus; *ectodermal* - skin, hair follicle, dura mater, and dental pulp; *prenatal and perinatal tissues* - umbilical cord, umbilical cord blood, amniotic fluid and placenta (Kuhn and Tuan, 2010).

Although still controversial, literature exists demonstrating the potential of MSCs to differentiate along non-mesodermal lineages, such as ectodermal skin and neurons and endodermal hepatocytes (Figure 3). MSCs are still correctly defined as "multipotent" rather than "pluripotent" despite these reports of their potential capability to differentiate into derivatives of all three germ layers.

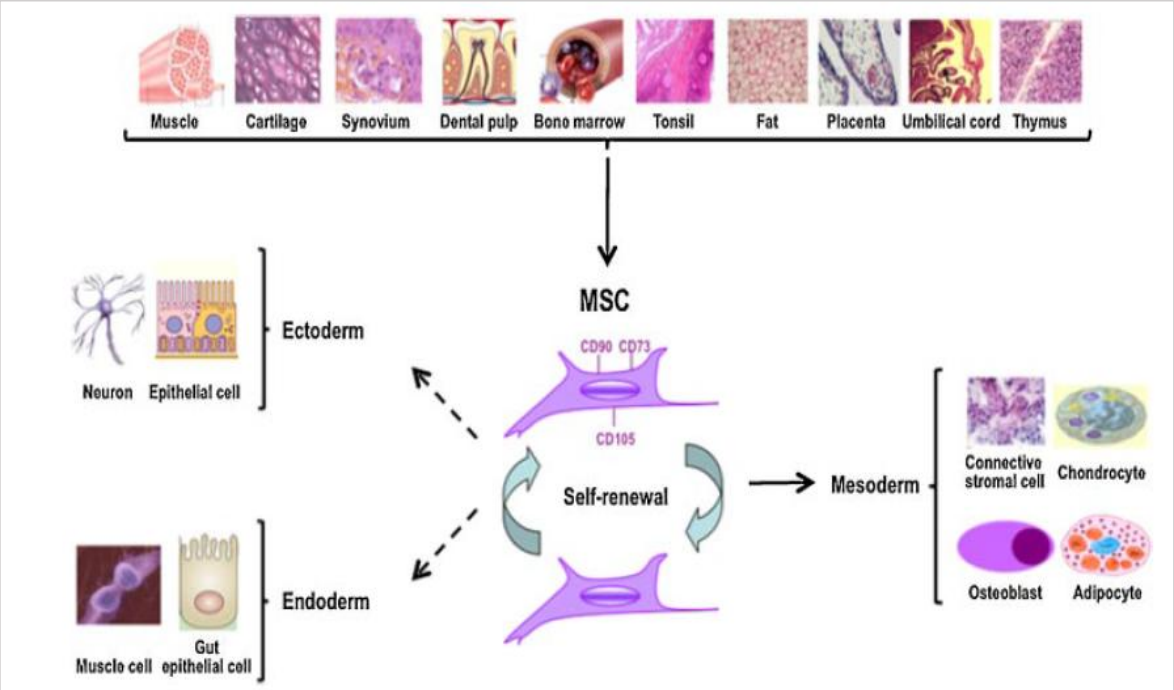



**Figure 3:** Identification and characterization of mesenchymal stem cells. Cells with MSC-like characteristics have been isolated and from several adult tissues. The most common phenotypical markers are CD90, CD73, and CD105. MSCs have the capability to self-renew and exhibit multilineage potential into many mesodermal cell types. Other studies have described the potential of MSCs to differentiate into ectodermal and endodermal lineages.

Most of the adult sources, including large volumes of normal bone marrow, are relatively difficult to access as a tissue source for the isolation of MSCs. Conversely, birth-associated tissues, including placenta, are readily and widely available. However, the bone marrow appears to be the most commonly exploited source of MSCs for most preclinical and clinical studies.

It is necessary to highlight that although MSCs can be isolated from these different tissues and can develop into bone, cartilage, or fat, they are distinctive and reflects aspects peculiar to their tissue of origin: as a matter of fact, human pellets of MSCs from bone marrow have shown to be able to make cartilage extracellular matrix through chemical exposition to TGF-β (Johnstone B *et al*, 1998), while mesenchymal progenitors from adult fat require both TGF-β and BMP-6 in order to produce cartilage (Yoo JU *et al*, 1998; Estes BT *et al*, 2006).

## *Dental pulp stem cells (DPSCs)*

Multipotent adult stem cells, as MSCs, are present in all mature tissues of the human body and are thought to reside in a specific area of each tissue where they may remain quiescent (non-dividing) for many years until they are activated by disease or tissue injury. MSC-like stem cells have been isolated – according to the standard criteria of mesenchymal stem cells illustrated above (Friedenstein AJ *et al*, 1976; Prockop DJ, 1997; Caplan AI, 1991; Pittenger MF *et al*, 1999; Dominici M *et al*, 2006) - from different tissue sources including bone marrow, adipose tissue, bone, periosteum, synovium of the joints, skeletal muscle, skin, pericytes of blood vessels, peripheral blood, periodontal ligament, umbilical cord, and dental pulp of permanent and deciduous teeth (Mizuno H *et al*, 2012).

Lately, human dental pulp has been attracting interest for its ease of collection from permanent and deciduous teeth through minimally invasive and safe procedure.

Dental pulp is a loose vascular connective tissue, surrounded by dentin (Figure 4) and consisting of a heterogeneous population of cells: the potential preodontoblasts, fibroblasts, stromal cells, endothelial and perivascular cells, neural cells and others (Pierdomenico L *et al*, 2005).

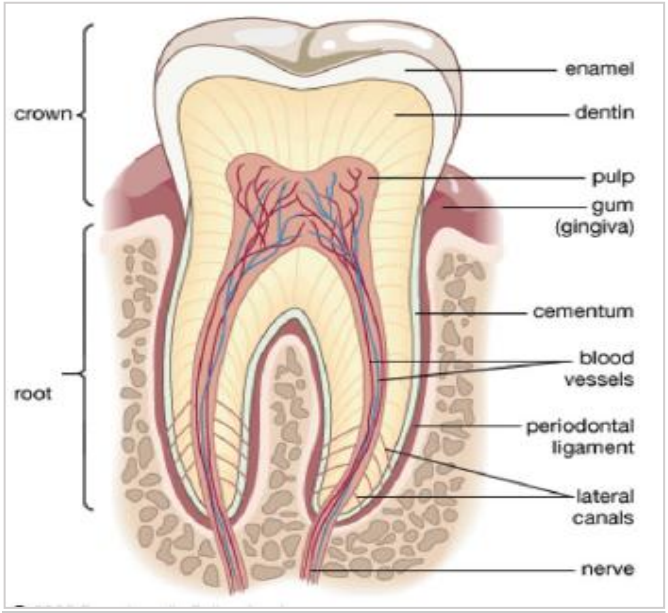



**Figure 4:** The main structures of the tooth. Dental pulp is part of the center of the tooth, is contained within the pulp chamber and harbors vessels and nerves.

To date, multiple stem/progenitor cells have been isolated from human dental pulp, characterized, and classified. The postnatal stem cells first isolated from dental pulp were termed "dental pulp stem cells" (DPSCs) (Gronthos S *et al*, 2000). Besides this group, three more types of dental cell populations were isolated and characterized: stem cells from exfoliated deciduous teeth (SHEDs) (Miura M *et al*, 2003), periodontal ligament stem cells (PDLSCs), dental follicle progenitor cells (DFPCs) (Morsczeck C *et al*, 2005) and stem cells from apical papilla (SCAPs) (Sonoyama W *et al*, 2008).

In particular, stem cells from human dental pulp of impacted third molars (also known as "wisdom teeth") show a greater advantage for the ease and low invasivity of recruitment, respect to the mesenchymal stem cells derived from bone marrow, adipose tissue, peripheral blood, and umbilical cord blood.

DPSCs are an attractive alternative source of mesenchymal stem cells since they show a wide panel of favorable properties:

- Easy surgical access and low invasiveness for the patient: the extraction of one or more molars, in healthy individuals, is a widespread and routinary procedure among the population.
- Very low morbidity of the anatomical site after the collection of the pulp.
- High efficiency of the extraction procedure of the stem cells from the pulp tissue.
- Minimal tooth processing may be required for banking of samples with no immediate plans for DPSC expansion and use, which subsequently would limit costs and favor the clinical banking of these cells.
- Cells expanded in culture can be preserved and stored for at least six months, and likely longer, at −85°C with regards to the qualitative ability to differentiate in at least a tri-lineage manner. Therefore, they can be cryopreserved, retaining multi-differentiation potential (Graziano A *et al*, 2008).
- No teratomas have been observed after *in vivo* transplantation.
- Mesenchymal stem cells from the dental tissue have been shown to possess immunosuppressive properties. These findings have elicited further interest in these cells as alternative cell sources able to modulate alloreactivity and tissue regeneration, following transplantation into human leukocyte antigen-mismatched donors (Wada N *et al*, 2013).

***Dental pulp embryogenesis***

In vertebrate embryos, a group of cells, called neural crest (NC) cells, separate from the neural tube and migrate to re-aggregate with other cells. In the developing embryo, almost

all organs, glands, and tissues - such as craniofacial skeleton, cornea, teeth/dentin, thyroid gland, thymus, cardiac septa, adrenal gland, melanocyte, autonomic nerve, sensory nerve, and Schwann cells - have these basic cells (Crane and Trainor, 2006; Alberts B *et al*, 2008; Lee G *et al*, 2010). The contribution from NC cells in building of the head of vertebrates has been considered to be a turning point in the evolution of the vertebrates (Gans and Northcutt, 1983). Although NC cells are of ectodermal origin, it has been suggested to call them "mesectoderm" or "ectomesenchyme", since they undergo "mesenchymalization." This property is important with regard to mesenchymal stem cells, since their origin is the mesenchyme, which is derived from the mesodermal germ layer. On the other hand, along with the cranial skeleton and other tissues of head and neck, odontoblasts and tooth papillae are derived from mesectoderm, or ectomesenchyme (Le Douarin NM *et al*, 2004). Oral ectomesenchymal and ectodermal interactions phylogenetically precede the origin of odontogenesis (Moss ML, 1969).

During the sixth week of embryogenesis, the ectoderm covering the stomodeum starts to proliferate, giving rise to the dental laminae. Reciprocal interactions between ectoderm and mesoderm layers lead to placode formation. One of these thick, ovoid ectodermal structures develops into tooth germs, where cells, belonging to the neural crest, will differentiate into the dental germ, containing both dental papilla and follicle. Therefore, dental pulp is made of ecto-mesenchymal components, containing neural crest-derived cells, which display plasticity and multipotential capabilities (Sinanan AC *et al*, 2004). Pulp is externally separated from dentin by odontoblasts and by Hohl's subodontoblastic cells, that are pre-odontoblasts (Goldberg and Smith, 2004). Adjacent to this layer, the pulp is rich in collagen fibers and poor in cells. Then, another, more internal layer, contains progenitor cells and undifferentiated cells, some of which are considered stem cells (Jo YY

*et al*, 2007). From this layer, undifferentiated cells migrate to various districts where they can differentiate under different stimuli and make new differentiated cells and tissues. The final, innermost layer is the core of the pulp; this area comprises the vascular plexus and nerves. Up to more recent discoveries (Gronthos S *et al*, 2000; D'Aquino R *et al*, 2007), researchers hypothesized that DPSCs were present in this layer (Fitzgerald M *et al*, 1990). Actually, only undifferentiated perivascular cells can be found in it.

The third molar tooth germ begins development around the sixth year of life. Until this time, embryonic tissues of dental lamina remain quiescent and undifferentiated within the jaw of the child. Even though crown mineralization begins during the eighth year of life, often third molar roots are still incomplete at the age of 18. This means that the structure of those teeth is still immature at this age, and a conspicuous pool of undifferentiated cells, resident within the "cell rich zone" of the dental germ pulp, are needed for development.

***DPSCs characterization***

During the characterization of these newly identified dental stem cells, certain aspects of their properties have been compared with those of bone marrow mesenchymal stem cells (BMMSCs).

Differences have been noted between the dental stem cell populations and BMMSCs.

One important feature of pulp cells is their odontoblastic differentiation potential. Human pulp cells can be induced *in vitro* to differentiate into cells of odontoblastic phenotype, characterized by polarized cell bodies and accumulation of mineralized nodules (Tsukamoto Y *et al*, 1992; About I *et al*, 2000; Couble ML *et al*, 2000). DPSCs isolated with enzyme treatment of pulp tissues form CFU-Fs with various characteristics (Gronthos S *et al*, 2000; Huang GT *et al*, 2006a). Colonies show different cell densities, suggesting

that each cell clone may have a different growth rate, as reported for BMMSCs (Gronthos S *et al*, 2002). Within the same colony, different cell morphologies and sizes may be observed also. If seeded onto dentin, some DPSCs convert into odontoblast-like cells with a polarized cell body and a cell process extending into the existing dentinal tubules (Huang GT *et al*, 2006a; Huang GT *et al*, 2006b). In addition to their dentinogenic potential, subpopulations of hDPSCs also possess adipogenic and neurogenic differentiation capabilities, by exhibiting adipocyte- and neuronal-like cell morphologies and expressing respective gene markers (Gronthos S *et al*, 2002). More recently, DPSCs were also found to undergo osteogenic, chondrogenic and myogenic differentiation *in vitro* (Laino G *et al*, 2005; Zhang W *et al*, 2006; D'Aquino R *et al*, 2007).

Current evidence suggests that biochemical pathways involved in the differentiation of DPSCs into functional odontoblasts are similar to differentiation pathways of BMMSCs into osteoblasts (Shi S *et al*, 2001): odontoblasts and osteoblasts express similar mineralized matrix proteins, such as dentin matrix protein 1, fibronectin, collagen type I, alkaline phosphatase, osteonectin, osteopontin, bone sialoprotein, and osteocalcin (Nakashima M *et al*, 1994; Shiba H *et al*, 1998; Kuo MY *et al*, 1992; Tsukamoto Y *et al*, 1992; Buurma and Rutherford, 1999). Therefore, DPSCs actually share a similar pattern of protein expression with BMMSCs *in vitro*.

To date, dental-tissue-derived stem/progenitor cells have been used for tissue engineering studies in large animals to assess their potential in pre-clinical applications (Sonoyama W *et al*, 2006; Liu Y *et al*, 2008).

## *Amniotic fluid stem cells (AFSCs)*

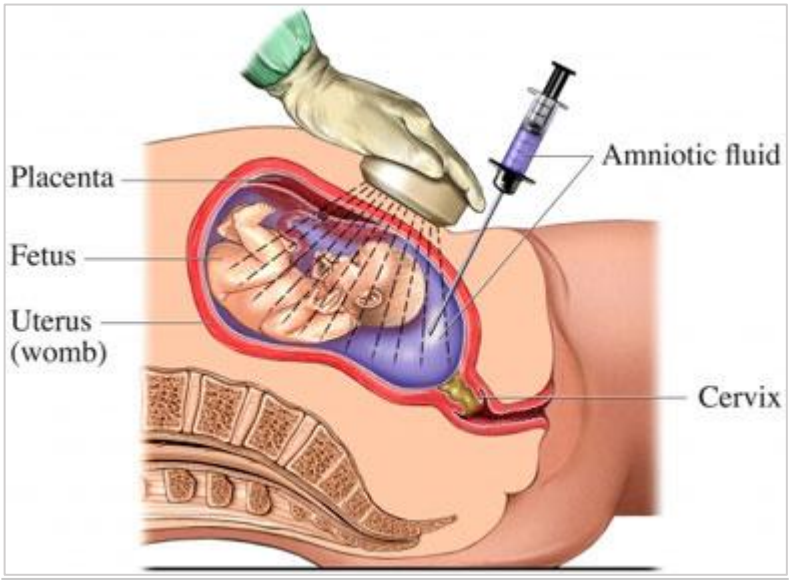



**Figure 5:** amniotic fluid aspiration during amniocentesis at 16-18 weeks of gestation.

Since decades, cells from cord blood, amniotic fluid, and the chorion were used in perinatal medicine mainly for invasive diagnostic purposes, such as detection of foetal infections, rare metabolic diseases, and mainly foetal karyotyping. With the introduction of the use of umbilical cord stem cells for the treatment of haematologic diseases many years ago, a new future for the use of perinatal cells has begun, namely the use of perinatal (amniotic, placental, chorionic, umbilical) stem cells for therapeutic purposes.

In normal pregnancies, amniotic cells can be collected by amniocentesis, between 16$^{th}$ and 18$^{th}$ week of gestation. In routine amniocentesis 16-20 ml of amniotic fluid are commonly sampled (Figure 5).

Amniotic fluid contains heterogenous population of cells, which are contributed mainly from the foetal skin, and possibly from placenta as well. Amniotic fluid-derived stem cells (AFSCs) have shown physical characteristics of both embryonic and adult stem cells.

According to their morphological and growth characteristics, amniotic fluid cells can be classified into three types: epithelioid, amniotic-fluid specific, and fibroblastoid (Milunsky A, 1979).

The first evidence of human amniotic fluid containing stem cells came from Prusa *et al*. in 2003. They identified **Oct-4** expression in human amniotic fluid and proposed that human amniotic fluid might be a new source for pluripotent-like stem cells without raising any ethical debates associated with human embryonic stem cells (Prusa AR *et al*, 2003). In the same year, In't Anker *et al*. demonstrated that human amniotic fluid was an abundant source of foetal MSCs, and these cells exhibited a phenotype and multilineage differentiation potential that appears to be similar to that of BMMSCs. They suggested that these AF-derived MSCs could be used in co-transplantation together with umbilical cord blood derived hematopoietic stem cells (In't Anker PS *et al*, 2003). In 2004, Tsai MS *et al.* successfully isolated AF-derived MSCs using a new two-stage culture protocol, and their study showed that these cells could be expanded rapidly and maintained the ability to differentiate towards multiple cell types, such as adipocytes, osteoblasts and neurons *in vitro* (Tsai MS *et al*, 2004). Following, in 2007, Atala and colleagues isolated the **c-kit** (CD117) positive population by immunoselection, from second trimester amniotic fluid samples and demonstrated that AFSCs are "broad-spectrum multipotent" (that is, pluripotent) - similarly to embryonic stem cells - and can differentiate into adipogenic, osteogenic, myogenic, endothelial, neurogenic, and hepatic lineages, including the representatives of all three germ layers (De Coppi P *et al*, 2007).

### *AFSCs isolation*

Currently, there are three main techniques for the isolation of AFSCs from human amniotic fluid. The first one is the direct culture from native amniotic fluid obtained from amniocentesis specimens, as described by In't Anker *et al.* (In't Anker PS *et al*, 2003). The second one is the immunoselection, through the use of magnetic beads, of the c-kit positive cell population from amniocentesis samples, which allowed to sort cells that are easily expandable in culture as stable lines (De Coppi P *et al*, 2007). The third one is a two-stage culture protocol performed by Tsai *et al.* using non-adhering amniotic fluid cells (NA-AFCs) of the primary culture to isolate AFSCs without interfering with the ordinary procedure of foetal karyotyping (Tsai MS *et al*, 2004): in this protocol, NA-AFCs are collected from the supernatant of primary amniocytes in culture (first stage) in serum free Chang's medium (Chang and Jones, 1985), then these NA-AFCs are plated for the expansion of AFSCs after completing the foetal chromosome analysis (second stage). The main difference of this protocol, compared to the other two, is that it isolates the non-adhering cells which had been left without any added nutrition for 7 to 10 days, a condition similar to serum deprivation that might enhance the expression of some embryonic genes by the stem cells (Pochampally RR *et al*, 1994).

### *AFSCs characterization*

The AFSCs are positive for HLA I antigens (HLA-ABC), and negative for HLA II antigens (HLA-DR, DP, DQ).

AFSCs are negative for markers of the hematopoietic lineage (CD45) and of hematopoietic stem cells (CD34, CD133). Nevertheless, AFSCs also show phenotypic characteristics similar to those of MSCs derived from other sources, such as umbilical cord blood,

placenta and first-trimester foetal tissues (blood, liver and bone marrow), which are positive for SH2, SH3, SH4, CD29, CD44, and CD166, and are negative for CD10, CD11b, CD14, CD34, CD117 and EMA (Colter DC *et al*, 2001; Pittenger MF *et al*, 1999; Young HE *et al*, 2001). Most importantly, AFSCs show the expression of Oct-4 mRNA and Oct-4 protein, a transcription factor expressed by embryonic cancer cells, embryonic stem cells, and embryonic germ cells, thus reflecting a key role in the maintenance of pluripotency of mammalian stem cells, *in vitro* and *in vivo* (Cogle CR *et al*, 2003; Henderson JK *et al*, 2002; Jiang Y *et al*, 2002).

Therefore, it is clear that human amniotic fluid contains a subpopulation of stem cells characterized by a high potential, that arise from embryonic and extra-embryonic tissues during the process of foetal development and growth.

The AFSCs can be expanded rapidly and can maintain the capability to differentiate towards multiple cell types *in vitro*. As mentioned above, their expression of the transcription factor Oct4 suggests that they represent an intermediate stage between pluripotent ESCs and lineage-restricted adult stem cells (De Coppi P *et al*, 2007).

It was observed indeed that AFSCs are able to differentiate along adipogenic, osteogenic, myogenic, endothelial, neurogenic and hepatic pathways. The acquisition of lineage-specific functionality was shown by AFSCs differentiated *in vitro* toward neurons, osteoblasts and hepatocytes. Unlike ESCs, AFSCs do not form tumors *in vivo* (De Coppi P *et al*, 2007).

Based on these findings, amniotic fluid may provide a promising alternative source for investigation of human “broadly multipotent” stem cells without raising the ethical controversies correlated with embryonic stem cells.

# *Bone regeneration*

- ***Bone composition***

Bone is a dynamic, highly vascularized connective tissue with the unique capacity to heal and remodel, depending on the line of stress (Jayakumar and Di Silvio, 2010). Bone is constituted by cells and an extracellular mineralized matrix within which the majority of cells are contained. Osteoblasts, along with osteocytes, osteoclasts, cells of the neurovascular supply, and cells of the periosteum, endosteum, and marrow, constitute the cellular component of bone (Jayakumar and Di Silvio, 2010).

*Osteoblasts* are cells originally derived from osteoprogenitor cells of mesenchymal origin located in bone marrow and connective tissues. They are responsible for forming bone by generating, secreting, depositing, and mineralizing bone matrix. Osteoblasts contain active cytoskeletal proteins for maintaining of their structural integrity as well as to facilitate motility and attachment to surfaces. Their contact interactions with the surrounding osteoblasts and osteocytes at intercellular gap junctions are achieved by the extensions created by their plasma membranes. Osteoblasts primarily synthesize and secrete organic matrix, predominantly collagen type I and trace quantities of collagen type V as monomers that undergo extracellular configuration as triple helical procollagen. They produce a variety of other factors and glycoproteins and also express genetic markers.

Osteoblasts deposit a premineralized bone matrix, osteoid, and subsequently facilitate its mineralization. The mineralization process involves supersaturation of extra-cellular fluids at local zones and increased osteoblastic alkaline phosphatase (ALP) activity, which raises local calcium and phosphate concentrations. Osteoblasts also produce osteocalcin, which binds calcium, further concentrating local calcium levels.

*Osteocytes* are essential bone maintenance cells derived from osteoblasts and are surrounded by newly formed osteoid matrix; they are spread within matrix, helping to preserve it, but are not as active as osteoblasts in the generation of osteoid and mineralized matrix.

*Osteoclasts* are the primary cells involved in bone resorption, by causing demineralization. They are specialized in local removal of bone during growth and during remodeling of osteons and bone surfaces. Osteoblastic formation and osteoclastic resorption are two sides of a finely "coupled" process in normal bone.

