

**Alma Mater Studiorum – Università degli Studi di Bologna**

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**Scuola di Dottorato in Scienze Mediche e Chirurgiche Cliniche**

Dottorato di Ricerca in Scienze Morfologiche Umane e Molecolari

Settore disciplinare BIO/16

Dipartimento di Anatomia ed Istologia

Università di Modena e Reggio Emilia

**Ciclo XXIII**

**STEM CELLS FROM ALTERNATIVE SOURCE IN TISSUE  
ENGINEERING: DPSC OSTEOGENIC DIFFERENTIATION  
ON 2D AND 3D SCAFFOLDS**

**Tesi di Dottorato**

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**XXIII Ciclo**

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**Anno Accademico 2009/2010**

**A mio nonno**

**“ Quanto manca alla vetta?”**

**“Tu sali, e non pensarci!”**

F. W. Nietzsche

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## **Abstract**

The aim of this thesis was to characterize the *in vitro* osteogenic differentiation of dental pulp stem cells (DPSCs) in 2D cultures and 3D biomaterials. DPSCs, separated from dental pulp by enzymatic digestion and isolated by magnetic cell sorting, were differentiated toward osteogenic lineage on 2D surface by using an osteogenic medium. During the differentiation process, DPSCs expressed specific bone proteins like Runx-2, Osx, OPN and OCN sequentially, as occurring during osteoblast differentiation, and produced extracellular calcium deposits *in vitro*. DPSCs were then cultured on 3D distinct bioscaffolds, Matrigel<sup>TM</sup>, Collagen sponge, P(d,l)LA and Silk fibroin, and analyzed for their ability to differentiate into osteoblastic cells *in vivo*. With the addition of a third dimension, osteogenic differentiation and mineralized extracellular matrix production significantly improved. In particular, in Matrigel<sup>TM</sup> DPSCs differentiated with osteoblast/osteocyte characteristics and formed calcified nodules with a 3D intercellular network. Furthermore, DPSCs differentiated in collagen sponge actively secreted human type I collagen micro-fibrils and formed calcified matrix containing trabecular-like structures. The effect of pre-differentiation of the stem cells in bioscaffolds on the subsequent bone formation *in vivo* was determined in a rat subcutaneous model. This thesis demonstrated the strong potential of DPSCs to produce 3D mineralized bioengineered constructs *in vitro* and *in vivo* and suggested that fibroin may be an effective tool for functional repair of critical size bone defects.

Among the many cell types that may be useful in regenerative medicine, mounting evidence suggests that human term placenta-derived cells will join the list of significant

contributors. The chorionic villi of human term placenta are a rich source of stem cells, and the stem cell “niche” within the chorionic tissue regulates how stem cells participate in placental tissue generation, maintenance and repair even if the anatomic location of the niche has not been defined.

After enzymatic digestion, stem cells were isolated from human term placenta, using MACS® selection: c-Kit<sup>+</sup> population grew on plastic culture ware and had a fibroblast-like morphology. PCR and WB analysis showed the expression of Oct4, a pluripotent marker, in cultured cells. Using immunohistochemical protocols, we studied the expression of c-Kit, Oct4 and Stella in chorion-side of placenta. Cytoplasmatic and nuclear staining revealed different morphological localization of stem cells: 1) the cytoplasm of perivascular cells were c-Kit<sup>+</sup>; 2) big cells with round morphology had a nuclear positivity for Oct4 and a cytoplasmatic positivity for c-Kit; 3) cells localized close to syncytiotrophoblast layer had a nuclear positivity for Oct4 and Stella. Taken together, our results indicated that chorion-derived placental cells are a convenient source of stem cells for cell-based therapy, as well as an ideal target for *in utero* foetal gene therapy.

### **Riassunto**

Le DPSCs, estratte dalla polpa dentale a seguito di digestione enzimatica e isolate mediante Magnetic cell sorting (MACS®), sono state indotte a differenziare in senso osteogenico. Il medium differenziativo e' stato aggiunto alle cellule in cultura su vetrini Termanox, e le cellule sono state fissate a tempi di coltura diversi, per valutarne il grado differenziativo. Durante il processo di differenziamento in senso osteogenico, le DPSCs esprimono specifici fattori di trascrizione, quali Runx2 e Osx, e bone proteins, quali