Premineralized matrix laid down by osteoblasts occurs in the early stages of bone formation. Osteoblasts produce most of the organic components of matrix, including collagenous and non-collagenous matrix components. Collagen synthesized by osteoblasts polymerizes from tropocollagen, at extracellular level. The molecular structure of collagen in bone is more covalently crosslinked than in other connective tissues, and crosslinking progresses with maturation, making the configuration stronger. Non-collagenous matrix components include proteoglycans, matrix proteins, growth factors, and cytokines. These complex macromolecules attach to collagen fibers and bone crystals. Among the non-collagenous matrix proteins are osteocalcin, osteonectin, osteopontin, and thrombospondin. Osteocalcin is required for the bone mineralization, by binding calcium and hydroxyapatite, and is a specific marker of bone formation. Osteonectin is a phosphorylated protein which binds to collagen and hydroxyapatite, and may be a factor in cell adhesion and initiating the crystallization of hydroxyapatite. Osteopontin and thrombospondin are bone sialoproteins and bind to integrins on osteoclasts, mediating their adhesion to bone surfaces before taking resorptive action.

Undifferentiated MSCs form osteoprogenitor cells which subsequently form osteoblasts in low-strain and high-oxygen-tension environments. Osteoprogenitor cells are also located in periosteum, endosteum, and Haversian canals and are in a standby state, ready for a stimulus to proliferate and differentiate into osteoblasts before forming bone (Jayakumar and Di Silvio, 2010).

- ***Osteoblastogenesis regulation and bone related proteins expression***

Bone formation is a tightly regulated process which is characterized by a sequence of events starting by the commitment of osteoprogenitor cells, their differentiation into pre-osteoblasts and in mature osteoblasts whose function is to synthesize the bone matrix that becomes progressively mineralized. Osteoblast commitment, differentiation and function are all governed by several transcription factors, resulting in expression of phenotypic genes and acquisition of the osteoblast phenotype (91 Marie, 2008).

**Runx2** (CBFA1) has been identified as the major transcription factor controlling osteoblast commitment and differentiation. It is a member of the Runt family of transcription factors, is expressed by mesenchymal cells at the onset of skeletal development and is present in osteoblasts throughout their differentiation (Marie PJ, 2008). Molecular studies and genetic manipulation of Runx2 in vivo indicated that the expression of Runx2 is both necessary and sufficient for mesenchymal cell differentiation towards the osteoblast lineage (Karsenty and Wagner, 2002; Komori T, 2006). As a member of the Runx family of transcription factors, Runx2 operates in bone lineage cells by binding to the Runx consensus sequence (PuACCPuCA), first named the osteoblast specific element.

The Runx regulatory element can be found in the promoter of all major osteoblast genes controlling their expression, including type I collagen alpha 1 chain, osteopontin, bone

sialoprotein and osteocalcin, resulting in the establishment of an osteoblast phenotype. In addition, Runx2 was found to negatively control osteoblast proliferation by acting on the cell cycle (Pratap J *et al*, 2003).

Recent studies indicate that Runx2 interacts with several regulatory proteins within the nuclear architecture, resulting in activation or repression of genes which control the program of osteoblast proliferation and differentiation (Stein GS *et al*, 2004). This indicates that Runx2 can control osteoblastogenesis through multiple mechanisms.

Molecular and genetic studies revealed that **Osterix** (**Osx**) is a zinc finger transcription factor specifically expressed by osteoblasts which is important for osteoblast differentiation. Osx-deficient mice show absence of osteoblasts and defective bone formation (Nakashima K *et al*, 2002). However, Runx2 is expressed in Osx-deficient mice, indicating that Osx acts downstream of Runx2.

Osx transcription is positively governed by Runx2 (Celil AB *et al*, 2005) and acts by directing pre-osteoblasts to immature osteoblasts. Although little is known on the mechanisms of action of Osx, this protein was found to form a complex with the nuclear factor of activated T-cells (NFAT), resulting in activation of COLIA1 promoter activity (Koga T *et al*, 2005). Accordingly, constitutive activation of NFAT activates the Wnt signaling pathway, bone formation and bone mass (Winslow MM *et al*, 2006).

Significantly, Osx modulate the expression of important osteoblast proteins such as OPN, OCN, bone sialoproteins and Col I. **Osteopontin** (**OPN**) - also known as secreted phosphoprotein-1 (SPP1), urinary stone phosphoprotein, uropontin, and early T-cell activator (ETA-1) - is a highly conserved multifunctional phosphorylated glycoprotein which is expressed in many mineralized and soft tissues including bone, dentin, elastin, muscle, tumors, and in body fluids (milk, inner ear, and urine). It is also a major

component of the calcium carbonate–containing eggshell (Gericke A *et al*, 2005). In bone, OPN is produced by osteoblastic cells at various stages of differentiation, by differentiated osteoblasts and osteocytes, and also by osteoclasts. Studies on the temporal expression of OPN during the formation of bone *in vitro* and during the formation of intramembranous and endochondral bone *in vivo*, have revealed a pattern in which OPN is produced early in the osteogenic differentiation, in immature osteoblasts (McKee and Nanci, 1995), and then it is progressively down-regulated according to its role of mineralization inhibitor during the bone development (Hunter GK *et al*, 1996).

**Osteocalcin** (**OCN**) is the major bone-specific protein, produced by mature osteoblasts, and it is expressed only post-proliferative phase, during the differentiation of normal diploid osteoblasts, when mineralization of extracellular matrix occurs. OCN is the most abundant non-collagenous protein that constitutes 1-2% of the total bone protein or 20% of the non-collagenous protein, and is the seventh most abundant protein of the human body. The biological function of OCN has been correlated with bone turnover, and it has been used as a biochemical marker for the clinical evaluation of bone metabolism. Transcription of the OCN gene is controlled by modulatory organized basal regulatory sequences and hormone responsive enhancer elements: these include the TATA box, which contain sequences for glucocorticoid receptor binding.

Interestingly, many growth factors were found to control osteoblastogenesis by modulating transcription factors expression or activity. Notably, bone morphogenetic protein (BMP)-2 promotes Runx2 expression in mesenchymal osteoprogenitors and osteoblastic cells (Lee KS *et al*, 2000; Lee MH *et al*, 2003a; Haÿ E *et al*, 2004). BMP2 also promotes Osx and Dlx5 expression in osteoblastic cells (Miyama *et al*, 1999; Lee MH *et al*, 2003b). Although transforming growth factor – β (TGF-β) was found to inhibit Runx2 activity in vitro (Kang

JS *et al*, 2005), TGF-β promotes bone formation in vivo by increasing Runx2 and decreasing PPARγ2 expression (Ahdjoudj S *et al*, 2002). Another anabolic factor, insulin-like growth factor-1, promotes Osx in osteoblastic cells (Celil and Campbell, 2005). The anabolic factor fibroblast growth factor-2 (FGF-2) increases Runx2 phosphorylation and activity (Kim HJ *et al*, 2003). Consistently, activation of fibroblast growth factor receptor 2 (FGFR2) expression or signaling results in increased Runx2 expression and enhanced osteoblast differentiation (Guenou H *et al*, 2005; Chen L *et al*, 2003; Tanimoto Y *et al*, 2004).

- ***Tissue engineering as a novel approach for bone healing***

The majority of bone lesions, such as fractures, heal well under standard conservative or surgical therapy. Severe bone defects can be treated through bone grafting, distraction osteogenesis, or the use of bone-replacing materials.

The basis of the understanding of bone regeneration are some fundamental principles.

***Osteoinduction*** is the ability to induce bone formation by attracting and stimulating bone forming cells of the recipient. This phenomenon features in most of bone healing processes, and osteoinductive materials provide a biological stimulus for induction, recruitment, stimulation, and differentiation of primitive, undifferentiated, and pluripotent stromal cells into osteoblasts or preosteoblasts, the initial cellular phase of a bone-forming lineage. Osteoinductive materials include autografts, demineralized bone matrix, and specific BMPs that naturally form bone within the skeleton as well as extra-skeletally (Hotz and Herr, 1994).

***Osteoconduction*** describes the capacity of a material to guide bone-forming tissue from the periphery to a given defect. Osteoblasts from the edges of the defect that is being

grafted use the bone graft material as a framework upon which to spread and generate new bone, by taking advantage of the composition, shape, and surface texture of the graft material. Examples of exclusively osteoconductive biomaterials are hydroxyapatites (Cornell and Lane, 1998).

***Osteogenic*** materials are defined as those that contain living cells and are capable to form bone.

***Osseointegration*** is the process of achieving stable direct anchorage and contact between bone and implant. This process was first described by Brånemark as "direct structural and functional connection between ordered, living bone and the surface of a load-carrying implant" (Brånemark PI, 1983).

It has also been defined at the histological level as the direct anchorage of an implant by formation of bone tissue around the implant without the growth of fibrous tissue at the bone‑implant interface.

Nowadays, despite the current therapeutic measures, large bone defects, due to traumas, infections or cancer, represent a primary problem in orthopaedic, trauma and craniofacial surgery. Autologous bone grafting is currently considered as the 'gold standard' for osteogenic bone replacement (118 Gazdag AR *et al*, 1995). It is highly efficient in filling defects, even under problematic conditions. However, the use of autologous bone grafts is limited by a significant donor-site morbidity, and by the need for vascularization which increases with the amount of bone transplanted. Moreover, bleeding, haematoma, infection, and chronic pain are common complications of bone-graft harvest, and occur in up to 30% of such procedures (Hill NM *et al*, 1999; Seiler and Johnson, 2000).

Allogenic bone can also be used for the repair of bone defects when autologous bone grafts are not available (Gazdag AR *et al*, 1995). The initial biomechanical properties resemble

the autologous bone grafts, but the allogenic bone is not osteogenic and, therefore, is less efficient in bone formation than the autologous bone.

Lastly, synthetic materials are mainly used as bone cements, in fact they are widely used for the fixation of total joint prosthesis and for craniofacial bone defects. Nevertheless, these materials are neither degraded nor integrated over time, hence long-term results are not always satisfying.

Bone tissue engineering is a state-of-the-art science designed to overcome the issues related to the conventional approaches. A successful bone tissue engineering requires a well coordinated interaction between cells, growth factors, and scaffold biomaterials in order to produce a skeletal tissue environment that is biologically and mechanically consistent with the original. MSCs have been widely used in experimental bone tissue engineering and currently hold promise for fracture repair and treatment of critical mass bone defects, osteoporosis, tumours, osteonecrosis (Panetta NJ *et al*, 2009; Lee K *et al*, 2009; Gordeladze JO *et al*, 2009; Clines GA, 2010; Bernstein P *et al*, 2009; Abdallah and Kassem, 2008; Oreffo and Triffitt, 1999).

In particular, *in vitro* expanded MSCs are commonly combined with a scaffold complex in order to be implanted at a given site, and the choice of the scaffold type itself relies on the site of application.

While materials intended for implantation were in the past designed to be "bio-inert", the focus has been recently shifted toward the design of deliberately 'bioactive' materials that integrate with biological molecules or cells and regenerate tissues (Langer and Vacanti, 1993; Hench and Polak, 2002). In the case of bone, optimal materials should preferably be both osteoinductive, osteoconductive and capable of osseointegration.

Many bone substitute materials intended to replace the need for autologous or allogeneic bone have been evaluated over the last two decades. In general, they consist of either bioactive ceramics, bioactive glasses, biological or synthetic polymers, and composites of these (Hench and Polak, 2002; Kretlow and Mikos, 2007; Liu and Czernuszka, 2007). The ideal basic premise, if following the tissue engineering paradigm, is that the materials will be resorbed and replaced over time by, and in tune with, the body's own newly regenerated biological tissue (Langer and Vacanti, 1993).

## *Substitutes for foetal bovine serum as supplements to culture media*

Recently, researchers and clinicians have addressed great interest on the identification of the optimal conditions for cell culture medium. The development of techniques to grow *in vitro* human mesenchymal stem cells, and the deeper understanding of cell differentiation pathways have expanded the horizon of prospective therapeutical applications. The level of concern about potential contamination and damage to cells/tissues depends on how and how much they have been handled and manipulated. When stem cell-based products involve more than minimal manipulation - such as expansion or differentiation - the cells are usually grown in *in vitro* culture, and the standard culture medium contains bovine serum. This common process, which involves the use of non-human serum, may be the source of possible contamination or immune reaction to foreign proteins, such as xenogenic bovine serum antigens, underlining the necessity for a more careful evaluation of culture media.

Foetal bovine or calf serum (FBS and FCS, respectively) are the most widely used growth supplements for cell culture, primarily because of their high levels of growth stimulatory factors and low levels of growth inhibitory factors. At the moment, most isolation and expansion protocols for clinical scale production of stem cells use culture media frequently supplemented with FCS, which may contain potentially harmful xenogeneic compounds. In particular, bovine serum proteins may be internalized in stem cells stimulating immunogenicity (Martin MJ *et al*, 2005; Spees JL *et al*, 2004); consequently, immunological reactions may occur due to the bovine protein attachment to cells in culture that act as antigenic substrates once transplanted (Heiskanen A *et al*, 2007). A wide variety

of polypeptides contained in bovine serum may attach to cells, inducing significant metabolic/morphologic changes; for example, bone marrow derived mesenchymal progenitor cells show a different behavior in culture performed in the presence of FCS with or without heat inactivation (Bruinink A *et a*l, 2004); a significant antibody response to bovine lipoproteins is observed using autologous T cells grown in FCS-supplemented medium (Tuschong L *et al*, 2002), linked to the observation that FCS contains a low density lipoprotein, which, in turn, binds to its human receptor (Haylett and Moore, 2003). These evidences allow to assume that FCS is unsuitable for the culture of stem cells to be used in cellular therapy, suggesting that cell culture medium composition should not include animal products (Dimarakis and Levicar, 2006; Sekiya I *et al*, 2002).

Several studies are in progress in order to define safer culture conditions for stem cells, indeed it is believed that the serum replacement medium should ideally be replaced by one containing only human proteins, hormones and growth factors.

Cultivation in serum-free conditions has demonstrated contradictory results, and no breakthroughs have yet been reached. In particular, some studies revealed that human MSCs from bone marrow and umbilical cord blood were efficiently ex vivo expanded in serum-free medium (Liu CH *et al*, 2007; Pochampally RR *et al*, 2004). Nevertheless, additional studies demonstrated that serum-free media cannot promote human MSCs growth without the addition of cytokines and/or growth factors (Gronthos and Simmons, 1995; Kuznetsov SA *et al*, 1997), likely due to the fact that serum induces intracellular calcium oscillations, which are vital to stem cell proliferation and differentiation (Foreman MA *et al*, 2006).

Using human blood derivatives should eliminate or reduce the risk of secondary effects due to FCS constituents. Various human plasma derivatives have been purposed as

alternatives to FCS to supply nutrients, adhesion and growth factors. These alternative supplements include autologous or allogenic human serum, human plasma, human platelet lysates and their released factors (Schallmoser K *et al*, 2007; Stute N *et al*, 2004; Kocaoemer A *et al*, 2007).

In the last years platelet-rich plasma was largely used to grow and differentiate MSC but its role in MSC osteogenic differentiation is debated (Feng Y *et al*, 2010; Gruber R *et al*, 2006; Tomoyasu A *et al*, 2007). Human platelet lysate and platelet-rich plasma have been shown to be suitable culture supplements to replace FCS, for the expansion and *in vitro* osteogenic differentiation of hDPSCs (Lee UL *et al*, 2011; Govindasamy V *et al*, 2011).

Apart from human plasma, in which have been identified factors that might be relevant for the differentiation of adipocyte precursor cells (e.g., growth hormone and insulin) (Sypniewska G *et al*, 1987), many studies have focused the attention on the use of autologous serum. Several papers evidenced that autologous serum/plasma preserves the differentiation capacity, promotes a greater cell amplification, and enhances motility of stem cells, improving cellular and genetic therapies (Shahdadfar A *et al*, 2005; Oreffo RO *et al*, 1997; Yamamoto N *et al*, 2003; Stute N *et al*, 2004; Lin HT *et al*, 2005; Kobayashi T *et al*, 2005; Gregory CA *et al*, 2006; Mizuno N *et al*, 2006); evidence in favour of using autologous serum exists for the expansion of human bone marrow MSCs, indeed it results as effective as supplementing the culture medium with FCS, and cells appear transcriptionally more stable over many cell doublings (Shahdadfar A *et al*, 2005). On the other hand, autologous serum might be a scarcely available or qualitatively affected source, particularly in the case it must be taken from some classes of patients, such as children, elderly, anaemic and with ongoing inflammatory processes individuals (Tateishi K *et al*, 2008).

Therefore, allogenic human serum, potentially from blood type AB healthy donors, might be investigated as a preferable alternative to autologous serum for the expansion and differentiation of human MSCs.

## *Skeletal muscle regeneration*

- ***Structure of skeletal muscle***

Skeletal muscles are composed of multinucleated myofibers organized into fascicles that are grouped together to form individual muscles. Individual myofibers develop by end-to-end fusion of mononucleate myoblasts to form a multinucleate syncytium. Each mononucleate cell is a contractile unit known as sarcomere, bounded by Z discs, that is, structures anchoring the actin filaments into each end of the sarcomere. The sarcomere shortens when the actin filaments slide along the thick myosin filaments toward the middle of the sarcomere (Ham and Cormack, 1987; Alberts B *et al*, 2008). Each myofiber is wrapped by endomysium, multiple myofibers are organized into fascicles surrounded by perimysia, and the fascicles collectively constitute the muscle, which is surrounded by an epimysium (Figure 6).

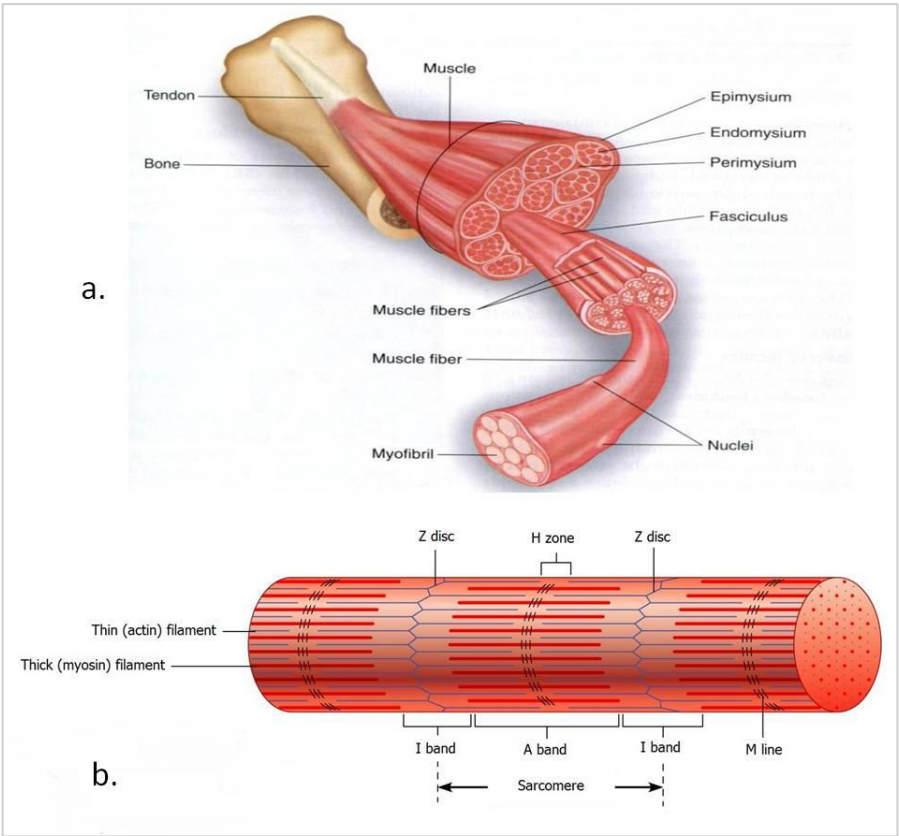



**Figure 6:** a. skeletal muscle structure; b. contractile unit of a myofibril.

Skeletal muscles are highly vascularized within and between the mysial sheaths and are heavily innervated at specialized contacts called neuromuscular junctions. At their ends, muscles grade into fascia or tendons, which attach them to bone. Fibroblasts of the endomysium synthesize an extracellular matrix consisting of a typical basement membrane made of fibronectin, laminin, and collagen IV that surrounds each myofiber, as well as collagen I and sulfated proteglycans outside the basement membrane, including a muscle specific sulfated proteoglycan (Caplan AI, 1991). In addition, the endomysium synthesizes tetranectin, a protein that acts as an interactive agent with other extracellular matrix proteins, cell surface receptors, cytokines, and proteases (Wewer UM *et al*, 1998). Tetranectin binds sulfated polysaccharides, suggesting that it might interact with the GAG chains of proteoglycans. It is particularly prominent at myotendinous junctions, which are considered to be the equivalent of focal adhesion sites in muscle (Wewer UM *et al*, 1998). The actin cytoskeleton of the myofibers is linked to the extracellular matrix by a dystrophin-glycoprotein complex (DGC), a multi-subunit complex comprised of intracellular dystrophin and syntrophins, and three types of sarcolemmal proteins: dystroglycans, sarcoglycans, and sarcospan. Disruption of this linkage by mutations in dystrophin or the sarcoglycans results in sarcolemmal damage during contraction, rendering the myofibers susceptible to necrosis, which is a feature of muscular dystrophy (Cohn RD *et al*, 2002).

- ***Muscle development (myogenesis)***

In vertebrates, muscle development occurs in three distinct phases: embryonic, fetal and postnatal. During embryonic development, muscle progenitors are derived from somites, which are compartmentalized into dermomyotomes (giving rise to skeletal muscle and

parts of the dermis) and sclerotomes (from which bones and ribs are derived). Expression of the paired-box/homeodomain transcription factors Pax3 and Pax7 marks myogenic stem and progenitor cells within the developing dermomyotome (Kassar-Duchossoy L *et al*; 2005; Relaix F *et al*, 2005). These Pax3/7-positive cells initiate expression of muscle regulatory factors (MRFs), including Myf5 (myogenic factor 5) Mrf4 (Myf6), MyoD (Myod1, myogenic differentiation 1) and Mef2c (myogenic enhancer factor 2C), which drives progenitors to the muscle lineage (Rudnicki MA *et al*, 1993; Edmondson DG *et al*, 1994; Kassar-Duchossoy L *et al*, 2004). Subsequently, the muscle progenitors exit the cell cycle, express the transcription factor myogenin and fuse to form multinucleated myotubes (Buckingham and Vincent, 2009). At late fetal stages, Pax3/7-positive cells relocalize to a specific microenvironment adjacent to muscle fibers under the basal lamina (Kassar-Duchossoy L *et al*, 2005). These cells, which originate from the Pax3/7 positive cells of the dermomyotome, persist in a quiescent state in the adult, where they are known as **satellite cells** (SC) and marked by continued expression of Pax7 (Seale P *et al*., 2000; Schienda J *et al*., 2006) (Figure 7).

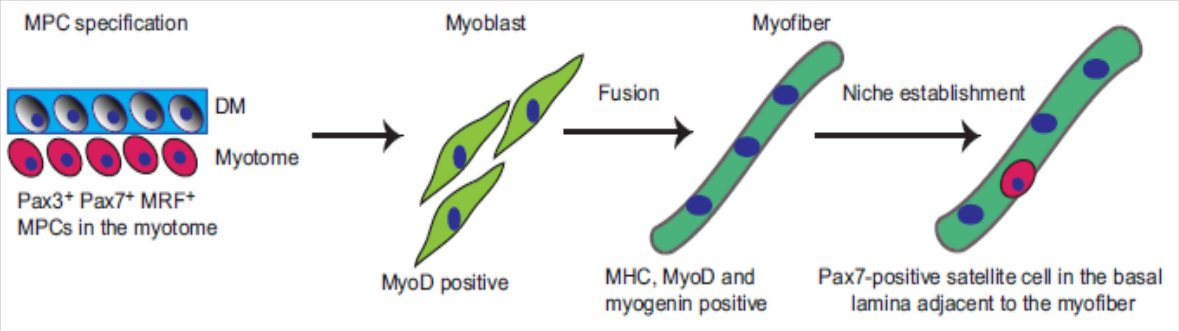



**Figure 7.** During myogenesis $Pax3^{+}Pax7^{+}$ muscle progenitor cells (MPCs) give rise to undifferentiated MyoD-positive myoblasts. Further fusion of myoblasts generates multinucleated myofibers expressing differentiation markers such as myosin heavy chain (MHC), MyoD and myogenin. The basal lamina adjacent to differentiated myofibers acts as a niche for satellite cells during postnatal myogenesis. DM, dermomyotome *(figure revised from Koch U et al, 2013).*

Recent studies have shown that Pax7-positive satellite cells exhibit SC properties as they give rise to viable muscles when transplanted into the tibialis anterior muscles of Pax7DTR/– mice (Sambasivan R *et al*, 2011). Moreover, these cells are crucial for muscle regeneration: injury drives quiescent satellite cells back into the cell cycle, inducing myoblast proliferation and differentiation, and hence regeneration of lost muscle fibers. During this process, new satellite cells are formed to replenish the SC pool and ensure muscle cell homeostasis (Sambasivan and Tajbakhsh, 2007; Brack and Rando, 2012).

Injured muscle tissue fails to regenerate following ablation of Pax7-positive satellite cells, highlighting the role of these cells in muscle regeneration (Sambasivan R *et al*, 2011).

- ***Molecular mechanisms modulating satellite cells: quiescence, activation, replenishment***

The activation of satellite cells is a multistep process. Ultimately, the activating signals received by quiescent satellite cells lead to the transition from G0 to G1 phase of the cell cycle, i.e. exit from the quiescent state. Once the satellite cells have entered into their first cell cycle, they proceed through a highly proliferative intermediate progenitor stage, characterized by high levels of Pax3 expression (Conboy and Rando, 2002). From this stage, the cells progress to the myoblast stage, thus completing the lineage progression from the quiescent, undifferentiated progenitor to the cell that will undergo myogenic differentiation and fusion.

A primary candidate for inducing satellite cells to break quiescence is the hepatocyte growth factor (HGF)–c-met pathway as HGF is present in an inactive form in the extracellular matrix surrounding satellite cells and c-met is expressed on quiescent satellite cells (Cornelison and Wold, 1997; 149 Tatsumi R *et al*, 1998). However, several factors,

both in vivo and in vitro, stimulate satellite cells and might serve as redundant pathways. Different members of the fibroblast growth factor (FGF) family are present in muscle tissue and released upon injury (Johnson and Allen, 1995), and various FGF receptors, notably FGF-R1 and FGF-R4, are expressed by quiescent satellite cells (Cornelison DD *et al*, 2001; Kästner S *et al*, 2000). The activation of the Notch signaling pathway is also important for satellite cell activation as inhibiting Notch signaling prevents satellite cell activation and proliferation (Conboy and Rando, 2002). This pathway is activated in muscle in response to injury by the upregulation of the Notch ligand, Delta-1, thus activating the Notch receptor that is expressed on satellite cells and inducing cell proliferation. One particularly intriguing finding is that Delta is upregulated on myofiber membranes distant from a muscle injury site (Conboy IM *et al*, 2003), and this could explain the "activation at a distance", that has been long recognized to be a property of satellite cells (Schultz and McCormick, 1994).

It is likely that the satellite cell niche is not only important in maintaining quiescence but also in determining the kinetics of satellite cell activation. This might be particularly relevant to the effect of aging on satellite cell activation. It has long been known that satellite cells isolated from aged animals display a delay of cell cycle entry in vitro (Schultz and Lipton, 1982). However, once activated, the cells proliferate normally. Furthermore, the age-related decline in satellite cell activation can be modified by extrinsic influences (Conboy IM *et al*, 2003; Conboy IM *et al*, 2005), indicating that the cellular environment profoundly influences the behavior of quiescent satellite cells. These findings suggest that the age-related decline in tissue regenerative capacity might not be due to irreversible changes in satellite cells but, rather, to modifiable changes in the stem cell niche. Once activated, satellite cells undergo rapid proliferation and progress along a

myogenic lineage. The proliferative expansion of the intermediate progenitor population - characterized by declining levels of CD34 and high levels of Pax3 expression - is strongly regulated by the Notch signaling pathway (Conboy and Rando, 2002). Inhibition of Notch signaling induces exit from this progenitor stage to the myoblast stage, and constitutively high levels of Notch signaling maintain cells in the intermediate progenitor stage.

The number of satellite cells in an adult muscle remains relatively constant through repeated bouts of injury and regeneration (Schultz and McCormick, 1994), thus assuring a sufficient reserve for future needs (Schultz, 1996). It has long been assumed that replenishment comes from the ability of satellite cells to self-renew. Although direct evidence has been lacking, in a recent publication, definitive evidence of satellite cell self-renewal was demonstrated by the transplantation of single fibers with their associated satellite cells from one mouse strain to another (Collins CA *et al*, 2005): using a genetic lineage marker, the donor satellite cells were shown to expand and generate new satellite cells in the host, adopting the appropriate anatomical position, expressing the appropriate biochemical markers and, most importantly, mediating subsequent regenerative responses in the host. The molecular mechanisms that regulate satellite cell self-renewal remain to be determined.

One interesting question concerning the transition of myogenic progenitor cells from a proliferative state to G0 phase is the role of MyoD expression. The levels of MyoD fluctuate through the cell cycle, with the highest levels observed during G1, where it can trigger differentiation in a mitogen-poor environment (Kitzmann M *et al*, 1998). Given the strong ability of MyoD to induce myogenic differentiation, it is clear that there are potent inhibitory activities that maintain the cells in a proliferative state in the presence of high levels of MyoD (Zhang JM *et al*, 1999). If proliferative signals are present, MyoD is kept

inactive (Wei and Paterson, 2001), the progression to S phase and the loss of signal-responsiveness follow, and MyoD expression declines. On the contrary, in response to differentiation cues during G1, cells exit the cell cycle, MyoD levels increase, and the differentiation-inducing activity of MyoD becomes evident with the upregulation of key targets, such as the cyclin-dependent kinase inhibitor p21, cyclin D3, Rb and myogenin, that combine cell cycle exit to tissue-specific gene expression (Kitzmann and Fernandez, 2001). The increase of MyoD, myogenin and other MRFs initiates the terminal myogenic differentiation program by upregulating genes such as those encoding creatine kinase, myosin heavy chain and acetylcholine receptor subunits that underlie the functional properties of mature muscle (Hawke and Garry, 2001). Hence, withdrawal from the cell cycle during differentiation represents a transition that assures both irreversible arrest and myogenic differentiation. On the other hand, the entry in G0 phase, following quiescence-inducing stimuli, does not involve either irreversible arrest or myogenic differentiation. In this case, MyoD declines instead of increasing, and there is no induction of MyoD differentiation-inducing activity or targets such as p21, Rb, or other MRFs (Milasincic DJ *et al*, 1996; Sachidanandan C *et al*, 2002). A general conclusion is that there is a different constellation of growth-suppressive molecules in reversibly, versus irreversibly, arrested cells.

- ***Muscular dystrophy***

Muscular dystrophy is a diverse set of genetic myopathies characterized by progressive muscle weakness, atrophy, and replacement of myofibers by fat and scar tissue (Bushby K, 2000; Burton and Davies, 2002). Several forms of the disease are caused by mutations in components of the dystrophin-glycoprotein complex (DGC), which links the cytoskeleton

of the myofiber to the basement membrane. The DGC plays an important role in protecting the sarcolemma against muscle contraction-induced injury. There are three mouse models of muscular dystrophy, the dystrophin-deficient *mdx* mouse, the β or γ sarcoglycan-deficient (SGD) mouse, and the muscle creatine kinase-dystroglycan (MCK-DG) null mouse, in which the dystroglycan gene is inactivated by a *Cre-lox* construct driven by the MCK promoter (Cohn RD *et al*, 2002). All these models maintain muscle mass by regeneration from satellite cells during the early stage of the disease, but since the SCs carry the genetic defect as well, the regenerated *mdx* and SGD mouse myofibers are dystrophic too. Eventually the muscles exhaust their pool of SCs and become fatty and fibrotic (Cossu and Mavilio, 2000; Heslop L *et al*, 2000).

Recent achievements in the treatment strategy for DMD - which affects nearly 1 in 3000 male births annually - have been encouraging. Clinical trials for exon skipping have yielded promising results in several dystrophic patients (Wood MJ *et al*, 2010); however, studies attempting muscle stem cell engraftment have been notably disappointing in the clinical scenario (Skuk and Tremblay, 2011).

Failure of satellite cell integration into the dystrophic or diseased muscle could be attributed to the limited ability of the human tissue to home grafted cells, an event that probably does not occur in experimental murine models for dystrophies, where the implanted satellite cells not only just integrated and produced myofibers in the degenerative tissue, but also led to the production of dystrophin protein which was intrinsically lacking in the *mdx* mice. Importantly, the grafted satellite cells also yielded donor-derived satellite cells in the engrafted muscle, proving their stem cell ability of self renewal and niche establishment (Sacco A *et al*, 2008; Cerletti M *et al*, 2008; Partridge TA *et al*, 1989). The fairly poor yield of functional muscle in clinical studies following cell

replacement has been also proposed to be due to loss of stemness by satellite cells expanded in culture systems, a consequence that appear to be related to their dependence on their niche (Cosgrove BD *et al*, 2009). Recent findings demonstrated that satellite cells cannot currently be isolated and expanded *in vitro* since they rapidly differentiate into myoblasts, the more committed muscle precursors marked by the irreversible expression of Myf5, MyoD, and myogenin (Boonen and Post, 2008). Another issue in such therapeutic settings is the small amount of satellite cells that can be collected versus the difficulty of grafting several hundreds of muscles all over the body.

Initial clinical trials with allogeneic myoblasts injection into the muscles of non-immunosuppressed DMD patients indicated a transient restoration of dystrophin positive fibers and improvement of muscle strength, which then was lost (Law PK, 1982; Huard J *et al*, 1992; Karpati G *et al*, 1993; Mendell JR *et al*, 1995; Gussoni E *et al*, 1992; Beauchamp JR *et al*, 1997).

Reasonably, the limits emerged from these therapeutical approaches, have triggered extensive research over the years. One of the attempted approaches has been to transplant bone marrow cells into the dystrophic muscle (Saito T *et al*, 1995; Ferrari G *et al*, 1998; Gussoni E *et al*, 1999): the bone marrow MSCs showed indeed the capacity to differentiate into muscle, and were also reported to be non-immunogeneic, since they do not constitutively express MHC class II antigens or the T-cell co-stimulatory molecule B7 (McIntosh and Bartholomew, 2000). More recent studies have allowed to identify several cell sources that may act as potential muscle stem cells, either within the muscle tissue itself or in other tissues (Mitchell KJ *et al*. 2010; Sampaolesi M *et al*. 2003; Torrente Y *et al*. 2006).

# MATERIALS AND METHODS

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## *1.1 In vivo implantation of stem cells-scaffold constructs*

### Cell isolation from human dental pulp and amniotic fluid

Cells were isolated from dental pulp as formerly described by Laino *et al*. (Laino G *et al*, 2006). Human dental pulp was extracted from third molar or permanent teeth of adult subjects after informed consent of patients undergoing routine extractions. Dental pulp was removed from the teeth and then immersed in a digestive solution (3 mg/mL type I collagenase plus 4 mg/mL dispase in α-MEM) for 1 h at 37°C. Once digested, pulp was dissociated and then filtered through 100 μm Falcon Cell Strainers to obtain a cell suspension. Cells were then plated in Petri dishes and cultured in culture medium (α-MEM with 20% FBS, 100 μM 2P-ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin; all from Invitrogen), at 37°C and 5% $CO_2$.

Supernumerary amniocentesis samples were collected with informed consent of the patients according to Italian law and ethics committee guideline. Amiotic fluid stem cells were isolated as previously described by De Coppi *et al*. (De Coppi P *et al*, 2007): backup human amniocentesis cultures were harvested by trypsinization.

After isolation, the cells obtained from human dental pulp (hDPSCs) and amniotic fluid (hAFSCs) underwent magnetic cell sorting by using MACS technology (Miltenyi Biotec) and the STRO-$1^+$ hDPSCs and c-$kit^+$ hAFSCs sub-populations were obtained.

Following the immune-selection, hDPSCs and hAFSCs were expanded in the culture medium described above.

## Preparation of fibroin scaffolds

Sponges with randomly distributed pores have been produced by solvent casting particulate leaching starting from fibroin water solution, as follows. Bombyx mori cocoons purchased from Cooperativa Sociolario were degummed by treating twice with 1,1 g/l $Na_2CO_3$ water solution at 98°C for 1 h, washing in deionized water, and air-drying. After degumming, silk was dissolved in 9.3M (20% w/v) LiBr (Sigma-Aldrich) at 65°C for 2 h. To eliminate LiBr, the solution was then dialyzed in a Slide-ALyzer Cassette (Pierce; MWCO3500 Da) against distilled water for 3 days; the fibroin concentration was afterward adjusted to 8% w/v. Silk fibroin scaffolds were prepared by adding 8 g of granular NaCl (particle size ≈ 315–425 μm) into 4 ml fibroin solution, and then poured in cylindrical polystyrene plates (3,5 cm in diameter and 1.5mm in height). The containers were covered, left at room temperature (RT) for 24 h in order to induce gelation, and then immersed in deionized water for 3 days with water change every 6 h in order to remove the salt (Wang Y *et al*, 2010). All samples were sterilized by Cobalt-60 gamma rays using facilities and control procedures validated in conformity with UNI EN ISO 9001, UNI EN ISO 13485, UNI EN ISO 11137, and GMP by Gammarad Italia S.p.A. with a standard cycle of 25.00 kGy and a dosimeter batch Red Perspex 4034.

## Scaffold's physical characterization

Environmental scanning electron microscopy. The morphology of the silk fibroin scaffolds was evaluated using the Field Emission-Scanning Electron Microscope (FE-SEM) SUPRA 40 (Zeiss Company). Samples were completely dried at RT and gold sputtered before analysis. Images were acquired at 2 kV.

## Water content and open porosity

For water absorption evaluation, three specimens of each material were cut in disks (1 cm in diameter), weighed in dry state ($M_{dry}$), and then immersed in distilled water for 2 days at RT.

Samples were then blotted dry on filter paper to remove excess water and weighed ($M_{wet}$). The percent water content, $W_{water}$ referred to the weight of the wet sample, was calculated as:

$$W_{water} = \frac{M_{wet} - M_{dry}}{M_{wet}} \times 100$$

The open porosity of the SF sponges was quantitatively evaluated on three samples using the principle of liquid displacement. Hexane (n-Hexane; Sigma-Aldrich) was used, since it is able to permeate the completely submerged fibroin scaffold without swelling. The scaffold was immersed in a known volume of hexane $V_1$ in a graduate cylinder for 10 minutes. The volume $V_2$ of the hexane and the sponge was measured. Finally, the sponge was removed and the residual volume of hexane $V_3$ was registered. The total volume of the scaffold can be obtained as follows: $V= (V_2 - V_1) + (V_1 - V_3) = V_2 - V_3$, where $V_2 - V_1$ is the volume of the bulk structure of the sponge and $V_1 - V_3$ is the volume of the hexane retained in the open porosity of the scaffold. The porosity can be calculated from the following equation:

$$\varepsilon(\%) = \frac{V_1 - V_3}{V_2 - V_3} \times 100$$

This ratio measures the percentage of the free volume of the scaffold with respect to the total volume of the scaffold.

## Cell seeding on fibroin scaffolds

Discs with a height of 1,5mm and diameter of 13mm were cut from fibroin scaffolds and placed in 24-well tissue culture plates. All the scaffolds were then washed twice with culture medium (1 h for each rinse). Cells were seeded on each scaffold at density of 1500 cell/$mm^3$ and cultured for 24 h with 2 ml of culture medium. After 24 h culture medium was replaced by osteogenic medium (culture medium supplemented with 10% FBS, 100 nM dexamethasone, 10mM β-glycerophosphate, and 50 μg/ml ascorbic acid-2-phosphate; all from Sigma-Aldrich). Cells/scaffolds cultures were then maintained in osteogenic medium for 10 days in an incubator at 37°C with 5% $CO_2$, in order to obtain a cell population committed to osteogenic differentiation yet still able to proliferate. Some samples were stained with 6-CFDA (Sigma-Aldrich) to test the viability of seeded cells.

## Surgery and transplantation procedure

To study the bone-forming ability of hAFSCs and hDPSCs on fibroin scaffolds, a critical-size bone defect was obtained by a full-thickness dissection of both the internal and external tables of compact bone and the trabecular diploe constituting the parietal skeletal segment, according to previous studies (de Mendonça Costa A *et al*, 2008; Kim J *et al*, 2007). Constructs were then implanted into a critical-size parietal defect up to 4 weeks (Figure 8 and Table 1). None of the animals died of infection or any other complication.

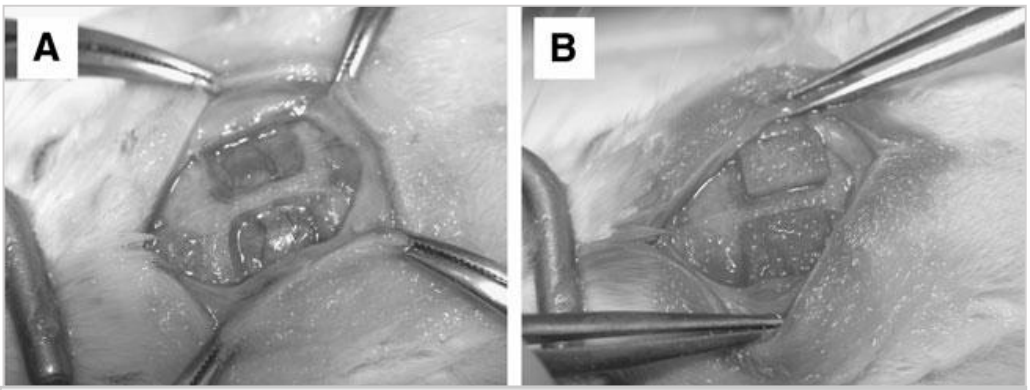


**Figure 8.** Cranial defects (5 x 8mm) induced in the parietal cranial bones in the rat with (B) or without (A) cell/scaffold implant.

**Table 1.** Description of the treatment groups of impant.

| | *Left parietal* | *Right parietal* |
|---|---|---|
| I group (5 rats) | Fibroin | Fibroin + DPSC |
| II group (5 rats) | Fibroin | Fibroin + AFSC |
| III group (5 rats) | Bone defect | None |

In this study outbred male Sprague-Dawley rats with age ranging between 12 and 14 weeks (Charles River Laboratories) were used. The animals were anesthetized with an intraperitoneal injection (0,2 ml/100 g of body weight) of ketamine hydrochloride (5%). A midline skin incision was performed from the nose-frontal area to the external occipital protuberance.

The skin and underlying tissues, including the periosteum, were reflected laterally to expose the full extent of the calvaria. Two symmetric full-thickness cranial defects of 5 x 8mm size were done on each parietal region of 15 animals by a micromotor drill under constant irrigation with sterile physiological solution to prevent overheating of the bone. The underlying dura mater was undisturbed (Figure 8). One scaffold of 5 x 8 x 1.5mm size

was implanted into each cranial defect and adapted to fill the entire defect area (Figure 8). Each animal received two constructs except for the animals of group III. After the scaffold implantation, the incisions were sutured with prolene 4-0 sutures (Ethicon). Animals were immunocompromised by means of Cyclosporine A at a dosage of 15mg/kg body weight (b.w.), administered 4 h before transplantation and then daily for 2 weeks.

During the last 2 weeks the daily dosage was reduced gradually up to 6 mg/kg b.w. Four weeks later the rats were sacrificed and the calvarias were rapidly explanted and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 3 h. All animal procedures were performed according to the guidelines approved by the Committee of Use and Care of Laboratory Animals of the University of Modena e Reggio Emilia.

## Radiography

Explanted calvaria samples were radiographed by a Kodak RVG Digital Radiography Systems.

## Histology

Fixed samples were decalcified in 0,5M EDTA (pH 8.3), then rinsed in PBS, dehydrated with graded ethanol, diaphanized, and embedded in paraffin. Transversal serial sections (10 μm thick) were cut through the parietal bones containing the implants. Routine hematoxylin/eosin (H&E) staining was performed in order to analyze the morphological details. Images of histological samples were obtained by a Zeiss Axiophot microscope equipped with polarizer filters and with a Nikon DS-5Mc CCD color camera.

## Confocal microscopy

Histological sections were processed as previously described (Maraldi T *et al*, 2009). Mouse anti-human mitochondria (Millipore) diluted 1:100 in PBS containing 3% bovine serum albumin (BSA) for 1 h at RT was used as primary antibody (ab). Secondary ab was diluted 1:200 in PBS containing 3% BSA (sheep anti-mouse-FITC; Jackson Immuno Research). After washing in PBS samples were stained with 1 µg/ml DAPI in PBS for 3 minutes and then mounted with anti-fading medium (0,21 M DABCO and 90% glycerol in 0,02 M Tris, pH 8.0). Negative controls consisted of samples not incubated with the primary Ab.

Confocal imaging was performed on a Leica TCS SP2 AOBS confocal laser scanning microscope as previously described. The confocal serial sections were processed with ImageJ software to obtain 3D projections (Maraldi T *et al*, 2009). The image rendering was performed by Adobe Photoshop software.

## *1.2 Human serum for osteogenic differentiation of human DPSCs*

### Preparation of human serum

Human serum was obtained from a blood collection by healthy male volunteers who underwent written informed consent. The whole blood was drained into 8 ml tubes containing silica beads for clot activation (Greiner Bio-one), then was stored at room temperature for 3 hours and centrifuged at 1400 x *g* for 15 minutes, to separate erythrocytes and coagulum contents. The collected serum was heat inactivated at 56°C for 30 minutes, then filtered with a 0,22 µm syringe-driven filters units (Millipore). Aliquots of sterile human serum were stored at – 20°C.

### Cell culture

Human dental pulp was extracted from the enclosed third molar of teenage subjects undergoing a routine tooth extraction, after written informed consent of their parents. Human DPSCs were isolated from dental pulp, as described above. Briefly, cells obtained from dental pulp were plated at clonal density (1,6 cells/$cm^2$) and after 6 days of culture, nodules originated by single cells were isolated from the culture plate, re-plated and expanded. The STRO-$1^+$ hDPSC sub-population was obtained by magnetic cell sorting (MACS; Miltenyi Biotec) using an anti-STRO-1 antibody (Santa Cruz). Two different culture media were used during isolation steps and throughout the subsequent cultures: α-MEM supplemented with heat inactivated 10% Foetal Calf Serum (FCS-medium; Euroclone) or Human Serum (HS-medium), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, at 37°C and 5% $CO_2$.

## Cell counting

The proliferation rate was analyzed on the same hDPSCs population, seeded in 60 mm Petri dishes at the density of 4000 cells/cm$^2$ and cultured for 1 week until reaching the confluence. Cell counting was performed in three culture conditions: serum free culture medium; culture medium supplemented with FCS; culture medium supplemented with HS. Each day cells were stained with CFDA (6-Carboxyfluoresceine diacetate; Sigma Aldrich) vital dye to detect viable cells and observed by using a Nikon TE2000 inverted microscope with a 10x objective. For each experimental point, green fluorescent cells contained in 10 fields of 1 mm$^2$ were counted. The mean of cell number was calculated on three experimental samples for each condition and cell density was expressed as mean of cells/cm$^2$ ± standard deviation (SD). The population doubling time (PDT) was calculated in the phase of exponential growth by the following formula:

$$PDT = \frac{\log_{10}(2) \times \Delta T}{\log_{10}(N_{7d}) - log_{10}(N_{1d})}$$

$N_{7d}$ is the cell number at day 7 and $N_{1d}$ is the cell number at day 1. To determine the population doubling (PD) rate, hDPSCs were initially seeded at the density of 4000 cells/cm$^2$ in culture medium supplemented with FCS or HS. Cells were passaged and counted once they reached a sub-confluence of 80%. At each passage cell were re-plated at the initial density and culture were performed until passage 5. Three samples for each condition were used. The following formula was applied:

$$PD = \frac{\log_{10}(N) - log_{10}(N_s)}{\log_{10}(2)}$$

N is the harvested cell number and Ns is the initial plated cell number. Cumulative population doublings (CPD) index for each passage was obtained by adding the PD of each passage to the PD of the previous passages.