OCN e OPN, in una sequenza temporale, analogamente a ciò che avviene durante il processo di osteogenesi da parte degli osteoblasti. Una ulteriore conferma dello stato differenziativo è data dalla produzione di depositi extracellulari di calcio da parte delle dDPSCs (DPSCs differenziate). Per meglio mimare il contesto fisiologico, le dDPSC sono state coltivate su distinti supporti 3D: Matrigel™, spugna di collagene, P(d,l)LA e Fibroina. Con l'aggiunta della terza dimensione, il differenziamento osteogenico e la produzione di matrice extracellulare mineralizzata hanno ottenuto una notevole accelerazione rispetto alle cellule differenziate in 2D. In particolare, nel Matrigel™, le dDPSC con caratteristiche di osteoblasti/osteociti mostrano gap-junctions e formano noduli calcificati con un network intercellulare piuttosto sviluppato; se coltivate nella spugna di collagene secernono attivamente collagene di tipo I, formando micro fibrille e strutture simili alle trabecole ossee, circondate da matrice calcificata. Successivamente, i complessi cellule-biomateriali sono stati testati *in vivo* (impianti ectopici): studi preliminari hanno mostrato che lo scaffold costituito da fibroina + dDPSCs è risultato essere l'unico che maggiormente supporta il processo di osteogenesi/osteointegrazione nel sito di impianto, producendo matrice mineralizzata. Si può quindi concludere che, pur essendo ancora nella fase di risultati preliminari, gli scaffolds che meglio si integrano e favoriscono il processo di ossificazione, sono possibili supporti utilizzabili in medicina rigenerativa e come terapia tissutale al fine di sanare nei pazienti deficit scheletrici di diversa eziologia.

In ultimo, analogamente allo studio sulle DPSC, si sono isolate cellule staminali dalla placenta umana a termine, a seguito di digestione enzimatica e selezione mediante

MACS® per c-Kit. La placenta risulta essere una fonte di cellule staminali, ancora in fase di studio: si è quindi cercata una localizzazione morfologica (nicchie) in sezioni di chorion incluse in paraffina, di cellule presentanti markers della linea germinale (Oct4 e Stella) e marker di staminalità (c-Kit). La localizzazione di nicchie di cellule staminali nella placenta ci permette di selezionare una nuova fonte di cellule pluripotenti/multipotenti, da utilizzare nella terapia fetale o in utero, e nella medicina rigenerativa, utilizzando un tessuto considerato “ di scarto”, con potenzialità maggiori rispetto alle cellule adulte ma con minori problematiche etiche rispetto alle cellule germinali.



## **Introduction**

*The Greek Titan, Prometheus, is a fitting symbol for regenerative medicine. As punishment for giving fire to Humankind, Zeus ordered Prometheus chained to a rock and sent an eagle to eat his liver each day. However, Prometheus' liver was able to regenerate itself daily, enabling him to survive. The scientific researchers and medical doctors of today hope to make the legendary concept of regeneration into reality by developing therapies to restore lost, damaged, or aging cells and tissues in the human body.*

## **Regenerative medicine**

The use of one body part for another or the exchange of parts from one person to another was mentioned in the medical literature even in antiquity and captured the imagination of many people over time. The maintenance of organs in culture was a major area of inquiry in the early 1900s. The kidney was the first entire organ to be replaced in human, in 1955 (Guill WR.,1955); however, the lack of good immune-suppression and the inability to monitor and control rejection, spurred physicians and scientists to look for other alternatives. Synthetic materials were introduced (like Teflon and silicone), opened a new field that included the use of devices for human use. However, the functional component of the original tissue was not achieved. Meanwhile,

new techniques for cell harvesting, culture, and expansion were developed: the concept of cell transplantation took hold in the research arena, and culminated with the first human bone marrow cell transplant in the 1970s. At this time, researchers began to combine the devices and materials with cell biology concepts, creating a new field called *tissue engineering* (Atala A *et al.*, 2009).

In 1999, William Haseltine, the Scientific Founder and Chief Executive Officer of Human Genome Sciences, coined the term '*regenerative medicine*',(Haseltine *et al.*, 1999): *a new branch of medicine will develop that attempts to change the course of chronic disease and in many instances will regenerate tired and failing organ systems.* Regenerative medicine brings all these areas (tissue engineering, cell transplantation, stem cells), under one defining field. In the last two decades, scientists have attempted to grow native and stem cells, engineered tissues, and to design treatment modalities using regenerative medicine techniques virtually for every tissue of the human body.

Applications of regenerative medicine technology may offer novel therapies for patients with injuries, end-stage organ failure, or other clinical problems, and may resolve the severe shortage of donor organs that is worsening yearly as the population ages as well as the increase of new cases of organ failure. Scientists in the field of regenerative medicine and tissue engineering are now applying the principles of cell transplantation, material science, and bioengineering to construct biological substitutes that will restore and maintain normal function in diseased and injured tissues. The stem cells field is also advancing rapidly, opening new avenues for this type of therapy (Atala A *et al.*, 2009).