## Clonogenicity, senescence and cell death

In order to evaluate colony forming ability, hDPSCs cultured in HS-medium and in FCS-medium were seeded at clonal density (1,6 cells/$cm^2$) in 6-well plates and cultured for 10 days. Six samples for each culture condition were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) and stained with Toluidine blue. Counting was performed considering only colonies consisting of more than 10 cells. In order to evaluate the presence of senescent cells in hDPSCs maintained in HS-medium and in FCS-medium, cells at $5^{th}$ passage were seeded in 12-well plates and cultured until reaching the confluence. Samples were then processed by a senescence β-Galactosidase staining kit (Cell Signaling), according to manufacturer's instructions. Three samples for each culture condition were analyzed and percentage of senescent cells was calculated.

The presence of apoptotic cells in hDPSCs cultures was analyzed by detection of the cleaved form of poli(ADP-ribose) polymerase (PARP). Whole cell lysates of hDPSCs cultured in HS-medium and in FCS-medium at passages 1, 3 and 5 were processed for Western blot analysis and PARP was detected by an anti-PARP specific Ab (Santa Cruz).

*FACS analysis.* Isolated hDPSCs cultured for 5 passages in FCS-medium and HS-medium were assayed for the expression of Stro-1, c-Kit and CD34 surface antigens. Indirect staining was performed using mouse anti-STRO-1 IgM, rabbit anti-c-Kit IgG (Santa Cruz) and mouse anti-CD34 IgG (Millipore), followed by goat anti-mouse-IgG-Alexa488, goat anti-rabbit-IgG-Alexa488;

and goat anti-mouse-IgM-Alexa488 (Invitrogen). Non-specific fluorescence was assessed by using normal mouse IgG or IgM followed by the secondary antibody as described above. Samples were analyzed using a 16-parameters CyFlow ML flow cytometer (Partec GmbH), equipped with a 488-nm blue solid-state, a 635-nm red diode laser, a UV mercury lamp HBO, a 532 nm green solid state laser, a 405 nm violet laser, and a CCD camera. Data were acquired in list mode by using FloMax (Partec GmbH) software, and then analyzed by FlowJo 9.4.11(Treestar Inc.) under MacOS 10. A minimum of 20000 cells per sample were acquired.

## *In vitro* multilineage differentiation

Human DPSCs cultured in HS-medium and FCS-medium were tested for their ability to differentiate towards neurogenic, adipogenic and myogenic lineages. Three samples of each culture condition were used for each differentiation experiment.

Myogenic differentiation experiments were carried out according to Laino *et al*. (Laino G *et al*, 2006). In order to investigate the capability of hDPSCs to participate in myotubes formation, cells were seeded in direct co-culture with C2C12 mouse myoblast cell line. Human DPSCs and C2C12 cells were seeded in a 10:1 ratio in DMEM High Glucose, supplemented with 10% FBS or HS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, until confluence was reached. Upon confluence, growth medium was replaced with DMEM High Glucose supplemented with 1% FBS or HS and 10 nM insulin. Cells were maintained in co-culture for 2 weeks. Double immunofluorescence staining, by using an anti-human mitochondrial protein ab (anti-hMit; Millipore) and an anti-Myosin ab (Sigma Aldrich), was performed in order to verify the formation of myotubes with the

direct contribution of hDPSCs. Neurogenic and adipogenic differentiation were performed as described by Zhang *et al.* (Zhang W *et al*, 2006).

Neurogenic differentiation: cells were seeded on 6-well plates at 20000 cells/cm$^2$. After confluency was reached, cells were pre-induced with FCS-medium or HS-medium supplemented with 1 mM β-mercaptoethanol. After 24 h, the cells were washed and differentiated in serum free α-MEM containing 10 mM β-mercaptoethanol, 2% dimethyl sulfoxide and 200 µM butylated hydroxyanisole until neuronal morphology was detectable. Neuronal differentiation was assayed for expression of β3-Tubulin by immunofluorescence experiments using specific ab (Cell Signaling).

Adipogenic differentiation: cells were seeded on 24-well plates at 20000 cells/cm$^2$. Subconfluent cultures were incubated in the adipogenic medium (FCS-medium or HS-medium containing 0,5 mM isobutyl-methylxanthine, 1 µM dexamethasone, 10 µM insulin, 200 µM indomethacin, 50 mg/ml gentamicin) for 3 weeks. Medium was changed every 3 days. Afterwards, cells were stained with oil red O stain and counterstained with Harris haematoxylin.

## *In vitro* osteogenic differentiation

In order to obtain osteogenic differentiation on 2D surface, STRO-1$^+$ hDPSCs were seeded at approximately 3000 cells/cm$^2$ on culture dishes in two different osteogenic media (culture medium supplemented with 100 µM 2P-ascorbic acid, 100 nM dexamethasone, 10 mM β-glycerophosphate) containing 5% FCS (FCS-osteogenic medium) and 5% HS (HS-osteogenic medium), respectively. In particular hDPSCs cultured in HS-medium were differentiated in HS-osteogenic medium while cells cultured in FCS-medium were differentiated in FCS-osteogenic medium. The media were changed twice a week.

Collagen sponges (Condress, ABIOGEN PHARMA S.p.A.) were used as 3D scaffold in this study, for the subsequent *in vivo* implantation. Cells were seeded on collagen sponge samples (13 mm diameter; 1,5 mm height), into a 12 multi-well plate, in an adequate volume of culture medium to obtain a starting density of about $1 \times 10^6$ cells per sponge. After 8 hours from cell seeding, 2 ml of the two osteogenic media were added to each sample. Human DPSCs/collagen constructs were pre-differentiated for 10 days before *in vivo* implantation as described above in this section.

## Surgery and implantation procedure

The *in vivo* implantation has been realized in 14 weeks old Sprague-Dawley male rats and surgical procedure was performed as described above in this section.

In order to compare the bone forming ability of hDPSCs on collagen scaffolds in both pre-differentiation conditions, a critical size bone defect was generated by a full-thickness dissection of both the internal and external tables of compact bone constituting the parietal skeletal segment. The stem cell-scaffold complexes were then implanted into the critical size parietal defect up to 6 weeks. One scaffold $5 \times 8 \times 1,5$ mm size was implanted into each cranial defect and adapted to fill the whole defect area; each animal received two constructs: on the left parietal bone the construct pre-differentiated with FCS-osteogenic medium; on the right parietal bone the construct treated with HS-osteogenic medium. Control group received collagen scaffold alone. Six weeks after the surgical implantation the rats were sacrificed and the calvarias were rapidly explanted and fixed in 4% paraformaldehyde in PBS for 3 h. A total of 10 animals were used in this study (controls n= 4;

treated n= 6). All animal procedures were performed according to the guidelines approved by the Committee of Use and Care of Laboratory Animals of the University of Modena e Reggio Emilia.

## Histology, histochemistry and morphometry

Some samples of hDPSCs differentiated *in vitro* were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at pH 7.4 for 20 minutes and were processed for subsequent steps. For Alizarin red staining fixed cells were incubated for 5 minutes at room temperature in a solution containing 0,1% alizarin red and 1% ammonium hydroxide. Other samples were fixed in methanol-acetone 3:7 for 10 minutes at - 20°C, in order to perform the alkaline phosphatase assay. For this enzymatic assay fixed cells were processed by using Leukocyte Alkaline Phosphatase Kit (Sigma Aldrich), following the manufacturer's instructions. Images were collected by a CCD color camera equipped with a 90 mm macro photograph objective. Densitometry was performed on culture plates from three independent experiments by NIS software (Nikon). An equal area (ROI) was selected around the plate surface and the mean of gray levels (in a 0–256 scale) was calculated. Data were then normalized to values of background and expressed as mean ± SD. Fixed samples of explanted calvarias were immersed in 0,5 M EDTA pH 8.3 until the complete decalcification and then rinsed in PBS, dehydrated with graded ethanol, diaphanized and embedded in paraffin. Transversal serial sections (10 µm thick) of the parietal bones containing the implants, were cut. Routine H & E staining was performed in order to analyze morphological details. Images of histological samples were obtained with a Nikon Labophot-2 optical microscope equipped with a DS-5Mc CCD color camera.

Morphometry on regenerating bone areas was performed by NIS software (Nikon). Implant area and new-formed bone area were measured in 5 transversal sections of implanted parietal bone for each animal. Percentage of new-formed bone was calculated in comparison to the total area of implant. Vasa, identified by morphological criteria and by labeling with anti-von Willebrand factor ab, were counted in the area of not yet reabsorbed scaffold and in the area of new-formed bone for each animal and for each implant type. Data were normalized to areas of not yet reabsorbed scaffold and of new-formed bone respectively and were presented as mean ± SD of each experimental group (controls n = 4; treated n =6).

## Confocal microscopy

Fixed monolayer cells were permeabilized with 0,1% Triton X-100 in PBS for 5 minutes; samples were then blocked with 3% BSA in PBS for 30 minutes at room temperature and then incubated with the primary antibodies diluted 1:50 [rabbit anti-osteocalcin (OCN), mouse anti-osteopontin (OPN), rabbit anti-osterix (Osx), rabbit anti-Runx2, mouse anti-hMit, rabbit-anti myosin, mouse anti-β3-Tubulin] in PBS containing 3% BSA, for 1 hour at room temperature. After washing in PBS containing 3% BSA, the samples were incubated for 1 hour at room temperature with the secondary antibodies diluted 1:200 in PBS containing 3% BSA (goat anti-rabbit FITC, sheep anti-mouse FITC, donkey anti-rabbit Cy3). After washing in PBS samples were stained with 1 µg/ml DAPI in PBS for 1 minute, and then mounted with anti-fading medium (0,21 M DABCO and 90% glycerol in 0,02 M Tris, pH 8.0).

Histological sections were processed as described above. Primary antibodies (mouse anti-hMit, rabbit anti-OCN, Millipore; rabbit anti-von Willebrand factor, Sigma Aldrich) were diluted 1:80 in PBS containing 3% BSA for 1 hour at room temperature. Secondary antibodies were diluted 1:200 in PBS containing 3% BSA (goat anti-mouse alexa488, goat anti- rabbit alexa546, Life Technologies). Negative controls were samples not incubated with the primary antibody. The multi-labeling immunofluorescence experiments were carried out avoiding cross-reactions between primary and secondary antibodies.

Immunohistochemistry with ant-hMit revealed by a HRP-labeled anti-mouse secondary Ab (Pierce) was performed in order to confirm specificity of the labeling. HRP was revealed by a DAB based kit (Sigma Aldrich). Control without primary Ab was also performed. Fluorescent samples were observed by a Nikon A1 confocal laser scanning microscope as described below. For detection, the

samples were sequentially excited with the respective laser wavelength: 405 nm lines of a diode laser for DAPI; 488 nm lines of the argon laser for FITC or alexa488; 543 nm line of a HeNe laser for Cy3 or alexa546. The excitation and the detection of the samples were carried out in sequential mode to avoid overlapping of the two signals. Optical sections were obtained at increments of 0,3 µm in the z-axis and were digitized with a scanning mode format of 1024×1024 pixels and 4096 gray levels.

The confocal serial sections were processed with ImageJ software to obtain three-dimensional projections and image rendering was performed by Adobe Photoshop Software.

## Western Blot

Whole cell lysates were obtained at different times of differentiation by using an hypotonic buffer (30 mM Tris-Cl, pH 7.8, containing 1% Nonidet P40, 1 mM EDTA, 1 mM EGTA, 1 mM Na3VO4, and freshly added Sigma-Aldrich Protease Inhibitor Cocktail). Lysates were cleared by centrifugation and the total lysate was immediately boiled in SDS sample buffer. The protocols of the Western blot were performed as described by Sambrook *et al.* (Sambrook J *et al*, 1989).

Sixty µg of protein extract, quantified by a Bradford Protein Assay (Biorad), underwent SDS-polyacrylamide gel electrophoresis and were transferred to PVDF membranes. The following antibodies were used: rabbit anti-osteocalcin antibody diluted 1:500; mouse anti-collagen type I (Millipore), rabbit anti-PARP (Santa Cruz) diluted 1:1000; peroxidase-labeled anti-rabbit and anti-mouse secondary antibodies diluted 1:3000 (Pierce Antibodies, Thermo Scientific). Antibody dilution was performed in TBS-T, pH 7.6, containing 2% BSA and 3% free fatty milk. The membranes were visualized by using ECL (enhanced chemioluminescence, Amersham). Anti-actin antibody was used as control of protein loading. Densitometry of cleaved PARP bands was performed by NIS software (Nikon). An equal area was selected inside each band and the mean of gray levels (in a 0–256 scale) was calculated. Data were then normalized to values of background and of control actin band.

## Statistical analysis

All experiments were performed in triplicate. Quantitative or semi-quantitative data were expressed as mean ± standard deviation (SD). Differences between two experimental

points or between experiments were evaluated by paired Student's t-test. ANOVA test followed by Bonferroni post-test was used to analyze differences between three or more experimental points. In all analyses, values of $p<0,01$ were considered a significant statistical difference, while values of $p<0,001$ were considered a strong significant statistical difference.

### *1.3 Skeletal muscle regeneration by human DPSCs and AFSCs*

## Cell culture and sorting

Human dental pulp and amniotic fluid stem cells were isolated and immuno-selected as described in Materials and methods section, paragraph 1.1.

Both the cell populations, STRO-$1^+$ hDPSCs and c-$kit^+$ hAFSCs , were expanded in culture medium containing α-MEM plus 20% Foetal Calf Serum (FCS), 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma Aldrich), at 37° C and 5% $CO_2$.

## Myogenic differentiation *in vitro*

After cell expansion, in order to evaluate the myogenic potential of STRO-$1^+$ hDPSCs and c-$kit^+$ hAFSCs, these cells underwent two different differentiation protocols.

The first protocol consisted in a direct co-culture of hDPSCs/hAFSCs with C2C12 mouse myoblast cell line: human stem cells and mouse myoblasts were seeded on cover-slides into 6-well plates, at 10:1 cell seeding ratio, and maintained in an expansion medium containing DMEM High Glucose (DMEM-HG) plus 10% FCS, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, until confluence was reached. After reaching confluence, the expansion medium was replaced by fusion medium, containing DMEM-HG plus 1% FCS, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10nM insulin. Co-cultures were held in fusion medium for 14 days.

The second protocol, on the other hand, involved the myogenic differentiation of hDPSCs and hAFSCs by means of the DNA demethylating agent, 5-Aza-2'-deoxycytidine (5-Aza, Sigma Aldrich).

Cells were seeded on cover-slides into 6-well plates, without direct co-culture, at a cell density of 4000 cells/cm$^2$ in expansion medium (DMEM-HG plus 10% FCS, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin) upon reaching confluence, then the medium was replaced with DMEM Low Glucose (DMEM-LG), plus 10% horse serum, 0,5% chicken serum, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and additioned with 10µM 5-Aza for 24 hours. Subsequently, cells were rinsed twice in Phosphate Buffer Solution (PBS) and kept in myogenic medium containing DMEM-LG, plus 5% horse serum, 0,5% chicken serum, 2mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10nM insulin, for 24 hours. The day after, horse serum concentration in myogenic medium was reduced to 2%; half of the hDPSCs and hAFSCs was differentiated under these conditions, the other half underwent the same treatment, with the addition of conditioned fusion medium from differentiated C2C12 cells.

## In vivo transplantation of hDPSCs and hAFSCs in SCID/*mdx* mice

In order to evaluate whether the hDPSCs and hAFSCs are capable to regenerate injured skeletal muscle and restore dystrophin expression, both human stem cell lines formerly demethylated by means of 10µM 5-Aza and pre-differentiated *in vitro* for 2 weeks in myogenic medium, with and without the addition of conditioned fusion medium from differentiated C2C12 cells, were transplanted into an animal model of Duchenne Muscular Dystrophy, the SCID/*mdx* mouse.

Briefly, $5 \times 10^5$ pre-differentiated hDPSCs and hAFSCs, resuspended in 30µl of PBS, were injected into gastrocnemia muscles of male SCID/*mdx* mice, with age ranging between 8 and 10 weeks. Each animal received two injections: on the left gastrocnemius muscle, the cells pre-differentiated after demethylating treatment; on the right

gastrocnemius muscle, the cells pre-differentiated after demethylating treatment, with the addition of conditioned medium. Non-injected and PBS-injected muscles were taken as controls. The animals were euthanized at 7, 14, and 28 days after cell transplantation. The gastrocnemia muscles were rapidly harvested, and snap frozen in liquid nitrogen. Subsequently, transversal serial sections (8µm thick) of frozen muscles were cut by means of a H525 MICROM Cryostat, collected on Super Frost Plus slides (Thermo Scientific) and then stored at - 80°C. A total of 10 animals were used in this study (controls n=4; treated n = 6).

Animal procedures were performed in compliance to the guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pittsburgh, Pittsburgh, PA, USA.

## Immunofluorescence microscopy and histology

Fixed monolayer cells differentiated *in vitro* on cover-slides were permeabilized with 0,1% Triton X-100 in PBS for 5 minutes; samples were then blocked with 3% BSA in PBS for 30 minutes at room temperature. Mouse anti-human nuclei (Millipore), mouse anti-myogenin, rabbit anti-myosin heavy chain (MyHC), rabbit anti-desmin (all from Sigma Aldrich), diluted 1:50 in PBS containing 3% BSA for 1 hour at room temperature were used as primary antibodies (abs). Secondary abs were diluted 1:200 in PBS containing 3% BSA for 1 hour at room temperature (goat anti-mouse Alexa488, donkey anti-rabbit Alexa594; Invitrogen), after washing in PBS cells were counterstained with 1 µg/mL DAPI in PBS for 3 minutes, and then mounted with anti-fading medium (0.21 M DABCO and 90% glycerol in 0.02 M Tris, pH8.0). Negative controls consisted of samples not incubated with the primary antibody.

Histological unfixed sections from frozen muscle were processed as described above. Mouse anti-human mitochondrial protein (1:80, Millipore), rabbit anti-dystrophin (1:500, courtesy of Dr. Bing Wang, University of Pittsburgh, PA) and rabbit anti-human von Willebrand factor (1:100, Millipore), were used as primary abs. Secondary abs, diluted 1:200, were donkey anti-mouse Alexa488, donkey anti-rabbit Alexa594, donkey anti-mouse Alexa594 and goat anti-rabbit Alexa488 (Invitrogen). Negative controls were samples not incubated with the primary antibody. The multi-labeling immunofluorescence experiments were carried out avoiding cross-reactions between primary and secondary antibodies.

Immunofluorescence analysis was performed on a Nikon Eclipse E800 microscope (Nikon), with the use of Northern Eclipse imaging software (EMPIX Imaging). The image rendering was performed by Adobe Photoshop software.

Routine H & E staining was performed in order to analyze the morphological details. Moreover, trichrome staining was performed to detect the collagen content of the muscles. Slides were processed as detailed in the manufacturer's protocol (Masson's Trichrome stain kit; IMEB, Inc.). Images of histological samples were obtained with a Nikon Eclipse E800 microscope (Nikon).

# RESULTS

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## *1.1 In vivo implantation of stem cells-scaffold constructs*

In order to evaluate the capability of hDPSC-scaffold and hAFSC-scaffold complexes to repair critical size bone defects *in vivo,* these human stem cells were pre-differentiated towards osteogenic lineage *in vitro*, following the seeding on three different types of scaffolds: silk fibroin, equine collagen, and poly-D,L-lactic acid (PDLLA). After pre-differentiation of hDPSC- and hAFSC-scaffold constructs in osteogenic medium, a critical-size bone defect was obtained through a full-thickness resection of both the internal and external tables of almost 95% of the parietal bone of immunocompromised rats; then the constructs were implanted into the critical size parietal defect and 4 weeks later the rats were euthanized and the calvaria were explanted and processed for further evaluation by radiological, histological and confocal analysis.

- ***Scaffold characterization: morphology, water content, porosity and mechanical properties***

Scaffold morphology was evaluated by FE-SEM on dry samples. The micrographs of fibroin sponges are shown in Figure 8. Their morphologies are characterized by a high and interconnected porosity but with a different pore diameter range, from 10 to 250 μm; moreover, pores are randomly distributed.

The open porosity as measured by the hexane displacement test was evaluated around 85% ± 5%, while a water content equal 89% by wt. was measured for fibroin sponges. The data are very close so indicating that most of the water filled the pores and only a negligible amount swelled the

solid polymer. Compressive elastic properties of the sponges reflected the effect of material porosity, the absorbed water within the pores, but also the plasticization of the polymer.

In the wet state, however, fibroin sponges retained their mechanical consistence, with a measured compressive modulus of 25,69 ±1,61 kPa.

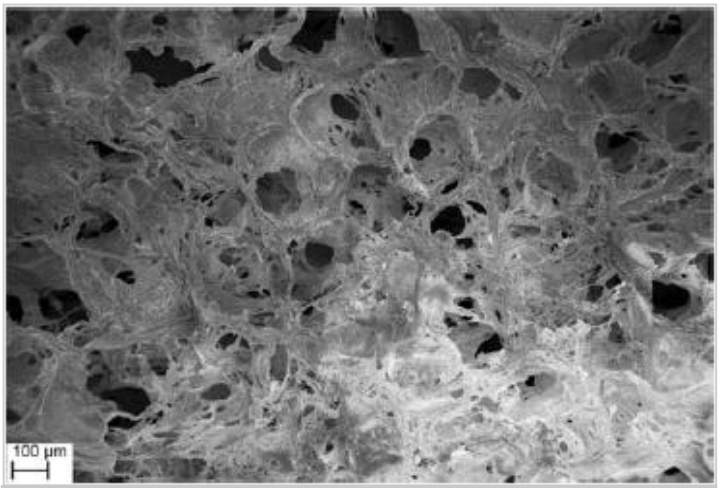


**Figure 8.** Scanning electron microscopy images of salt-leached fibroin scaffold.

- ***Radiological analysis***

First, the bone healing process at the cranial defects was evaluated by radiograph 30 days after surgery. Figure 9 shows that the scaffold alone is able to slightly repair the area removed by the drill. In particular, the fibroin scaffold healed the bone defect mainly in lateral areas, while no bone repair was observed in the central area of the implant.

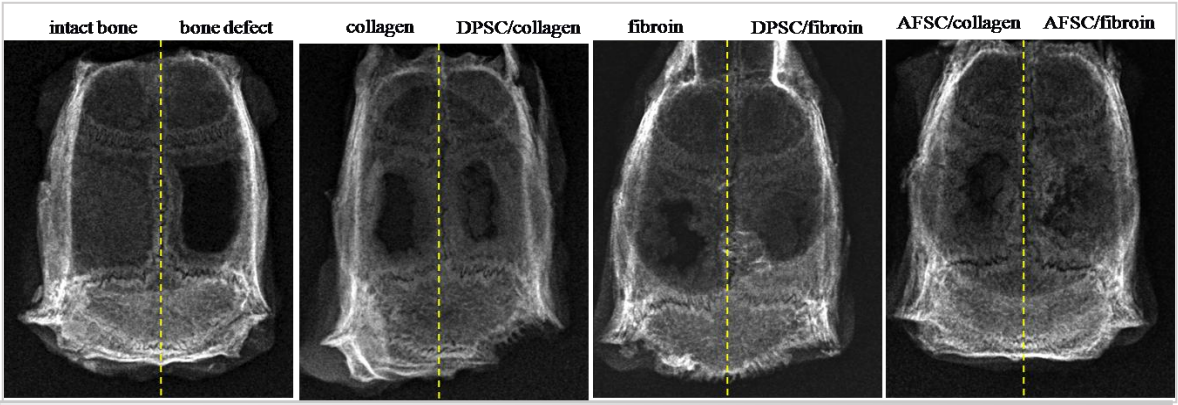


**Figure 9.** Radiological analysis of rat calvaria dissected 30 days after surgery. Images are representative of the bone healing in the cranial defects of fibroin scaffolds alone and fibroin scaffold seeded with human dental pulp stem cells (hDPSCs) or human amniotic fluid stem cells (hAFSCs).