## Cells for use in regenerative medicine

### - Native cells

Native cells used for tissue engineering are obtained from a small biopsy of tissue. When native cells are used for tissue reconstitution, donor tissue is dissociated into single cells, which are implanted directly into the host or expanded in culture, attached to a support matrix, and reimplanted after expansion. The implanted tissue can be heterologous, allogenic (donor derived) or autologous (the host's cells). Ideally, "autologous approach" allows lost tissue function to be restored or replaced *in toto* and with limited complications (Atala A *et al.*, 1997); autologous cells are not rejected by the immune system, and the use of immunosuppressant drugs is avoided. Native cells and tissues are usually preferable for reconstruction. In most cases, the replacement of lost or deficient tissues with functionally equivalent cells and tissues would improve the outcome for these patients. An advantage in using native cells is that they can be obtained from the specific organ to be regenerated, expanded, and used in the same patient without rejection, in an autologous manner. Bladder, ureter, and renal pelvis cells can be equally harvested, cultured, and expanded in a similar fashion (Cilento BG *et al.*, 1994; Liebert M *et al.*, 1997; Atala A *et al.*, 1993).

However, one of the limitations of applying cell-based regenerative medicine techniques to organ replacement has been the inherent difficulty of growing specific cell types in large quantities. Even when some organs, such as the liver, have a high regenerative capacity *in vivo*, cell growth and expansion *in vitro* may be difficult. By noting the location of the progenitor cells, as well as by exploring the conditions that

promote differentiation and/or self-renewal, it has been possible to overcome some of the obstacles that limit cell expansion *in vitro* (Atala A, 2009). Major advances in cell culture techniques have been made during the past decade, and these techniques make the use of autologous cells possible for clinical application. However, even now, not all human cells can be grown or expanded *in vitro*. Liver, nerve, and pancreas are examples of human tissues where the technology is not yet advanced to the point where these cells can be grown and expanded. Native cells and tissues are usually preferable for reconstruction. Furthermore, autologous cells are recognized as the ideal transplantation resource, some patients with end-stage organ disease or with not functioning organ because of congenital or acquired diseases, do not produce enough cells for transplantation. In these situations, allogenic cells may be advantageous: pluripotent/multipotent stem cells are envisioned as an alternative source of cells from which the desired tissue can be derived.

#### - **Stem cells**

“Stem cells are defined as clonogenic, self-renewing progenitor cells that can generate one or more specialized cell types” (Anderson *et al.*, 2001; also defined in Potten and Loeffler, 1990). The term “stem cell” can be traced back to the late 19th century, where it was primarily used to describe what are today called the germline stem cells (Haeckel, E., 1877; Ramalho-Santos M and Willenbring, H, 2007). Later, the term was used to describe stem cells of the hematopoietic system (Till JE *et al.*, 1961). Now, the identification of stem cells has progressed beyond embryologic systems and was found in almost every adult tissue. Stem cells are undifferentiated cells with the capacity to

self-renew, differentiate and repopulate a host in vivo (Kuhn NZ *et al.*, 2009). Regardless of their tissue of origin, human stem cells can be more broadly organized into a hierarchy based on their ontogenetic origin. Their plasticity or potency is ranging from:

- a) totipotent (differentiating into all cell types including placenta),
- b) pluripotent (differentiating into cells of the three germ layers, ectoderm, mesoderm and endoderm, but not trophoblastic cells),
- c) multipotent (differentiating into cells of more than one type but not necessarily into all the cells of a given germ layer) ,
- d) unipotent (differentiating into one type of cell only, e.g. muscle or neuron).

During mammalian development, the fertilized oocyte generates a complex organism comprising more than 200 different cell types. This developmental potential results from the capability of early embryonic cells to proliferate and to differentiate into all cell lineages including the germ line. ‘Totipotency’, defined as the ability to generate an entire organism independently, is retained by the zygote and its early progeny up to the 8-cell stage of the morula (Fig. 1).