Broadly, the presence of stem cells into collagen and fibroin scaffolds enhances the radiopacity of the bone defect area that appears similar to the intact bone (Figure 9).

With regard to the implant with PDLLA scaffold either alone or plus the cell, the radiological analysis did not show any significant bone regeneration (not shown).

- ***Histological analysis***

In order to analyze the bone reconstruction process through histological analysis, serial sections of the parietal bones containing the implants were stained by H&E labeling. The samples were then observed by white field and polarized light microscopy. The presence of vessels in the scaffold area shows that all implanted constructs have been successfully vascularised (Figure 10D, F, G, K, L).

The untreated critical-size bone defects, after 30 days, are filled by connective tissue only and lack a significant amount of bone tissue in comparison to the intact parietal bone (Figure 10A, B).

Radiographic data for fibroin are confirmed by histological analysis (Figure 10), since the implantation with the scaffold alone causes the formation of small mineralized spots spread

throughout the defect area (Figure 10C–E). This can be an adequate starting step for bone regeneration by stem cells. As a matter of fact, the samples containing hDPSCs and hAFSCs even more (Figure 10F, K) show a complete reconstruction of the internal table of parietal bone and an extensive but incomplete regeneration of the external one. Moreover, areas of spongy bone were present between the two tables of compact bone (Figure 10F, G, K, L). Polarized light microscopy analysis indicates that newly formed bone presents some areas with lamellar organization compared with pre-existing bone characterized by a clear lamellar structure (Figure 10H, M). Magnification images highlight area of active bone deposition, as evidenced by the presence of preosteocyte cells partially included in forming lacunae (Figure 10J) and fibroin remnant islands wrapped by the new mineralized tissue (Figure 10G, N, O). This means that a progressive resorption of fibroin leaded to ossification process. Fibroin colonized with hAFSCs seems to have a greater regenerative potential compared to hDPSC/fibroin complex, indeed it forms bone also in scaffold areas distant from dura mater (Figure 10F, K). The *in vivo* data showed that fibroin scaffolds provided hAFSCs and hDPSCs with an optimal microenvironment for their osteogenic differentiation and bone formation.

Radiographic data from the implant with collagen scaffold, with and without the cells, were confirmed by the histological analysis, in fact it did not produce any relevant bone reconstruction, as well as the implant with PDLLA scaffold did, either alone and with cells (data not shown).

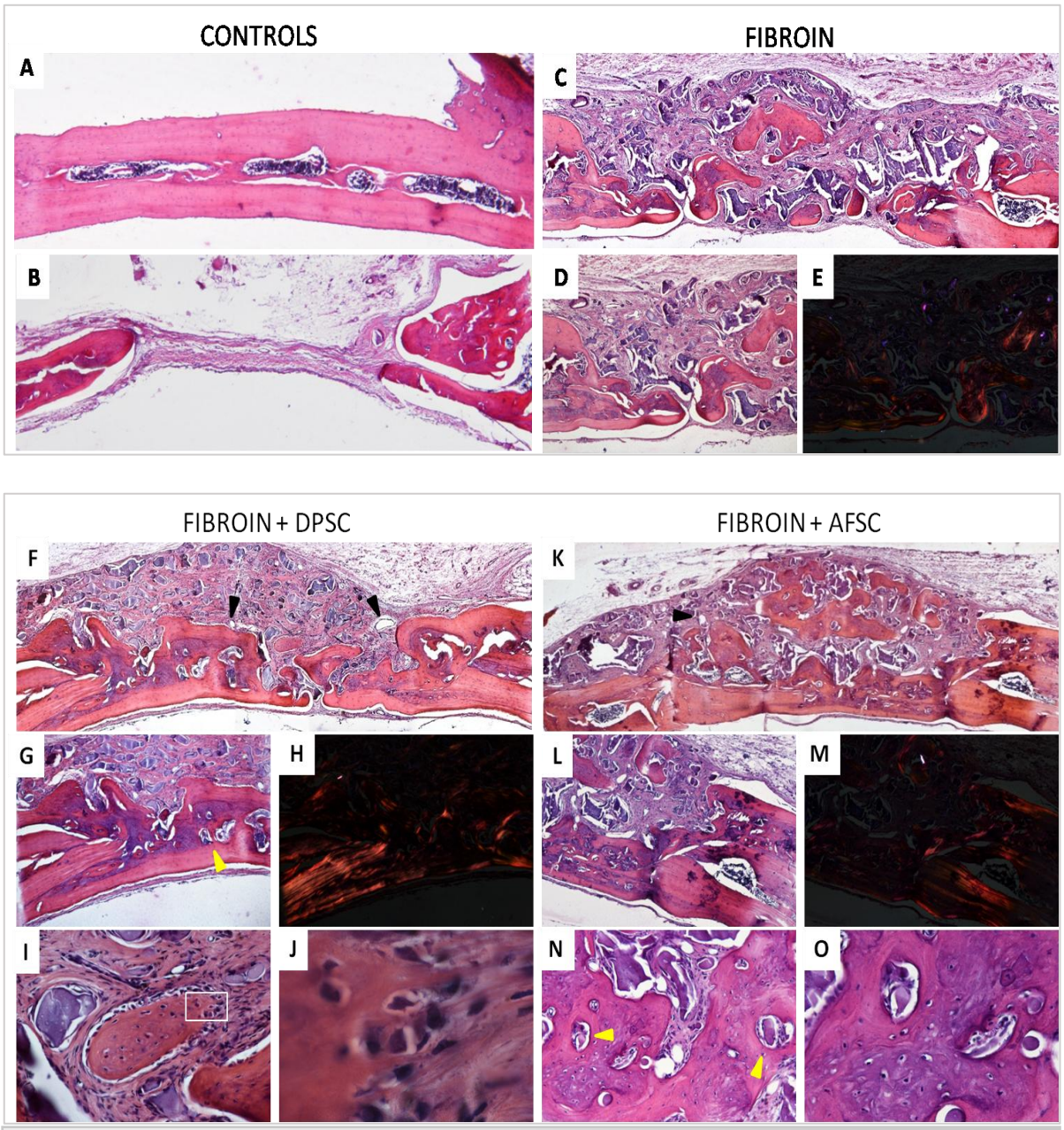


**Figure 10.** Comparative histologic analysis of the cranial defect reconstruction by fibroin constructs at 30 days post-surgery. Images show transversal sections carried out through the central area of the implants. H&E staining of parietal bone (A) and cranial defect: unfilled (B); filled with fibroin (C and magnification D); filled with fibroin + hDPSCs (F and magnifications G, I, J); filled with fibroin + hAFSCs (K and magnifications L, N, O). (E), (H), and (M) are the same field of (D), (G), and (L) observed by polarized microscopy. Black arrowheads indicate vasa; yellow arrowheads indicate fibroin remnants.

- ***Confocal analysis***

To check whether the cells inside the new bone were still from human origin and not from the host, immunofluorescence experiments with anti-human mitochondria antibody were carried out. This antibody recognizes only mitochondrial protein from human origin. In control samples (fibroin alone, Figure 11A, B) only DAPI staining (red signal) occurs, while human mitochondria (green signal) are not detectable.

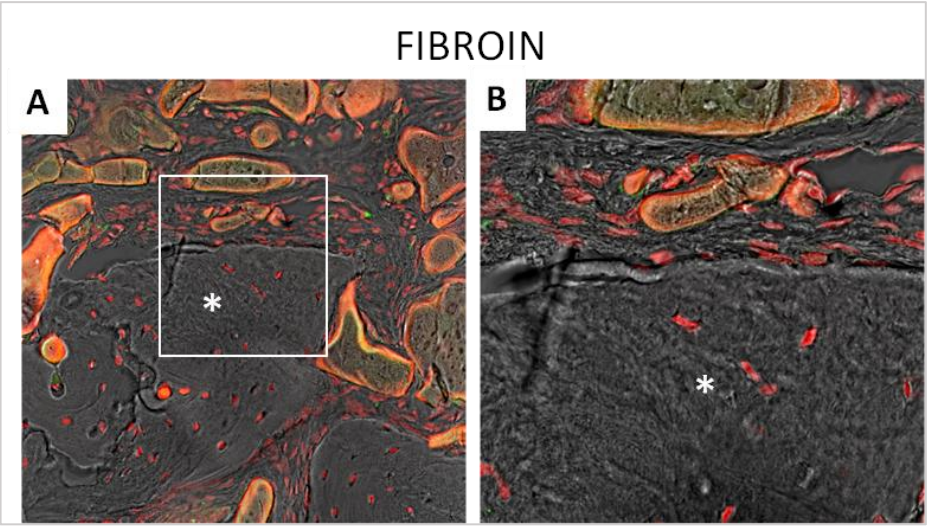


**Figure 11.** Confocal images of implants obtained 4 weeks after surgery. Double immunofluorescence staining with DAPI (red) and anti-human mitochondria protein antibody (green), the signals were superimposed to pseudo-phase contrast images. (A, B) Fibroin scaffold: no human stem cells were detectable within the fibroin scaffold alone. The square indicates the image area magnified on the right. Asterisks indicate bone tissue.

The autofluorescence phenomenon of fibroin is also evident. Images of stem cell/scaffold implants demonstrate that most of the cells are clearly labeled by anti-human mitochondria antibody (Figure 11C–G), indicating the survival of donor cells *in vivo*. In particular, Figure 11C, D, F, and G shows that human cells surround the bone tissue in growth. Furthermore, in Figure 11F, it is evident that the new-formed bone tissue wraps modified fibroin islands.

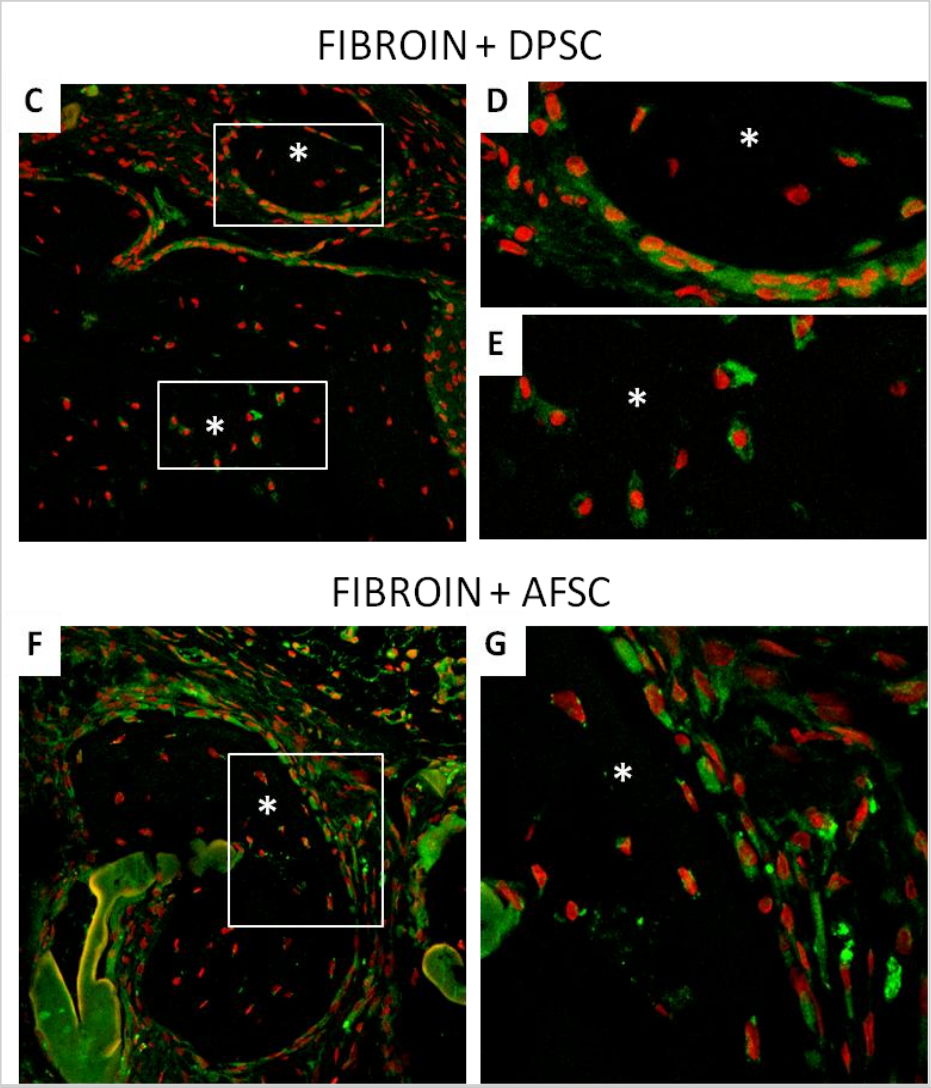


**Figure 11.** Confocal images of implants obtained 4 weeks after surgery. Double immunofluorescence staining was performed with DAPI (red) and anti-human mitochondria protein antibody (green). (C–E) fibroin colonized with hDPSCs; (F–G) fibroin colonized with hAFSCs. Squares indicate the image areas magnified on the right. Asterisks indicate bone tissue.

Moreover, Figure 11D, E, and G highlights that human cells are enclosed within bone lacunae. These data confirm the central role of human stem cells in bone regeneration after cell/scaffold implant in rat model of large cranial defects.

## *1.2 Human serum for osteogenic differentiation of human DPSCs*

- ***Cell Proliferation***

To evaluate the influence of HS or FCS on the growth of hDPSCs during routine cell culture, proliferation rate was evaluated by using 6-CFDA staining to detect viable cells in adherent culture at different times until confluence was reached. Human DPSCs were then grown in culture media without serum, or containing respectively 10% FCS and 10% HS, and cell counting was performed for up to 7 days (Figure 12A).

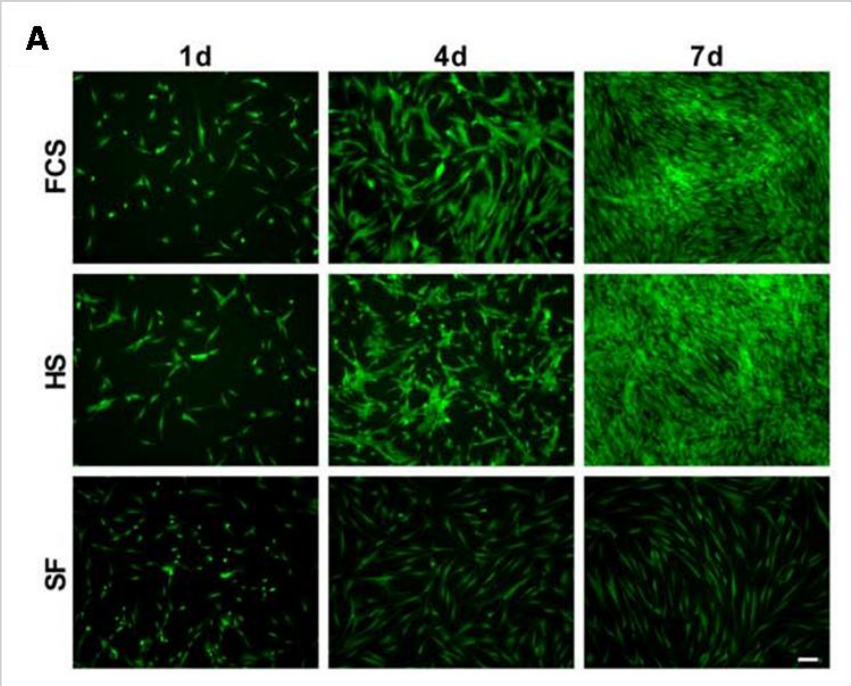


**Figure 12.** A: CFDA vital staining of hDPSCs cultured for 1, 4 and 7 days, in serum-free medium, HS-medium and FCS-medium. Green signal indicates viable cells. Bar: 100 µm.

Human DPSCs cultured in serum-free medium do not show any significant proliferation rate, indicating a stationary trend. Cells cultured with HS show, at the beginning, a slightly lower proliferation rate compared to hDPSCs growing in FCS-medium. From day 4

onward the proliferation rate of cells in HS-medium slightly exceeds, in significant manner, the one of hDPSCs growing in FCS-medium (Figure 12B). Furthermore the population doubling time of HS cultures is lower than FCS cultures (HS: 35,0 ± 2,31 hours; FCS: 40,3 ± 1,50 hours; $p < 0,01$). Cumulative population doubling analysis shows that HS culture proliferates more speedily, overtaking FCS culture by 13% at each passage (Figure 12C). Human DPSCs cultured in HS-medium or in FCS-medium for 5 passages were seeded at clonal density and cultured in 6-well culture plates for 10 days, in order to verify any differences in clonogenic ability. No significant differences were observed in the number of colonies formed by hDPSCs growing in the two culture conditions (HS 17,3 ± 4,8 colonies; FCS 18,7 ± 4,0 colonies).

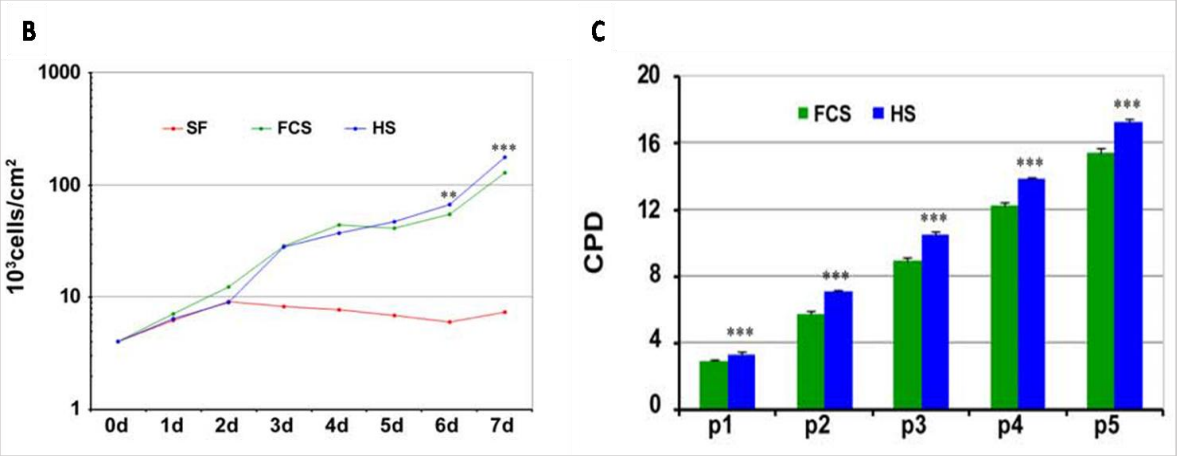


**Figure 12.** B: Proliferation rate of hDPSCs cultured for one week. Values are mean reported in a Log scale, n = 3; * indicates values of paired t-test HS vs. FCS (**p<0,01, ***p<0,001). C: Cumulative population doubling (CPD) of hDPSCs cultured for a total of 5 passages. At each passage cells cultured in HS-medium show a CPD significantly higher than FCS-medium cultured cells (n = 3; ***p<0,001).

- ***Senescence and apoptosis***

Cell senescence was evaluated by detection of β-galactosidase activity in confluent culture of hDPSCs grown for 5 passages in HS-medium or in FCS-medium. Very low levels of β-

galactosidase activity were detected in both culture conditions (Figure 12D arrowheads). In particular no significant differences were observed in the percentage of senescent cells detected by microscopic observation in the two culture conditions (FCS 0,044 ± 0,007%; HS 0.039 ± 0.004%; n= 3).

Western blot analysis of PARP carried out in hDPSCs at passages 1, 3 and 5, shows an equivalent amount of uncleaved PARP (116 kDa) in both culture conditions. On the other hand positive control (etoposide treated HL60 cell line), shows a clear band in correspondence of cleaved PARP form (89 kDa; Figure 12E) that is not appreciable in hDPSCs. Densitometry performed on the cleaved PARP bands did not show significant differences between HS and FCS cultured DPSCs.

These data indicate that HS-medium does not induce premature senescence and does not affect the survival of hDPSCs.

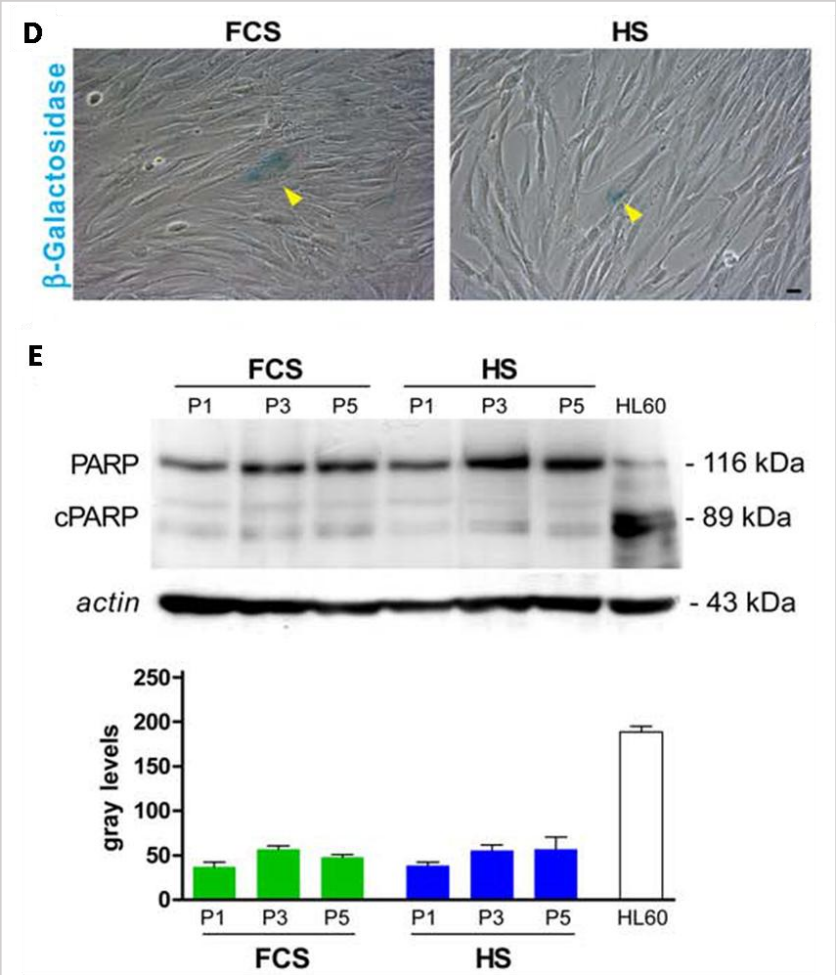


**Figure 12.** D shows b-galactosidase activity staining in confluent culture of hDPSCs grown for 5 passages in FCS-medium or in HS-medium as indicated. Arrowheads indicate cells positive to bgalactosidase activity staining. Bar 10 µm. E: western blot analysis of PARP in hDPSCs cultured in FCS-medium and in HS-medium at passages 1, 3 and 5. HL60, treated with etoposide, were loaded as positive control of the presence of cleaved PARP (cPARP). Actin bands were presented as control of the protein loading. Densitometry of cPARP bands was shown on the bottom of western blot images.

- ***Surface antigens expression and in vitro multi-lineage differentiation***

In order to verify whether HS affects the stemness of hDPSCs during *in vitro* culture, surface antigens expression and multi-lineage differentiation ability were evaluated in hDPSCs cultured in HS-medium and in FCS-medium. Stro-1, c-Kit, and CD34 are surface

antigens typical of hDPSCs (49 Graziano *et al*, 2008). FACS analysis performed in hDPSCs in both culture conditions after 5 passages, demonstrates that hDPSCs maintain the expression of the above mentioned antigens. Moreover, the fluorescence intensity of c-Kit labeling appears higher in HS-cultures, indicating that the whole population expresses this antigen at a dim level. Stro-1 and CD34 antigens appear equally expressed (Figure 13A).

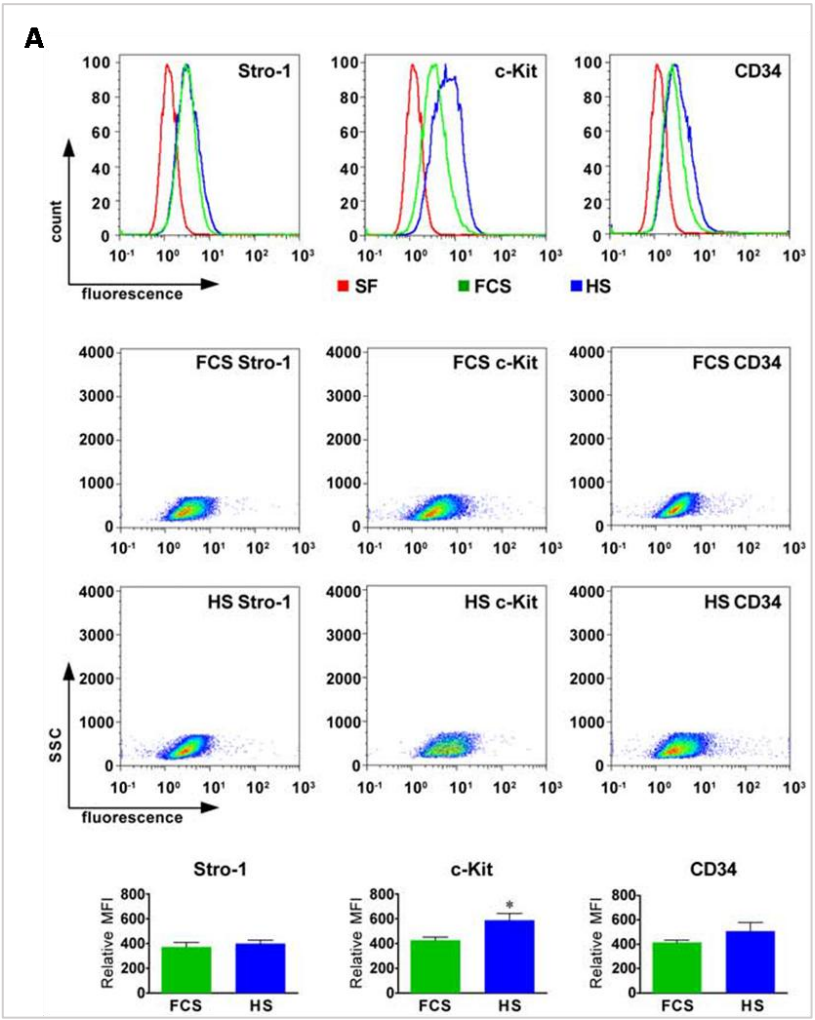


**Figure 13.** hDPSCs surface antigens expression. A: Cytofluorimetric analysis of Stro-1, c-Kit and CD34 expression in hDPSCs cultured in FCS-medium and in HS-medium. Dot plots reporting SSC vs fluorescence are shown. In the histograms, the net fluorescence value was calculated by linearizing the fluorescence value from the logarithmic scale and subtracting the linearized value of the unstained sample to the linearized value of the stained one. Data represent the mean ± SD of three different experiments. * indicates values of paired t-test HS vs. FCS (*p<0.05).

Human DPSCs cultured in both media were differentiated towards myogenic, adipogenic, neurogenic and osteogenic lineages as described in materials and methods.

The ability of hDPSCs to differentiate towards myogenic lineage was verified by co-culture with C2C12 mouse myoblasts. After 14 days of co-culture myotubes formation was observed both in HS co-cultures and in FCS co-cultures. Myotubes appear multinucleated indicating that cell fusion occurs. Labeling by anti-human mitochondria antibody (anti-hMit) demonstrates that hDPSCs are involved in myotubes generation. Double staining with anti-hMit and anti-myosin antibodies indicates that in both culture conditions mature myotubes were formed with the contribution of hDPSCs. Myotubes not labeled by anti-hMit antibody and therefore formed only by C2C12 cells were also present (Figure 13B). The percentage of myotubes formed with the contribution of hDPSCs respect to the total myotubes, was similar in both culture conditions (FCS 42,7 ± 6,4%; HS 46,3 ± 7,2%; n =3).

Human DPSCs cultured in adipogenic medium supplemented with HS start to form intracellular lipidic drops after three days of differentiating culture while cells differentiating in FCS adipogenic medium show the first intracellular lipidic drops at day 7. After two weeks of differentiation, hDPSCs in both conditions, show a similar morphology characterized by numerous lipidic drops accumulated in the cytoplasm and clearly stained by oil red (Figure 13B). The percentage of oil Red positive cells did not show significant differences between the two culture conditions (FCS 73,7 ± 8,1%; HS 75,7 ± 9,0%; n= 3).

Neurogenic differentiation starts with an initial cell detachment from culture plates probably induced by β-mercaptoethanol.

Remaining cells progressively assume neuronal morphology with multiple cellular processes and a defined cell body. Immunostaining with anti-β3-Tubulin after 20 days of culture shows the presence of neurotubules in hDPSCs pre-differentiated in FCS-medium as well as in HS-medium (Figure 13B). No particular differences, in the percentage of β3-Tubulin positive cells, were observed between the two experimental groups (FCS 64,3 ± 7,2%; HS 62,0 ± 7,6%; n= 3).

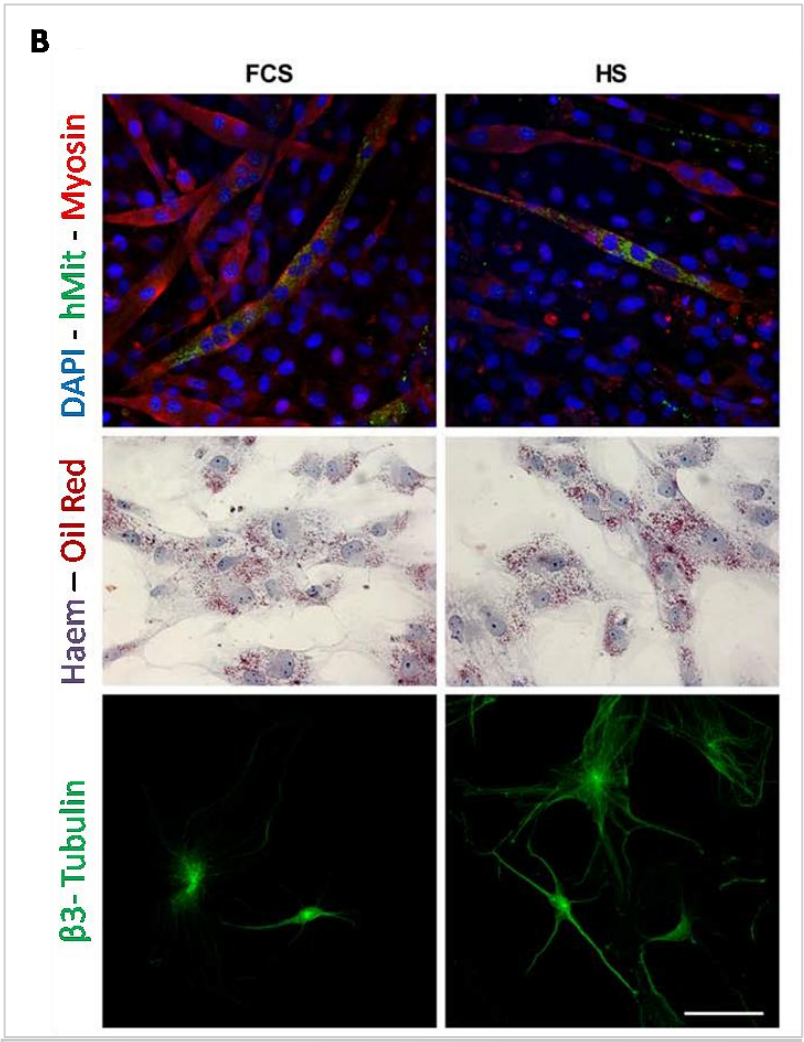


**Figure 13.** hDPSCs multi-lineage differentiation *in vitro*. B: In the first line are shown double immunofluorescence images of hDPSCs/C2C12 co-culture stained by anti-hMit (green) and anti-myosin (red) Abs. DAPI staining is shown in blue. The second line shows oil red staining of hDPSCs differentiated for two weeks towards adipogenic lineage with HS or FCS supplemented medium. Cells were counterstained with Harris haematoxylin. Images, in third line represent anti-β3-Tubulin immunofluorescence labeling on hDPSCs differentiated in neurogenic media supplemented with FCS or HS. Bar 50 µm.

Human DPSCs differentiating towards the osteogenic lineage start to form nodular aggregates at day 8. At day 24, mineral deposits appear in the extracellular space and in nodular aggregates (data not shown). In cells, both the differentiation conditions induce an intense positivity to Alkaline phosphatase assay, which is extended to the whole culture plate. The *in vitro* deposition of mineralized extracellular matrix was analyzed by Alizarin red staining: differences were observed in Alizarin red staining, in favour of hDPSCs treated with HS (Figure 14A), which show a greater deposition of mineralized extracellular matrix than hDPSCs differentiated with FCS, as confirmed by densitometric analysis.

A higher alkaline phosphatase activity was detected in HS-treated cells (Figure 14B). Controls of undifferentiated cells are negative for both the stainings (Figure 14A).

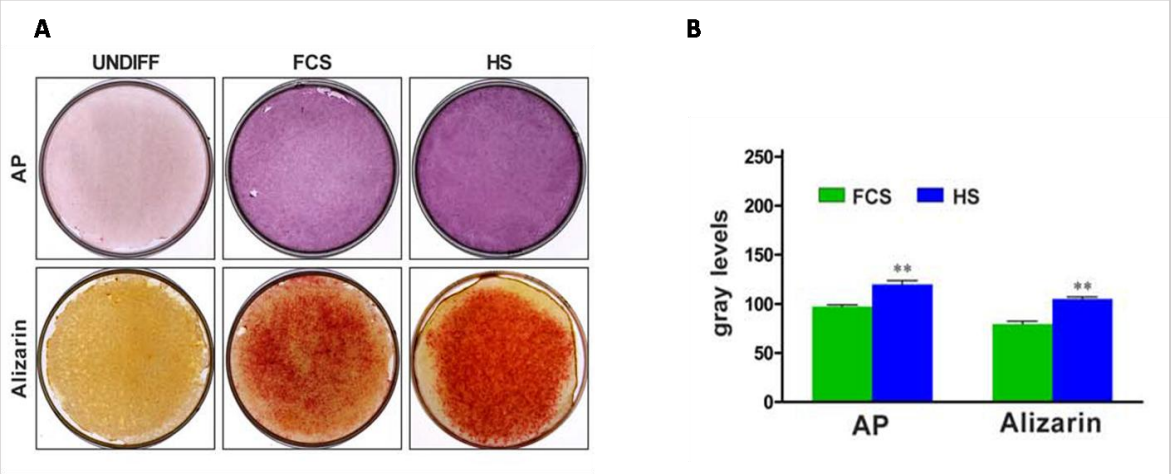


**Figure 14.** In vitro osteogenic differentiation of hDPSCs after 24 days of culture in osteogenic medium, supplemented with FCS or HS. A: Alkaline phosphatase assay (AP) and Alizarin Red staining; B: densitometric analysis of AP and Alizarin Red staining. Values are mean ± SD of gray levels (0–255 scale). HS n = 3; FCS n = 3; ** indicate values of paired t-test HS vs. FCS ($p<0.01$).

The confocal analysis at day 24 shows the expression of osteocalcin, together with other specific markers of osteogenic commitment, such as osteopontin (OPN), osterix (Osx) and Runx2. Double immunofluorescence labeling was carried out to analyze simultaneously

the localization of OPN and Runx2 in hDPSCs differentiated with FCS-medium or HS-medium (Figure 14C). OPN appears localized in the cytoplasm perinuclear region, that normally contains the rough endoplasmic reticulum, while Runx2 is localized in the cell nucleus as expected. OCN shows a typical cytoplasmic localization, strongly expressed in both the differentiation conditions. Signal from OCN was also detected in extracelluar matrix where it appears as spot localized in mineralization areas. Similarly to Runx2, Osx shows the typical nucleoplasmic localization, as demonstrated by the overlapping with DAPI signal (Figure 14C). No differences were observed between the two differentiation conditions. To confirm the differentiation of hDPSCs towards osteoblast-like cells, in both osteogenic media, the presence of type I collagen (Coll-I) and of the specific marker osteocalcin (OCN), was analyzed by Western blot in whole cell lysates of differentiating hDPSCs: Coll-I and OCN were detected in both the differentiating conditions, and show an increase of expression during the differentiation (Figure 14D).

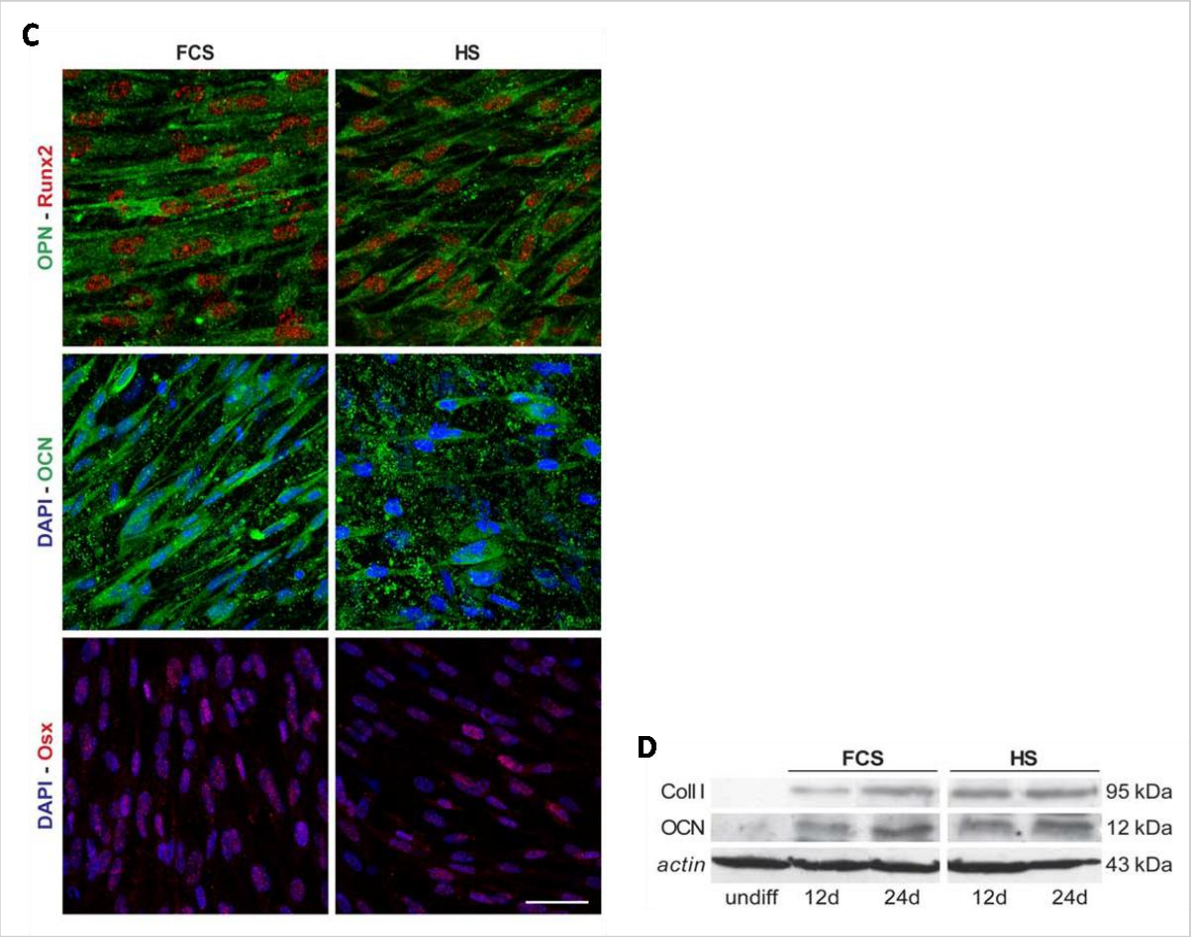


**Figure 14.** In vitro osteogenic differentiation of hDPSCs after 24 days of culture in osteogenic medium, supplemented with FCS or HS. C: Confocal analysis of osteogenic differentiation of hDPSCs. Double immunofluorescence confocal images showing signals from anti-OPN (green) and anti-Runx2 (red); DAPI (blue) and anti-OCN (green); DAPI (blue) and anti-Osx (red). Bar: 50 µm. D: Western blot (WB) analysis of Coll-I and OCN expression in whole cell lysates of differentiated hDPSCs. Whole cell lysates were collected from three plates of human dental pulp stem cells for each differentiation protocol. Actin bands demonstrate that an equal amount of protein was loaded in each line. Undiff samples show hDPSCs cultured in FCS-medium alone. The same results were obtained in HS-medium culture (data not shown).

- ***Osteogenic differentiation in vivo***

In order to evaluate whether the use of different sera (FCS and HS) during pre-differentiation of hDPSCs seeded in collagen scaffold, influences differently their ability to reconstruct critical size parietal bone defects, in vivo implants were performed as described in materials and methods section. After six weeks parietal bones containing the implants

were histologically processed and observed by white field microscopy. Figure 15 shows representative photomicrographs of engineered bone grafts *in vivo*. The hDPSCs/collagen constructs realized in both pre-differentiation conditions exhibit an appreciable contribution to regeneration of the resected bone area (Figure 15A, 15D). Magnification images show the presence of vessels indicating a successful vascularization of the implants (Figure 15: B, C, E, F, arrowheads) and areas of active bone deposition and rearrangement, particularly detectable in samples pre-differentiated with osteogenic HS-medium, where osteoblast layer surrounds islets of new-formed bone (Figure 15E, 15F, star). Morphometric analysis was carried out in order to evaluate the amount of new-formed bone areas in animals implanted with collagen scaffold colonized with FCS or HS pre-differentiated hDPSCs and in the control group implanted only with the collagen scaffold. The analysis revealed that animals implanted with scaffold colonized with hDPSCs pre-differentiated in FCS and in HS differentiating medium show a bone regeneration significantly higher when compared to the control group (FCS vs C $p<0.01$; HS vs C $p<0.001$; Figure 15G). Moreover, by comparing HS implants with FCS implants emerges that the new-formed bone area is greater in HS implants respect to the FCS implants (HS vs FCS $p<0.001$; Figure 15G). The number of vasa in the scaffold not yet reabsorbed appears higher in HS pre-differentiated cells suggesting a better angiogenic potential. On the other hand the number of vasa in new-formed bone does not show significant differences among the three experimental groups indicating that the new-formed bone structure was not affected by the different pre-differentiation conditions (Figure 15H).

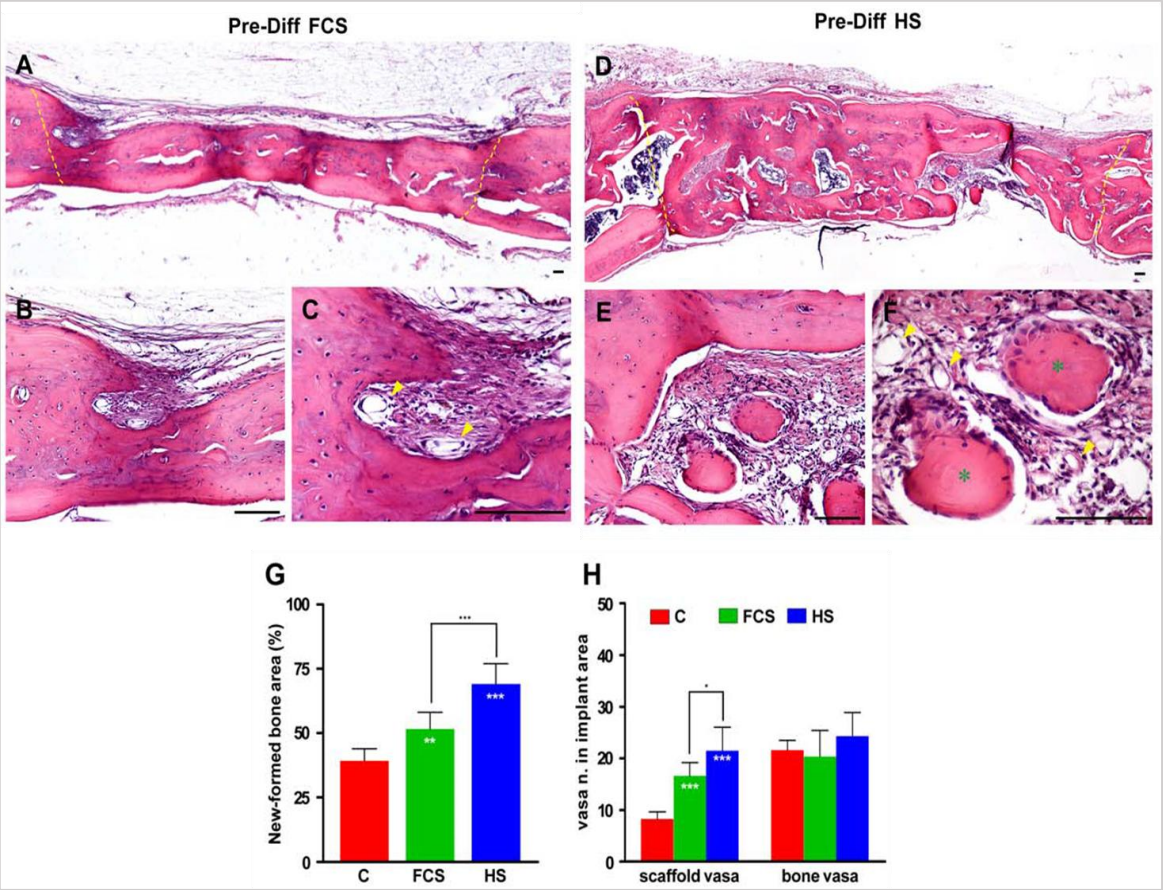


**Figure 15.** Comparative histological analysis (H&E staining) of the critical size cranial defect reconstruction by hDPSCs/collagen constructs 40 days post-surgery. Images show transversal sections carried out through the central area of the implants. A–C: cranial defect filled with hDPSCs/collagen pre-differentiated with FCS containing medium; D–F: cranial defect closed with hDPSCs/collagen pre-differentiated with HS containing medium; (dotted line delimitates the areas of bone resection; arrowheads indicate vasa; * indicate areas of active bone deposition). Bar: 100 µm. G: morphometric analysis of new-formed bone areas in controls (C), FCS and HS implants. Values are mean ± SD of the percentage of regenerated bone respect to the whole resected bone area. HS and FCS n = 6; C n = 4; white * inside the column indicate values of ANOVA test of HS and FCS vs. C (**p<0.01, ***p<0.001); black * indicate values of ANOVA test of HS vs. FCS (***p<0.001). Data, normalized to areas of the scaffold not yet reabsorbed and of new-formed bone areas respectively, were presented as mean ± SD (vasa number respect to the total implant area) of each experimental group (controls n = 4; treated n = 6). White * inside the column indicate values of ANOVA test of HS and FCS vs. C (***p<0.001); black * indicate values of ANOVA test of HS vs. FCS (*p<0.05).