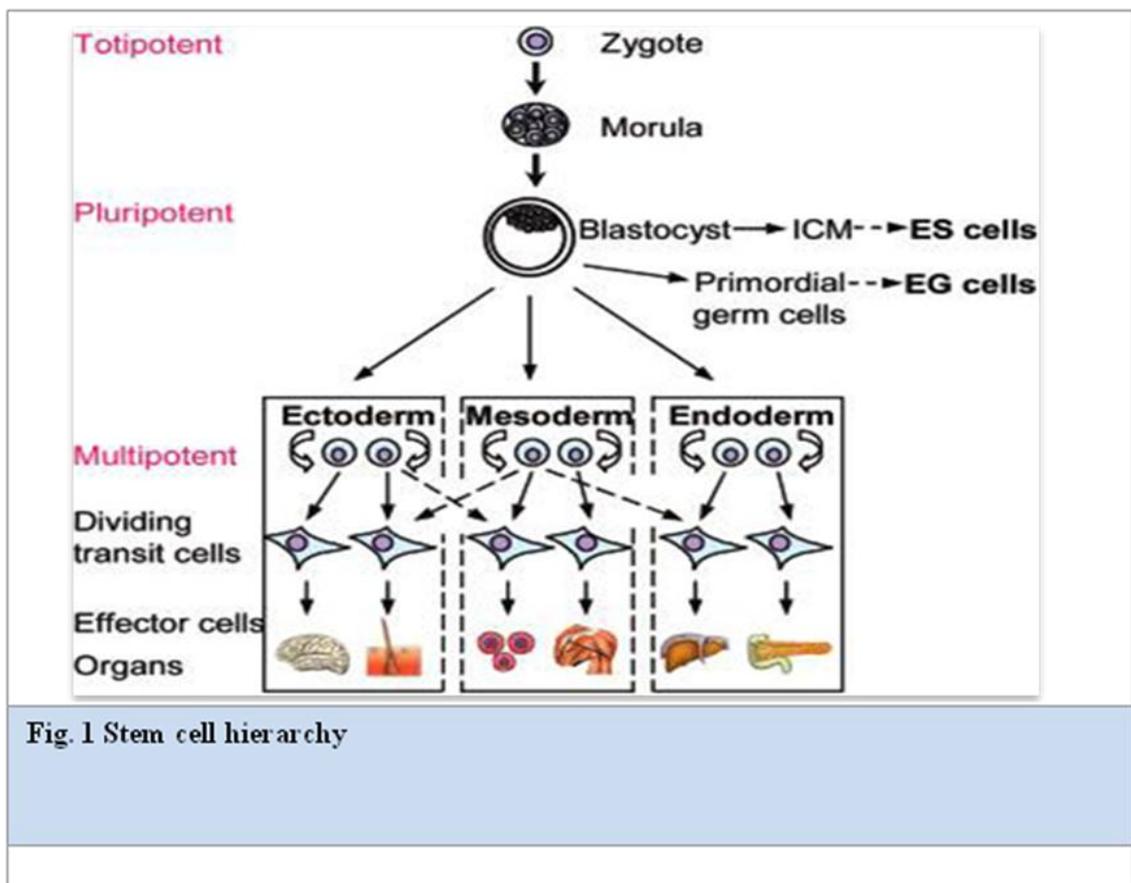


Fig. 1 Stem cell hierarchy

Thereafter, primary cell differentiation processes result in the formation of blastocyst composed of outer cells forming the trophoblast, whereas the inner cells, known as the ‘inner cell mass’ (ICM), remain undifferentiated. The cells of the ICM are no longer totipotent, but retain the ability to develop into all cell types of the embryo proper. The ICM cells of mouse embryos have been cultured *in vitro* as undifferentiated embryonic stem (ES) cell lines with no apparent loss of potential (Czyz J *et al.*, 2003).

Pluripotent stem cells can be derived from the inner cell mass of the pre-implantation embryo (i.e. embryonic stem (ES) cells) or isolated from the foetal primordial germ cell pool (PGC) above the allantois (i.e. embryonic germ (EG) cells and embryonic carcinoma (EC) cells; Thomson, JA., *et al.*, 1998). The destruction of the blastocyst or

early fetus necessary for their derivation/isolation raises ethical concerns, although recent work has shown that ES cells can be derived from single blastomeres isolated using procedures similar to those routinely used for pre-implantation genetic diagnosis. However, safety concerns remain because of the tumorigenicity of ES cells. The subsequent establishment of human embryonic stem (hES), and embryonic germ (hEG; Shambloott, MJ *et al.*, 1998) cells has evoked widespread discussion concerning the potential application of human embryonic stem cells in regenerative medicine. This debate has been enlivened further by a series of reports that stem cells continue to reside in various tissues and organs throughout the life of an organism, and that at least some of these ‘adult stem’ (AS) cells may cross lineage boundaries to generate a variety of tissue types.