To verify whether cells inside the new-formed bone were still from human origin and not from the host, immunofluorescence labeling with anti-hMit antibody was performed. This antibody recognizes only mitochondrial protein from human origin: images of stem cell-scaffold implants demonstrate that most of the cells are clearly stained by anti-human

mitochondria antibody (Figure 16 A–D) indicating survival of the donor cells in vivo, in both the pre-differentiation conditions.

Interestingly, human DPSCs are included within bone lacunae indicating the direct contribution to the osteocytes population of new-formed bone. Double immunofluorescence analysis shows that human cells express OCN, which was detectable also in new-formed bone matrix (Figure 16 E–H). Moreover cells localized in the inner layer of vascular canals show a clear staining by anti-human mitochondria antibody, suggesting a direct contribution of hDPSCs to the angiogenetic process that gives rise to endothelial cells of new-formed vasa. As a matter of fact, double labelling by anti-hMit and anti-von Willebrand factor indicates that hDPSCs contribute to the constitution of the endothelium of vasa localized in the new-formed bone (Figure 16 I–L).

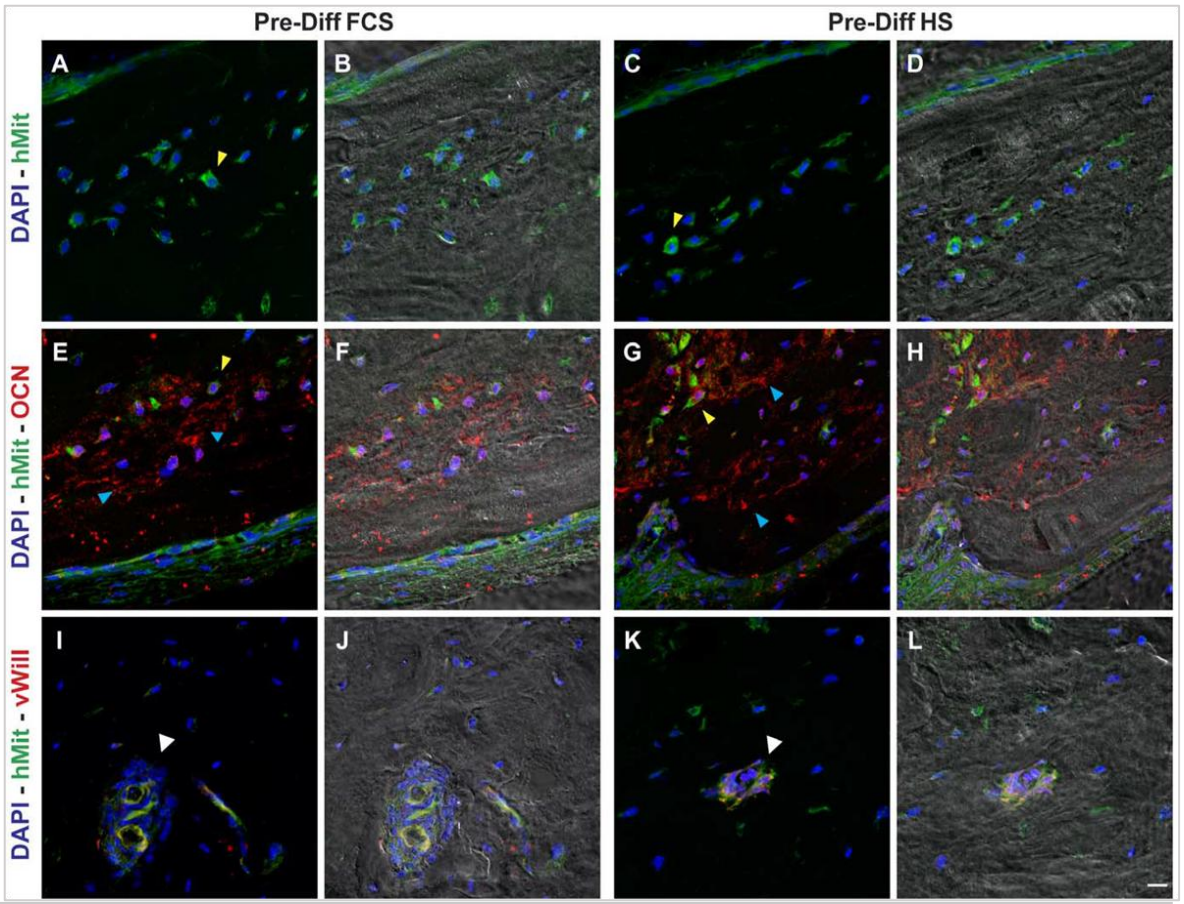


**Figure 16.** Confocal images of implants pre-differentiated in both the conditions. A–D: Double fluorescence signals from DAPI (blue) and anti-hMit Ab (green) superimposed to pseudo-phase contrast images (B, D). Arrowheads indicate cells entrapped in calcified bone matrix clearly stained by anti-human mitochondria antibody. E–H: triple fluorescence signals from DAPI (blue) and anti-hMit (green) and anti-OCN (red) Abs superimposed to pseudo-phase contrast images (F, H). Yellow arrowheads indicate osteocytes labelled by the two Abs; cyan arrowheads indicate OCN deposits in extracelluar bone matrix. I–L: triple fluorescence signals from DAPI (blue) and anti-hMit (green) and anti-von Willebrand factor (red) Abs superimposed to pseudo-phase contrast images (J, L). Arrowheads indicate vasa double stained by the two Abs. Bar: 10 µm.

## *1.3 Skeletal muscle regeneration by hDPSCs and hAFSCs*

- ***Myogenic differentiation in vitro***

In order to test their myogenic potential human AFSCs and DPSCs were induced towards the myogenic differentiation *in vitro* by means of two different protocols, based on a direct co-culture with C2C12 mouse myoblasts and on a DNA demethylating treatment with 5-Aza-2'-deoxycytidine, respectively.

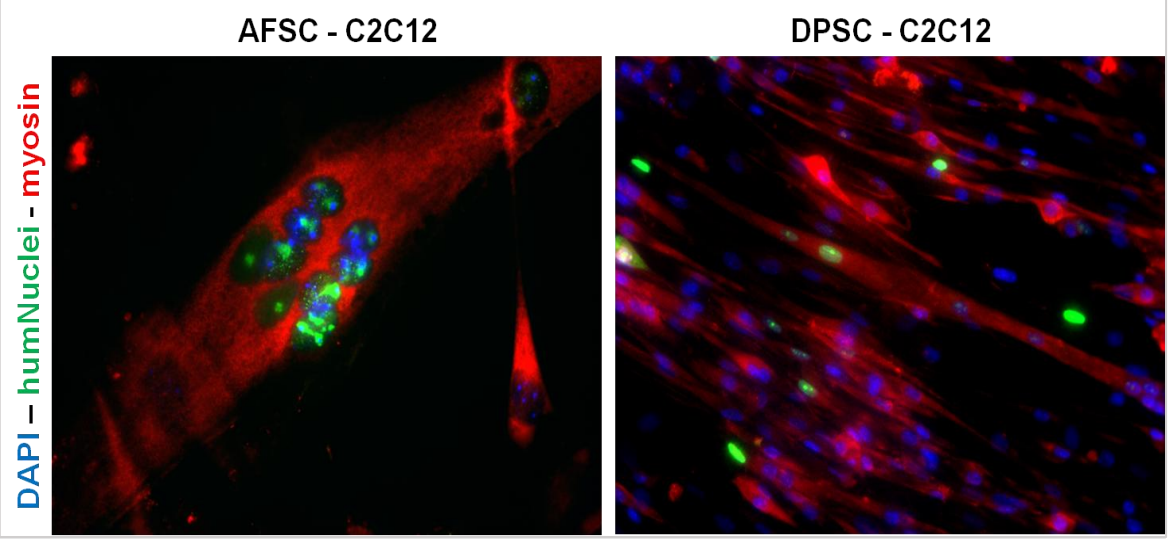


**Figure 17.** Immunofluorescence staining of direct co-cultures of hAFSCs and hDPSCs with C2C12 mouse myoblasts. New-formed myotubes are labeled with DAPI (blue), anti-human nuclei (green) and anti-myosin heavy chain (red) abs.

The immunofluorescence analysis performed on hDPSCs and hAFSCs after 14 days of direct co- culture with C2C12 cells revealed the formation of new myotubes showing a positive staining with both anti-human nuclei and anti-myosin heavy chain (MyHC) abs, as shown in Figure 17. In particular, new-formed hybrid myotubes containing central nuclei either of human and mouse origin were observed, thus demonstrating a shared participation of human stem cells and mouse myoblasts to the formation of functional new myotubes.

Immunofluorescent labeling was also carried out on hDPSCs and hAFSCs differentiated alone, after the DNA demethylation treatment with 5-Aza (Figure 18). Human AFSCs

underwent the myogenic commitment, after 14 days of induction, as demonstrated by the positive staining for myogenin, one of the myogenic regulatory factors (MRFs), peculiar of the myogenesis process (Figure 18A).

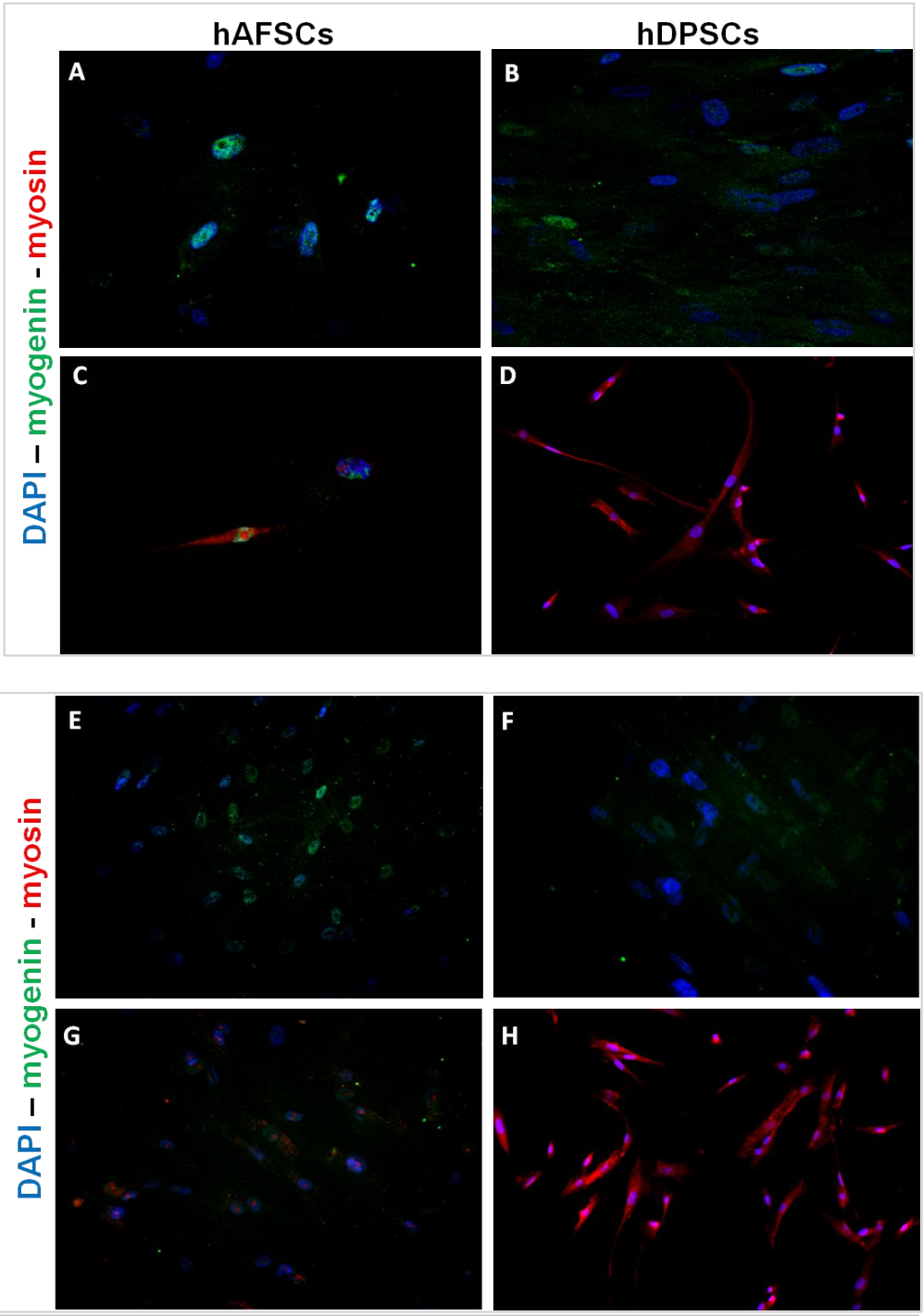


**Figure 18.** Immunofluorescence analysis of myogenic differentiation of hAFSCs and hDPSCs by means of demethylation treatment with 5-Aza. Immunofluorescence staining with DAPI (blue), anti-myogenin (green) and anti-myosin (red) abs. Myogenic differentiation was induced with (E-H) and without (A-D) the addition of conditioned fusion media from differentiated C2C12. Human AFSCs and DPSCs underwent myogenic commitment after 14 (A,C, E, G) and 21 (B, D, F, H) days of induction, respectively.

Moreover, at the same time point, some hAFSCs also expressed myosin heavy chain, confirming a terminal myogenic commitment (Figure 18C). At 21 days of induction – one week later than hAFSCs - hDPSCs expressed myogenin and myosin heavy chain as well (Figure 18B, 18D).

Similar results were observed for both hDPSCs and hAFSCs differentiated after the demethylating treatment, with the addition of conditioned fusion media from differentiated C2C12 (Figure 18E-H).

- ***Engraftment evaluation and muscle regeneration after in vivo transplantation***

Following the results observed *in vitro* the subsequent step of the study was to investigate the capability of human DPSCs and AFSCs pre-differentiated towards the myogenic lineage through the use of 5-Aza, with and without the addition of conditioned media from C2C12 cells, to regenerate the dystrophic skeletal muscle tissue of SCID/*mdx* mice, murine model of Duchenne muscular dystrophy.

The animals treated with human DPSCs and AFSCs via intramuscular injection were sacrificed at different time points, and the explanted gastrocnemia muscles were processed for further analyses, with the aim to evaluate the successfulness of the treatment on different aspects.

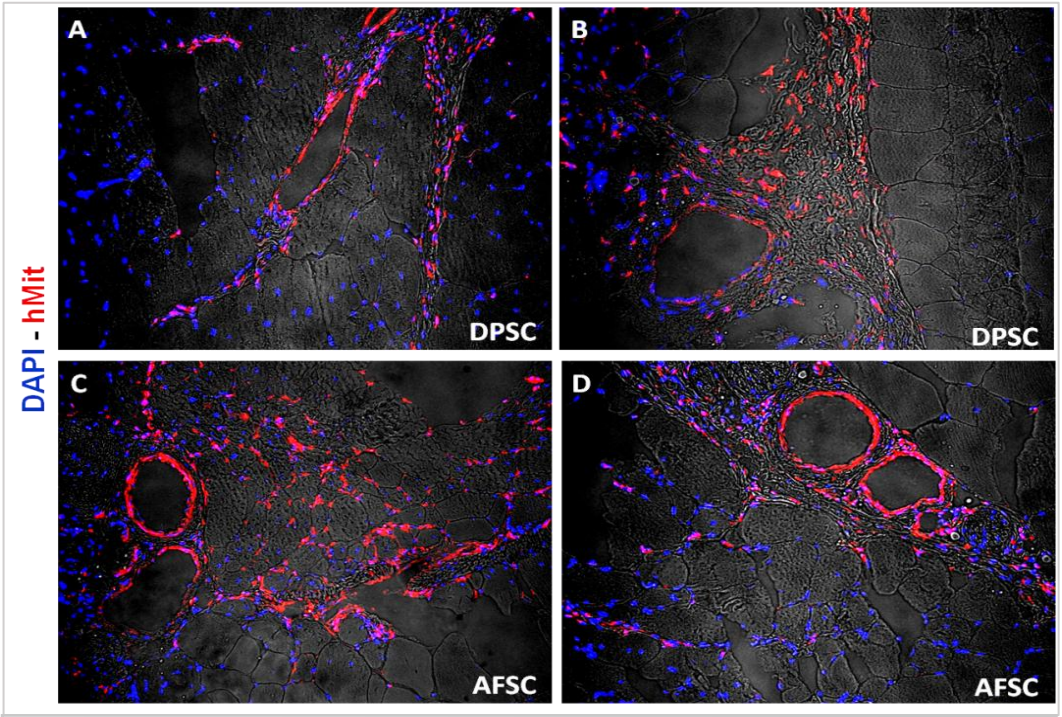


**Figure 19.** Engraftment evaluation at 7 days. Immunofluorescence staining with DAPI (blue) and anti-hMit ab (red) shows the presence of human DPSCs (A, B) and AFSCs (C, D) within the host dystrophic muscle, after the cell injection.
A, C: muscular cryosections immunostained after injection of human stem cells pre-differentiated through demethylation treatment.
B, D: muscular cryosections immunostained after injection of human stem cells pre-differentiated through demethylation treatment and addition of conditioned media from C2C12.

Immunofluorescence analysis shows that as early as 7 days after injection, human DPSCs and AFSCs are present and appear to be histo-integrated within the host muscle, as demonstrated by the positive staining against human mitochondrial protein (Figure 19A, 19C). Similar results were observed after both the pre-differentiating conditions (Figure 19B, 19D).

Furthermore, the double immunofluorescence staining, performed still 7 days after injection, with anti-human mitochondria (anti-hMit) and anti-von Willebrand factor (anti-vWill) abs revealed that cells positively stained as endothelial cells within the vasa walls correspond to the human DPSCs and AFSCs specifically recognized by the anti-human

mitochondria ab (Figure 20A, 20C). Similar results were observed after both the pre-differentiating conditions (Figure 20B, 20D).

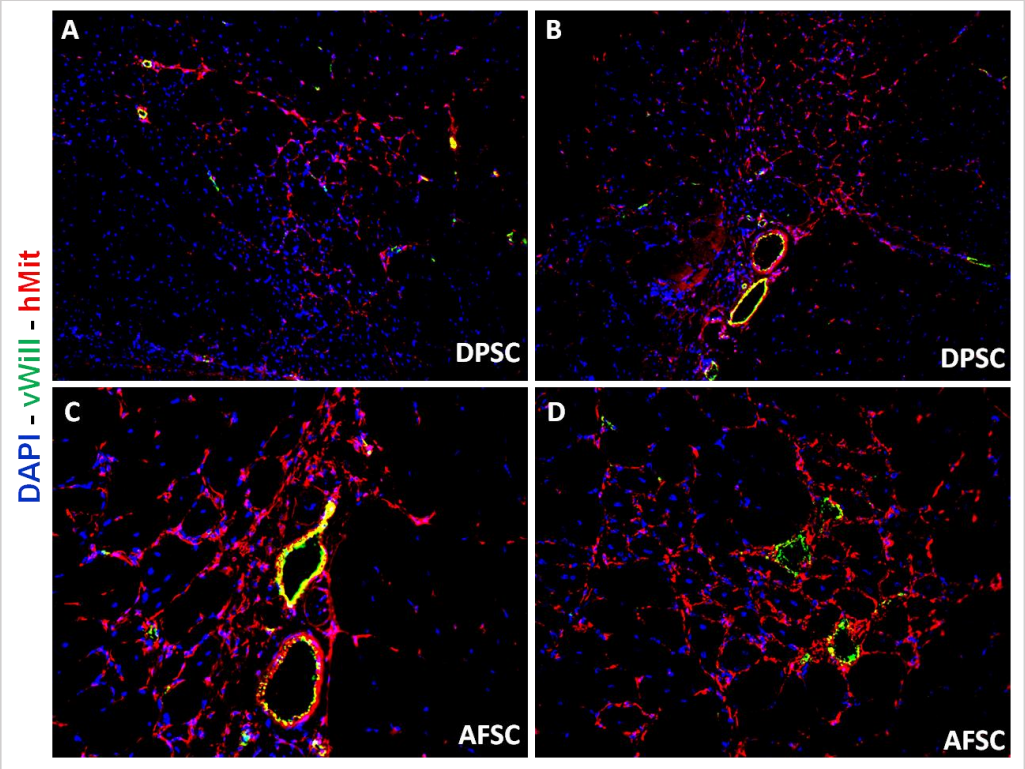


**Figure 20.** Evaluation of angiogenesis promotion by human DPSCs and AFSCs 7 days after the injection. Immunofluorescence labeling with DAPI (blue), anti-vWill (green) and anti-hMit (red) abs. Green and red signals appear co-localized showing that human stem cells are present within the endothelium of new vasa. A, C: muscular cryosections immunostained after injection of human stem cells pre-differentiated through demethylation treatment.
B, D: muscular cryosections immunostained after injection of human stem cells pre-differentiated through demethylation treatment and addition of conditioned media from C2C12.

Moreover, two weeks after the cell injection, the immunofluorescent labelling showed the restored expression of dystrophin within the mouse dystrophic skeletal muscle fibers, as demonstrated by the positive staining against dystrophin. Interestingly, the fibers expressing dystrophin are also positively stained against the human mitochondrial protein (Figure 21A, 21C). Similar results were observed after both the pre-differentiating conditions (Figure 21B, 21D).

Regenerated mouse muscle fibers positively labelled by anti-human mitochondria ab keep expressing dystrophin 4 weeks after cell injection (Figure 21E, 21G). Similar results were observed after both the pre-differentiating conditions (Figure 21F, 21H).

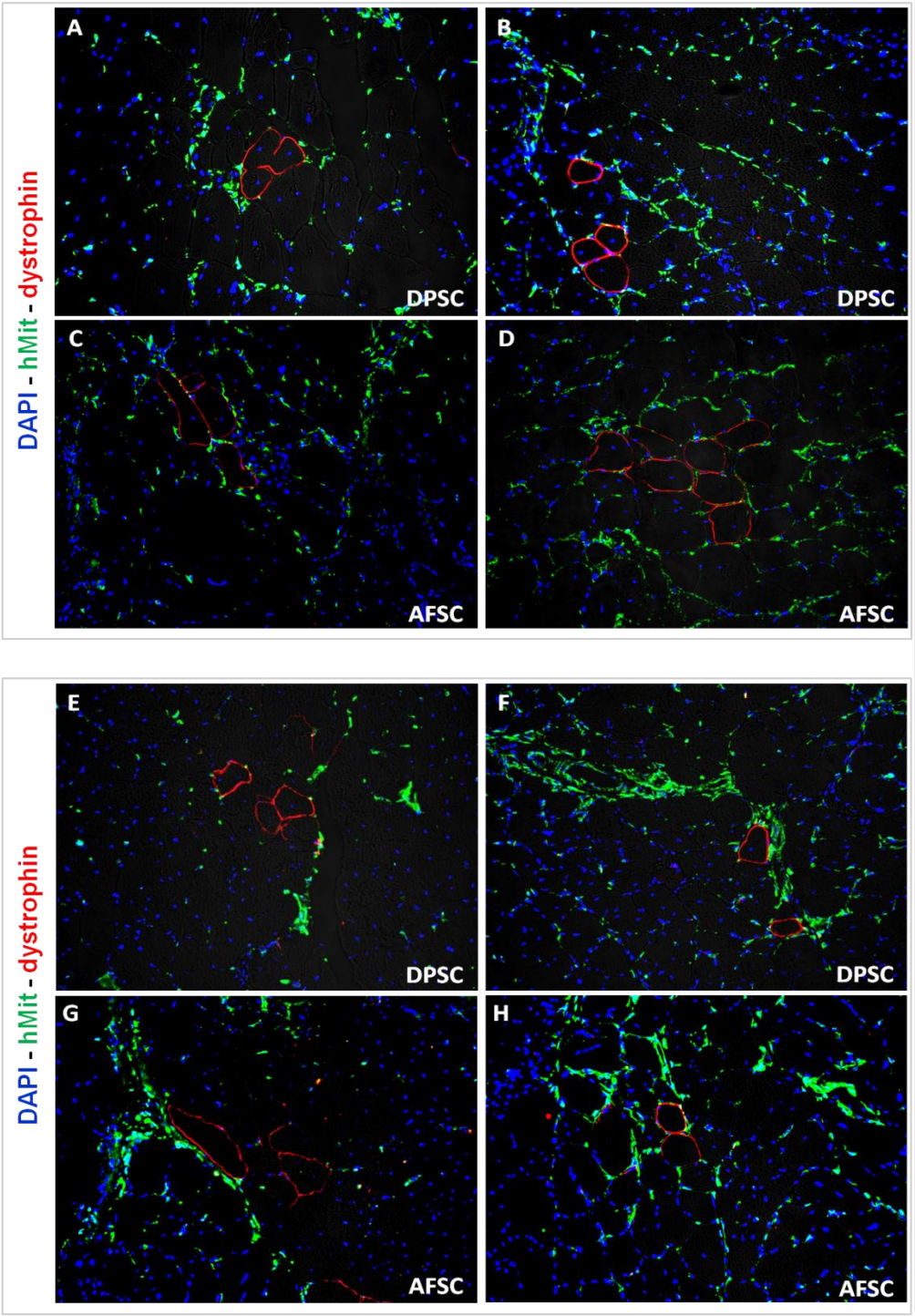


**Figure 21.** Evaluation of muscle regeneration after cell injection. Immunofluorescence staining with DAPI (blue), anti-hMit (green) and anti-dystrophin (red) abs shows the presence of human DPSCs (A, B) and AFSCs (C, D) within dystrophin positive muscle fibers, as early as 14 days after the cell injection. The restored expression of dystrophin by muscle fibers containing human DPSCs and AFSCs can be observed still 28 days after the treatment.
A, C, E, G: muscular cryosections immunostained after injection of human stem cells pre-differentiated through demethylation treatment.
B, D, F, H: muscular cryosections immunostained after injection of human stem cells pre-differentiated through demethylation treatment and addition of conditioned media from C2C12.