Adult stem cells can be found in almost all tissues examined, including brain, dental pulp, muscle, bone marrow, skin and pancreas and have been extensively characterized for their therapeutic potential. The adult stem cell could be multipotent, capable to differentiate in cells that belong to the same germ layer (e.g. hematopoietic stem cells (HSCs) giving rise to all blood cells and adherent stromal/mesenchymal stem cells (MSCs) that give rise to bone, fat, cartilage and muscle), or unipotent (e.g. progenitor cells), with limited capacity of self-renewal, that can differentiate only in one cell type.

### **Stem cells: Embryonic-adult-foetal**

#### **- Embryonic stem cells**

In 1981 pluripotent cells were found in the inner cell mass of the human embryo, and the term ‘human embryonic stem cell’ (hESC) was coined (Martin GR *et al.*, 1981).

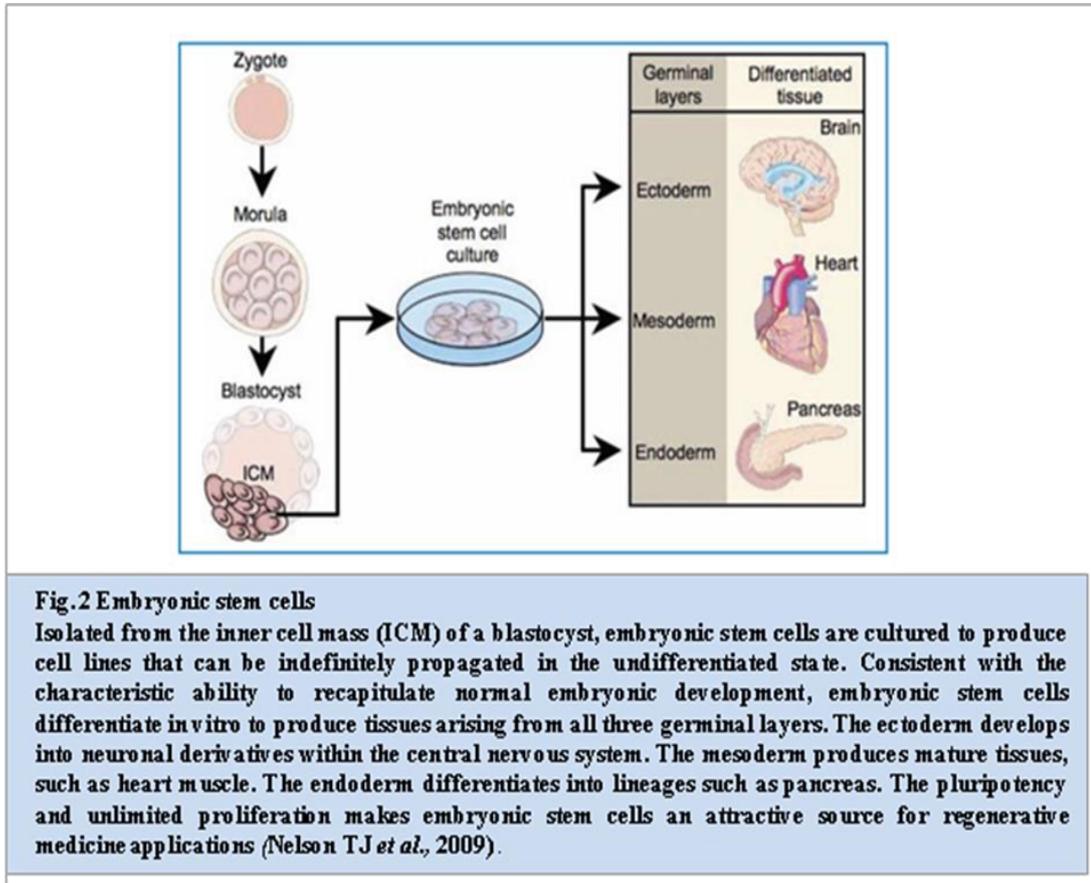
ESCs are generally isolated from the inner cell masses (ICMs) of blastocysts (five days post fertilization), which consist of pluripotent cell populations that are able to generate the primitive ectoderm during embryogenesis (Fig. 2). More specifically, in normal embryonic development, the primitive ectoderm gives rise during the gastrulating process to the primary germ layers, including ectoderm, mesoderm, and endoderm. These three germ layers might subsequently generate a variety of organized tissue structures involving complex epithelial- mesenchymal interactions (Mimeault M *et al.*, 2006).

ESCs are defined by their unique capacity to **self-renew indefinitely** in cell culture, long telomere length (high levels of telomerase expression), high nuclear/cytoplasmic ratio, and demonstrate an unparalleled differentiation capacity. In this matter, ESCs possess the dual ability to undergo unlimited self-renewal and to differentiate in all foetal and adult stem cells and their more differentiated progenitors (Nelson TJ *et al.*, 2009). Recent reports have shown that:

- skin and neurons have been formed, indicating **ectodermal** differentiation (Reubinoff BE *et al.*, 2001);
- blood, cardiac cells, cartilage, endothelial cells, and muscle have been formed, indicating **mesodermal** differentiation (Kaufman DS *et al.*, 2001);
- Pancreatic cells have been formed, indicating **endodermal** differentiation (Assady S *et al.*, 2001).

These cells have demonstrated longevity in culture by maintaining their undifferentiated state for at least 80 passages when grown using current published protocols (Reubinoff BE *et al.*, 2000) and lineage specific differentiation of ESCs

can be directed under specific culture conditions and by manipulating the microenvironment (Hwang WS *et al.*, 2005).



A potential limitation of ESCs with regard to regenerative applications relates to their inherent unrestricted growth potential with the associated risk of teratoma formation prominent once differentiation cues are evaded following transplantation. Given these considerations, therapeutic applications of ESC have so far been limited to preclinical studies (neurological disorders, such as Parkinson's disease, endocrine disorders, such as Type I diabetes, liver and renal failure, spinal cord injuries.). Their clinical

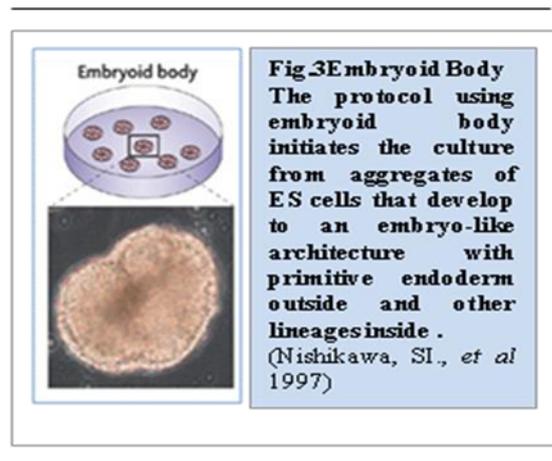
application is also limited because they represent an allogenic resource and thus have the potential to evoke an immune response (Hipp J *et al.*, 2008).

Human ESCs are also inherently connected to ethical issues because of their origin in a blastocyst, and the potential that the technology can be used to clone human beings. The International Society for Stem Cell Research has published ethical guidelines for human ESC research (Daley GQ *et al.*, 2008). To date, researchers should retain open communication with the public and policy makers with regard to the direction to be taken concerning stem cell- based therapies.

#### - **Embryo Bodies**

As further evidence of their pluripotency, when cultured in suspension without anti-differentiation factors, ES cells spontaneously differentiated *in vitro* and formed three-dimensional multi-cellular aggregates called Embryo Bodies (EBs). An EB consists of ectodermal, mesodermal, and endodermal tissues, which recapitulate many aspects of cell differentiation during early mammalian embryogenesis (Itskovitz-Eldor J *et al.*, 2000). As differentiation continues, a wide range of cell types is developed within the EBs environment (Zhou J *et al.*, 2010). Therefore, EBs formation has been widely utilized as a trigger during the process of ES cells differentiation *in vitro*. Because of the important role the EBs played in the *in vitro* differentiation system of ES cells, the quality of EBs formed from ES cells affects the induction efficiency of derivatives from the EBs in a subsequent differentiation culture (Koike M *et al.*, 2007). The partial disaggregating and subculture of EBs might allow the isolation and differentiation of a particular progenitor cell type that might be isolated on the basis of the expression of specific cellular markers. More particularly, the use of specific growth factors or

cytokines during the outgrowth of EBs in culture in vitro might induce their differentiation into the specific cell lineages (Fig. 3). One of the critical steps in the purification procedure appears to be the enrichment of EB-derived progenitor cells by the elimination of pluripotent and undifferentiated stem cells. Indeed, the elimination of undifferentiated stem cells that may form teratomas or teratocarcinomas in vivo appears to be essential for generating transplantable sources of differentiated stem cell progenitors for the treatment of diverse disorders. Therefore, they represent a useful source of stem cells for investigating the molecular events that are involved in normal embryogenesis and generating a large number of specific differentiated progenitors for cellular therapies.