- ***Histological analysis***

To evaluate the evolution of fibrosis within the mouse dystrophic skeletal muscle after the treatment with human stem cells, serial sections of the gastrocnemia muscles injected with hDPSCs and hAFSCs were stained with H&E and Masson's trichrome.

Analysis of Masson's trichrome staining in Figure 22 highlighted a reduction of fibrosis and necrosis processes within the muscle fibers treated with pre-differentiated hDPSCs and hAFSCs, compared to the controls (Figure 22A-C). At the same time, the histological analysis revealed that the muscles injected with human stem cells contain wide areas of muscle regeneration, as demonstrated by the high presence of centrinucleated muscle fibers (Figure 22D-F).

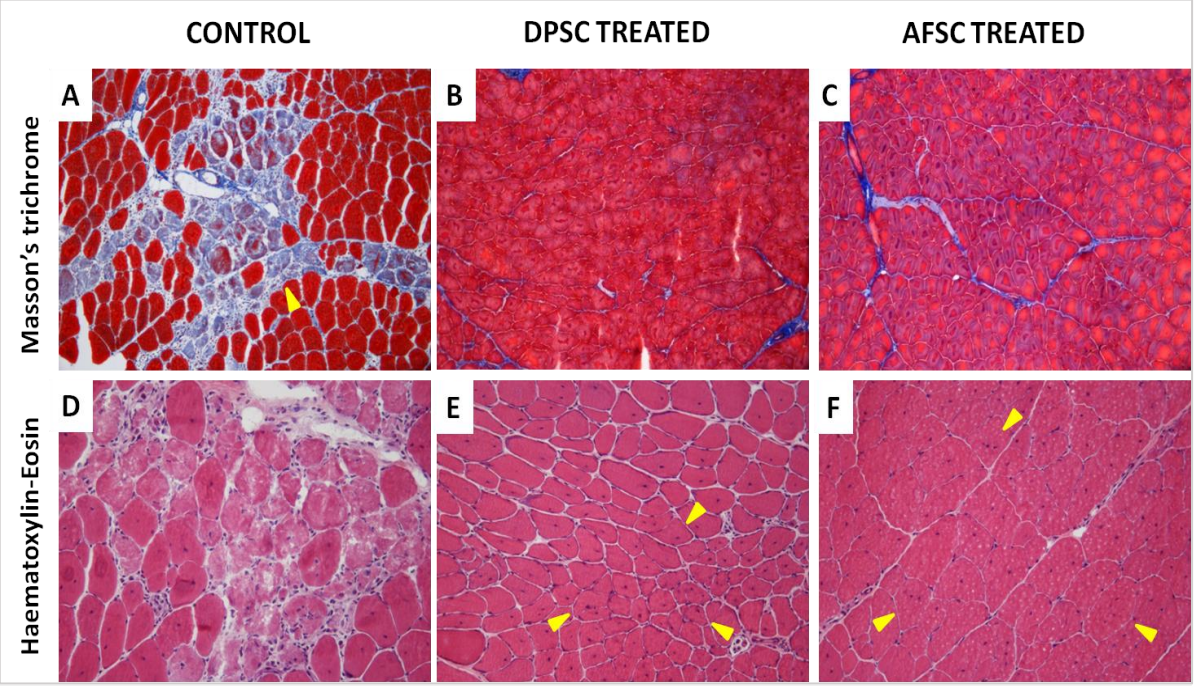


**Figure 22.** Comparison of fibrosis and necrosis processes between controls (non-injected muscles) and muscles injected with human DPSCs and AFSCs. A, B, C: Masson's trichrome highlights the reduction of fibrosis and necrosis within the muscles treated with human DPSCs (B) and AFSCs (C), while the controls (A) appear rich in fibrotic tissue (blue stained collagens). D, E, F: H&E shows an active muscle regeneration in progress within the muscles treated with human DPSCs (E) and AFSCs (F), as represented by a high number of centrinucleated muscle fibers (yellow arrowheads), whereas the controls (D) are characterized by a severe degeneration of the fibers.

# *Discussion*

## *1. Human DPSCs and AFSCs for bone tissue regeneration*

This section of the thesis investigated the future applications of human alternative stem cells in tissue engineering, including the application of human AFSC or DPSC/fibroin complexes to the repair of bone defects. An engineered osseous graft derived from easily accessible cell sources, such as the amniotic fluid and the dental pulp, would offer several advantages over techniques currently available for the repair of severe bone damages. The engineered approach affords much versatility, since a stem cell/scaffold implant can be crafted into any shape and size, following the anatomical details of the defect. This study evaluated the capability of human stem cells (hAFSCs and hDPSCs) seeded onto fibroin scaffolds, in inducing new bone formation in critical size calvaria defects.

Radiograph and histological analysis revealed that the highest bone formation occurred in fibroin, seeded with stem cells committed towards ostegenic lineage, whereas the scaffold alone resulted in much lower bone repair. Formation of bone with a very efficient union of the new bone with the margins of the defect area occurred after the use of hDPSC or hAFSC/fibroin constructs. As a matter of fact, this implant leaded to a very homogenous bone formation process, as both parietal tables are in reconstruction, indicating this as a promising approach. The defect was also healed with the use of fibroin only, but the ossification process was evidently incomplete when compared to the implant of fibroin plus human stem cells. This represents a further evidence of the osteoinductive properties of fibroin (Meinel L *et al*, 2006; Mieszawska AJ *et al*, 2010; Zhang Y *et al*, 2011).

Results obtained in our former study on ectopic implants (Maraldi T *et al*, 2011) enabled us to identify fibroin scaffold as a promising tool for osteogenic differentiation of human

stem cells. The present findings demonstrate that fibroin scaffold promotes human stem cell proliferation and differentiation in parietal bone graft in a rat experimental model.

Fibroin scaffold shows a higher biointegration with rat tissues, since a great vascularization and the homing of host cells inside the scaffold microenvironment occur.

Moreover, transplantation of human stem cells in cranial defect model leads to substantial increase in bone formation, and the presence of human cells 30 days after implantation demonstrates that stem cells, already committed to osteogenic differentiation *in vitro*, finally pilot the bone repair *in vivo*. Confocal analysis shows ossification areas reflecting physiological osteogenesis, since a layer of human cells is distributed on the new bone margin, suggesting a bone deposition by osteoblast apposition. At the same time, inside the bone synthesis area, human cells are progressively entrapped in lacunae showing a less intense staining for anti-human mitochondrial protein ab, indicating a lower metabolic activity, as in osteocytes occurs.

Moreover, this study provides additional insight into the potential use of fibroin scaffolds as cell carriers for implantation into skeletal connective tissue defects.

Mechanical strength is often a prerequisite for scaffolds destined to bone tissue engineering; however, most of the materials, although possessing the needed mechanical forces, are not optimal for cell growth and differentiation (Ben-David D *et al*, 2011). A major advantage of a mechanically stable scaffold is that it contrasts the deformation caused by adjacent tissues at the implant site. This deformation can lead to an implant compression within the defect or even a shift beyond the borders of the defect site (Meinel L *et al*, 2006). The data observed in this study indicate that fibroin scaffolds retain a high percentage of water maintaining their mechanical properties. Moreover, histological analysis demonstrates that fibroin maintains its shape after implant and has been

progressively reabsorbed during bone new-synthesis, while remains longer in areas where bone deposition may yet occur, according to data previously reported (Meinel L *et al*, 2006).

Taken these considerations, fibroin scaffold seems to be an appropriate solution for bone tissue engineering. These findings show that an optimal bone defect repair can be obtained by colonizing fibroin scaffold with hDPSCs and hAFSCs, considering that both present the advantage of an easy recruitment without ethical problems. Autologous transplantation might be performed with both cell types. Human AFSCs could be used in neonatal surgery to treat congenital malformations such as the large cranial defects occurring in AC (*aplasia cutis*). Human DPSCs, available mainly from the puberty onward, might be used to heal pathologies occurring in the adult life. With respect to other adult stem cell sources, such as adipose-derived stem cells, amniotic fluid and dental pulp are tissues largely available from routine medical practice and therefore suitable to be collected in stem cell banks in order to be employed in both autologous and allogenic transplantations. In fact, both the cell types present major histocompatibility complex (MHC) class I, but lack MHC II class molecules and seem to have immunosuppressive activity (De Coppi P *et al*, 2007; Kim J *et al*, 2007; Tsai MS *et al*, 2004; Pierdomenico L *et al*, 2005). Further evidence came from recents findings demonstrating that MSCs and DPSCs are able to induce T-cells apoptosis through Fas/Fas ligand pathway (Akiyama K *et al*, 2012; Zhao Y *et al*, 2012) All these data indicate that the progenitor cell association with an appropriate scaffold represents an optimal tool in many aspects of bone regeneration and tissue engineering applications.

## *2. Human serum is a suitable substitute for FCS to expand and differentiate human DPSCs*

The second section of the thesis was aimed to investigate a culture condition that may allow human DPSCs for cell-based therapy to be used in clinical application with minimal safety concerns. This goal assumes it is desirable and necessary to obtain a sufficient number of cells, as the result of expansion and differentiation conditions realized by using the most suitable reagents to promote cell proliferation and differentiation.

Foetal calf serum (FCS) has been widely used for this purpose over the past several decades. Anyway, some potential hazards related to the use of FCS in clinical settings have been raised in terms of potential disease transmission and xenogenic immunologic response (Spees JL *et al*, 2004).

Indeed previous works indicate that FCS proteins can be internalized in stem cells and involve the hazard of pathogen transmission: bovine proteins may also be recognized as antigenic substrates, leading to a xenogenic immune response (Shahdadfar A *et al*, 2005; Dimarakis and Levicar, 2006). For these reasons, in addition to good manufacturing practices (GMP), clinical protocols for cell therapy encourage the use of FCS substitute.

Even serum free media have been tested, but so far literature has demonstrated that these media do not constitute an adequate support for proliferation of mesenchymal stem cells, without the addition of exogenous growth factors (Lee JW *et al*, 2004; Foreman MA *et al*, 2006). Supplementation of media with small amount of serum and specific factor as EGF and PDGF could represent a valid perspective (Karbanová J *et al*, 2011).

Various human plasma derivatives have been purposed as alternatives to FCS to supply nutrients, adhesion and growth factors. These alternative supplements include autologous

or allogenic human serum, human plasma, human platelet lysates and their released factors (Schallmoser K *et al*, 2007; Stute N *et al*, 2004; Kocaoemer A *et al*, 2007). In the last years platelet-rich plasma was largely used to growth and differentiate MSC but its role in MSC osteogenic differentiation is debated (Feng Y *et al*, 2010; Gruber R *et al*, 2010; Tomoyasu A *et al*, 2007). The use of human serum, preferably autologous, appears to provide benefits for different aspects in culture and differentiation of human synovial MSCs and human bone marrow MSCs (Tateishi K *et al*, 2008; Nimura A *et al*, 2008; Aldahmash A *et al*, 2011).

Evidence in favour of using autologous serum also exist for the expansion of human bone marrow MSCs, indeed it results as effective as supplementing culture medium with FCS, and cells appear transcriptionally more stable over many cell doublings (Shahdadfar A *et al*, 2005). Nevertheless, autologous serum may also be a scarcely available or qualitatively affected source, particularly in the case it must be taken from some classes of patients, such as children, elderly, anaemic and with ongoing inflammatory processes individuals (Tateishi K *et al*, 2008). In these cases, allogenic human serum, potentially from blood type AB healthy donors may represent a preferable alternative to autologous serum.

To date, human platelet lysate and platelet-rich plasma have been shown to be suitable culture supplements to replace FCS, for the expansion and in vitro osteogenic differentiation of hDPSCs (Lee UL *et al*, 2011; Govindasamy V *et al*, 2011). In literature there is not yet evidence that human serum may constitute a suitable replacement to FCS for culture and differentiation of human DPSCs.

This study demonstrates that human serum (HS) is an appropriate supplement for the *in vitro* expansion of human DPSCs, as its addition to culture medium promotes a good proliferation rate, comparable to the one measured in culture conditions using FCS.

Furthermore, HS increases the hDPSCs doubling during culture, indicating this as an optimal condition to obtain a sufficient cell number soon and to consequently reduce the waiting for a therapeutical treatment. Otherwise, cells cultured in serum free medium showed a growth arrest. Moreover, HS does not induce cell senescence or apoptosis and does not affect the surface antigens expression and the multi-lineage differentiation ability of hDPSCs.

The expression of bone related proteins as well as the mineralization of the extracellular matrix analyzed in experiments of osteogenic differentiation *in vitro* indicate that the use of HS is adequate to osteogenic differentiation protocols for hDPSCs.

Moreover HS, compared to FCS, seems to induce a better mineralization of the extracellular matrix synthesized by differentiating hDPSCs.

When transplanted *in vivo*, in immunocompromised animals, by means of stem cell-scaffold constructs, hDPSCs pre-differentiated in both the osteogenic media show a significant contribution to the regeneration of critical size bone defect. Immunofluorescence staining with anti-human mitochondria antibody demonstrates that hDPSCs widely contribute to the osteocyte population of the new-formed bone and seem to participate to the angiogenetic process that gives rise to endothelial cells of the new-formed bone. Thus, these findings have demonstrated that human serum represents an eligible alternative to FCS, for successful expansion and differentiation of human DPSCs towards osteogenic lineage, finding, furthermore, a helpful application for *in vivo* transplantation.

In particular, the results obtained *in vivo* allow to propose human serum as additive for expansion and differentiation of human DPSCs to be applied in human cell therapy with

GMP-compatible protocols. Indeed, it matches with the criteria approved by the Committee for medicinal products for human use.

In this respect, it would be eventually conceivable to develop banks for the storage of human cryopreserved stem cells from different sources, which would collect not only stem cell samples, but also backup specimens of human serum, both of them obtained from the same donors, thus allowing optimal conditions for the expansion and following application of human stem cells in clinical cell-based therapies, under compliance with GMP-compatible methods.

## *3. Skeletal muscle regeneration by human DPSCs and AFSCs*

Duchenne Muscular Distrophy (DMD) is one of the most common (1 in 3500 live male births) and severe dystrophies, which occurs with a progressive degeneration of muscle fibers and results in death by the third decade of life (Emery and Muntoni, 2003).

Since the use of corticosteroids only delays the loss of muscle function and at this moment a therapy capable to stop the progression of muscle degeneration is lacking (Manzur AY *et al*, 2008), different therapeutic approaches have been tested in order to find the most suitable one for treating DMD.

Cell therapy may be used to reintroduce dystrophin and thus to repair damaged muscle fibers.

Recently, various stem cell lines were used to treat muscular dystrophy, although with narrow results. Within the skeletal muscle compartment, other than satellite cells, many adult multi-lineage progenitor cells show a myogenic potential: muscle derived stem cells (MDSCs), muscle side population (mSP) cells and muscle derived CD133+ progenitors (Qu-Petersen Z *et al*, 2002; Peault B *et al*, 2007; Torrente Y *et al*, 2004).

Aside from the muscular-resident cells, non-muscular stem cells, from different tissues, such as bone marrow, adipose tissue, umbilical cord, placenta, were tested for myogenic differentiation capacity (Ferrari G *et al* 1998; Pinheiro CH *et al* 2012; Brzóska E *et al*, 2006; Kawamichi Y *et al*, 2010). This thesis evaluated the myogenic potential of human dental pulp and amniotic fluid derived stem cells, by using different conditions, such as the direct co-culture with murine myoblasts, the use of the DNA demethylating agent 5-Aza-2'-deoxycytidine, and the addition of conditioned media from differentiated mouse myoblasts, with the aim to determine the optimal conditions for achieving the myogenic commitment of these human stem cells.

Previous studies demonstrated that the genes related to skeletal myogenic differentiation are controlled by DNA methylation and that the use of the demethylating agent 5-Aza was able to induce adult human bone marrow stem cells to differentiate towards the cardiomyogenic lineage (Ye NS *et al*, 2006; Antonitsis P *et al*, 2008).

The results from the direct co-culture of hDPSCs and hAFSCs with C2C12 mouse myoblasts have demonstrated that these human stem cells are actually able to participate and fuse with mouse myoblasts to form new hybrid myotubes, as clearly shown by the positive staining with anti-human Nuclei ab.

Even more importantly, after being differentiated without direct co-culture, by means of DNA demethylation treatment, hDPSCs and hAFSCs underwent myogenic commitment, even though myotubes formation was not observed. When conditioned fusion media from differentiated C2C12 cells were added to the myogenic medium, a quantitatively higher expression of muscle specific factors and markers by hDPSCs and hAFSCs occurred. These observations suggest that modulating the myogenic potential of these cells may be achieved by combining the demethylation treatment – which triggers the expression of those muscle regulatory factors that are fundamental for the myogenesis process – with the addition of conditioned fusion media, which contains soluble factors, such as insulin-like growth factor II (IGF-II), that have a role in promoting the myogenesis (Duan C *et al*, 2010).

Following the results obtained from the myogenic differentiation *in vitro*, this study was focused on the evaluation of the regenerative potential of hDPSCs and hAFSCs after cell injection into the dystrophic skeletal muscle of SCID/*mdx* mice, the murine model of DMD.

A good early engraftment was observed within the skeletal muscle fibers of the host, as confirmed by the high presence of human cells into the injected muscles, positively labelled by anti-hMit ab. This significantly demonstrated that these cells are able to survive and be histo-integrated after transplantation in the host tissue. In particular, human DPSCs and AFSCs show a direct participation in muscle regeneration as demonstrated by their contribution in promoting of new angiogenesis, which is a typical event of tissue regeneration processes.

Furthermore, the regenerative potential of these cells was clearly demonstrated by the restoration of dystrophin expression, which shows off as early as 14 days after the cell injection and keeps being detected 4 weeks after the transplantation.

These observations suggest that, once engrafted within the host muscle, human DPSCs and AFSCs actively contribute to regenerate the dystrophic skeletal muscles, either by promoting the angiogenesis and by restoring the dystrophin expression, that is maintained after a relatively long time.

While from a therapeutic standpoint this research has reached the objective to demonstrate the ability of human DPSCs and AFSCs to regenerate the dystrophic skeletal muscle and restore the dystrophin expression, a question mark might be raised on whether this is the consequence of a cell differentiation or a cell fusion.

According to the ontogeny, the skeletal muscle development is a tightly regulated process involving the specification of mesodermal precursors into myoblasts and following differentiation and fusion of these cells into multinucleated myotubes, therefore skeletal muscle formation is the result of cells whose derivation has been definitively established to imply cell fusion (Doherty JT *et al*, 2011; Eisenberg LM and Eisenberg CA, 2003).

What was observed in the direct co-culture with C2C12 demonstrates that a clear fusion process between human and mouse cells occurred, giving rise to new hybrid multinucleated myotubes, even though it was not possible to detect the formation of human myotubes, neither when human DPSCs and AFSCs were co-cultured with murine myoblasts, nor when when they were differentiated after the demethylating treatment. However, the treatment with 5-Aza allowed the human stem cells to be effectively committed towards the myogenic lineage, as clearly shown by the immunofluorescence microscopy analysis. The detection of human stem cells in the injected dystrophic skeletal muscle and, in particular, their presence within muscle fibers with a restored expression of dystrophin demonstrate that human DPSCs and AFSCs are able to histo-integrate in the muscle microenvironment, but yet preserve their genetic identity over a relatively long time, through the maintained expression of dystrophin, otherwise completely lacking in dystrophic skeletal muscles.

In light of these results, it may be reasonable to assume that human DPSCs and AFSCs underwent an event that can be defined halfway between the cell differentiation and the cell fusion processes.

Therefore, human DPSCs and AFSCs may be considered a suitable tool that could be very useful for translational strategies aimed to enhance the repair of injured skeletal muscle in DMD or trauma patients, besides representing an effective means to promote new vascularisation of different tissues.

## *Conclusions*

This thesis has highlighted the properties and the differentiation abilities of human dental pulp and amniotic fluid stem cells, that allow to propose them as a suitable non-controversial source of stem cells, for the application in regenerative medicine of different tissues.

With regard to the bone regeneration, the effective differentiation potential towards the osteogenic lineage shown by hDPSCs and hAFSCs is confirmed when these human stem cells are combined to fibroin scaffold in stem cells-scaffold complexes aimed to heal critical size bone defects *in vivo*; indeed, the three-dimensional structure of this fibroin support has shown to be an optimal microenvironment for the osteogenic differentiation of these human stem cells and for the subsequent bone tissue regeneration.

These results suggest the eventual applicability of human DPSCs and AFSCs for autologous transplants. Moreover, the need for development of optimal culture conditions without the use of animal products for future applications of cell therapy to humans, has leaded the research to investigate alternative human derivatives as supplements to culture and differentiation media for mesenchymal stem cells.

The results obtained in this study demonstrate that human serum not only promotes a good proliferation rate, but also increases the cell doubling during culture, does not induce cell senescence or apoptosis and does not affect either the expression of stemness surface antigens and the multi-differentiation ability *in vitro*. The mineralization of the extracellular matrix observed in the experiments of osteogenic differentiation *in vitro* indicate that the use of human serum is adequate to hDPSCs osteogenic differentiation protocols. The subsequent results have confirmed the successful use of human serum for

pre-differentiating of hDPSCs to be transplanted *in vivo* for the regeneration of critical size bone defects, by means of a stem cell-scaffold construct.

This study has also demonstrated that human dental pulp and amniotic fluid stem cells have the capability to be committed towards the myogenic lineage *in vitro* and to actively regenerate the skeletal dystrophic muscle of SCID/*mdx* mice, in particular by restoring the expression of dystrophin.

Moreover, human DPSCs and AFSCs have shown a high contribution in promoting new angiogenesis of the tissues in which they were implanted *in vivo*, an event that has never been implicated with tumorigenic processes in the experiments carried out so far.

Altogether these data allow to propose these two types of human stem cells for a future potential application in regenerative medicine of different tissue injuries, following traumas and diseases.

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