#### - Markers of hESC

Embryonic stem cell phenotype has been studied in detail and several markers have been reported as ESC specific antigens, including transcription factors *Oct4*, *Nanog*, *Sox2*, cell surface antigen SSEA-4, *Stella* and others (Adewumi O et al., 2007). For

characterization of hESCs, it is common to report one or more of the following: *Oct4* expression, alkaline phosphatase and telomerase activities; stage-specific embryonic antigens 3 and 4; hESC antigens TRA-1-60, TRA-1-81, GCTM-2, TG-30, and TG-343; and CD9, Thy1, and major histocompatibility complex class 1 (MHC-1) (Stojkovic M *et al.*, 2004). Other stem cell antigens are also sometimes reported, *e.g.*, AC133, **c-kit (CD117)**, and flt3 (CD135), but these are frequently only expressed in a proportion of the hESC population, making them potential derivatives of interest in the heterogeneous hESC cell population. **Oct-4** (octamer-binding transcription factor 4) also known as **POU5F1** (POU domain, class 5, transcription factor 1) is a protein that in humans is frequently used as a marker for undifferentiated cells. Oct-4 expression must be closely regulated; too much or too little will actually cause differentiation of the cells (Niwa H *et al.*, 2000). The transcription factor **Oct4**, coupled with Sox2, is a component of a network of transcription factors, including the homeobox protein Nanog, that cooperatively contribute to the maintenance of the undifferentiated state through fibroblast growth factor-4 (FGF-4), Wnt, and transforming growth factor- $\beta$  (TGF- $\beta$ ) dependent pathway. Oct-4 transcription factor is initially active as a maternal factor in the oocyte but remains active in embryos throughout the preimplantation period. Its expression is associated with an undifferentiated phenotype and tumors (Looijenga L *et al.*, 2003): in fact gene knockdown of Oct-4 promotes differentiation, thereby demonstrating a role for these factors in human embryonic stem cell self-renewal. Mouse embryos that are Oct-4-deficient or have low expression levels of Oct-4 fail to form the inner cell mass, lose pluripotency and differentiate into trophectoderm. Therefore, the Oct-4 expression level in mice is vital for regulating pluripotency and

early cell differentiation since one of its main functions is to keep the embryo from differentiating. Oct-4 has been implicated in tumorigenesis of adult germ cells. Ectopic expression of the factor in adult mice has been found to cause the formation of dysplastic lesions of the skin and intestine. The intestinal dysplasia resulted from an increase in progenitor cell population and the upregulation of  $\beta$ -catenin transcription through the inhibition of cellular differentiation (Hochedlinger K *et al.*, 2005).

Therefore, the presence of *Oct4* expression alone may be misleading, as this transcription factor takes some time to shut down RNA transcription in differentiating hESCs and is also found in other pluripotent cell populations (*e.g.*, embryonic germ stem cells), as well as some adult and foetal multipotential stem cells (Hoffman, LM., *et al.*, 2005).

**Nanog** is a transcription factor critically involved in self-renewal of undifferentiated embryonic stem cells. In humans, this protein is encoded by the *NANOG* gene (Mitsui K *et al.*, 2003). Human Nanog protein is a 305 amino acid protein with a conserved homeodomain motif localized in the nuclear component of cells. NANOG is a gene expressed in ESCs and is thought to be a key factor in maintaining pluripotency. Overexpression of Nanog in mouse embryonic stem cells causes them to self-renew in the absence of Leukemia inhibitory factor (Lif); in the absence of Nanog, mouse embryonic stem cells differentiate into visceral/parietal endoderm: loss of its function causes differentiation into other cell types. In human embryonic stem cells, its overexpression enables their propagation for multiple passages during which the cells remain pluripotent. Gene knockdown of Nanog promotes differentiation, thereby demonstrating a role for these factors in human embryonic stem cell self-renewal. It has

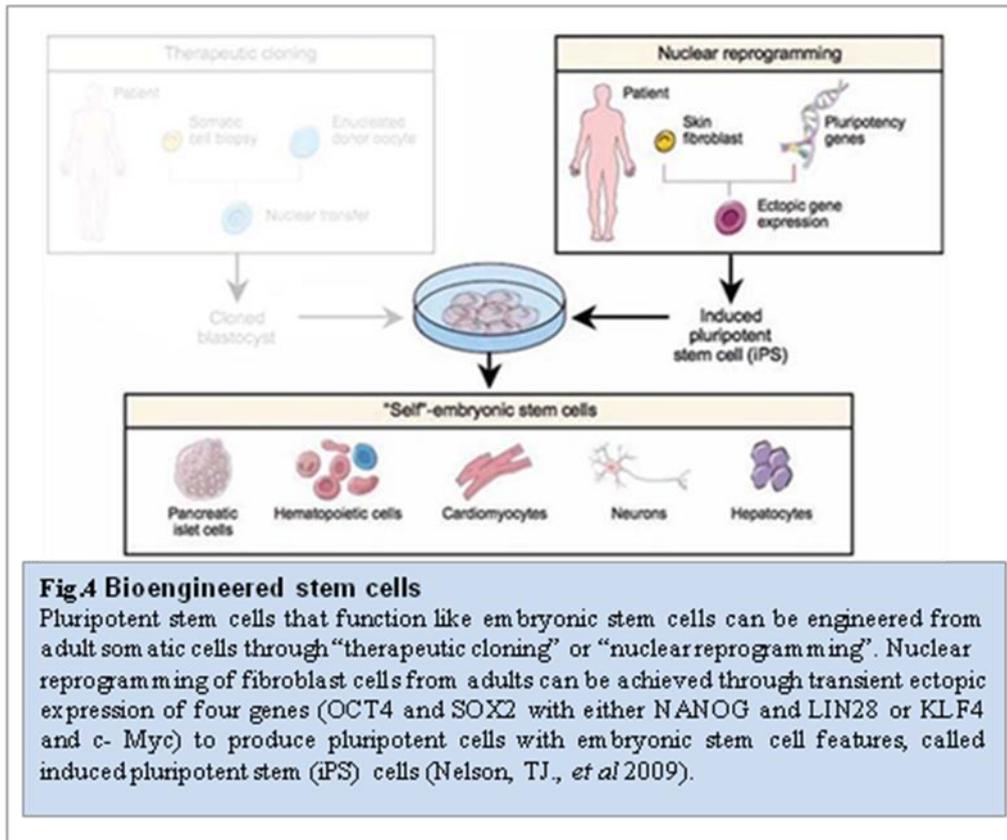
been shown that the tumor suppressor p53 binds to the promoter of *NANOG* and suppresses its expression after DNA damage in mouse embryonic stem cells. P53 can thus induce differentiation of embryonic stem cells into other cell types, which undergo efficient p53-dependent cell-cycle arrest and apoptosis.

*Stella* (*Pgc7*, *Dppa3*) is a maternal effect gene that encodes a protein with a SAP-like domain and a splicing factor motif-like structure and is specifically expressed in primordial germ cells, oocytes, preimplantation embryos and pluripotent cells (Payer B *et al.*, 2003). The loss of maternally inherited *Stella* leads to a reduced fertility in *Stella*-null females, while embryos devoid of *Stella* rarely develop to the blastocyst stage and are mainly blocked at the 2-cell stage (Payer B *et al.*, 2003). It has been recently demonstrated that *Stella*, aside from localizing into the nucleus, also contributes to maintain the methylation state of several imprinted genes (Nakamura T *et al.*, 2007). *Stella*, like all the other genes that localize within the *Nanog* locus, is co-regulated in the ES cells and the transcription factor Oct-4 regulates its expression, maintaining a higher order chromatin structure within this locus.

#### - **IPs**

Embryogenesis is a sequential process of differential gene expression dictated by the epigenetic environment. Exploiting epigenetic influence on phenotypic outcome, biotechnology platforms are developed for reversal of differentiation to achieve genetic reprogramming of adult sources back to embryonic state (Hochedlinger K *et al.*, 2006). Recently, exciting reports of the successful transformation of adult cells into pluripotent stem cells have been published. Nuclear reprogramming is a technique that involves de-differentiation of adult somatic cells to produce patient-specific pluripotent stem cells,

without the use of embryos, through ectopic introduction of a small number of pluripotency-associated transcription factors: Oct3-4, Sox2, Klf4 with (Yamanaka's group) or without (Thompson's group) the oncogene c-Myc and the transcription factor Nanog. Cells generated by reprogramming would be genetically identical to the somatic cells (and thus, the patient who donated these cells) and would not be rejected. Yamanaka was the first to discover that mouse embryonic fibroblasts (MEFs) and adult mouse fibroblasts could be reprogrammed into an 'induced pluripotent state' (iPS). The resultant iPS cells possessed the immortal growth characteristics of self-renewing ES cells, expressed genes specific for ES cells, and generated embryoid bodies in vitro and teratomas in vivo. When iPS cells were injected into mouse blastocysts, they contributed to a variety of cell types. However, although iPS cells selected in this way were pluripotent, they were not identical to ES cells. Unlike ES cells, chimeras made from iPS cells did not result in full-term pregnancies. Gene expression profiles of the iPS cells showed that they possessed a distinct gene expression signature that was different from that of ES cells. In addition, the epigenetic state of the iPS cells was somewhere between that found in somatic cells and that found in ES cells, suggesting that the reprogramming was incomplete (Zhao HX *et al.*, 2010).

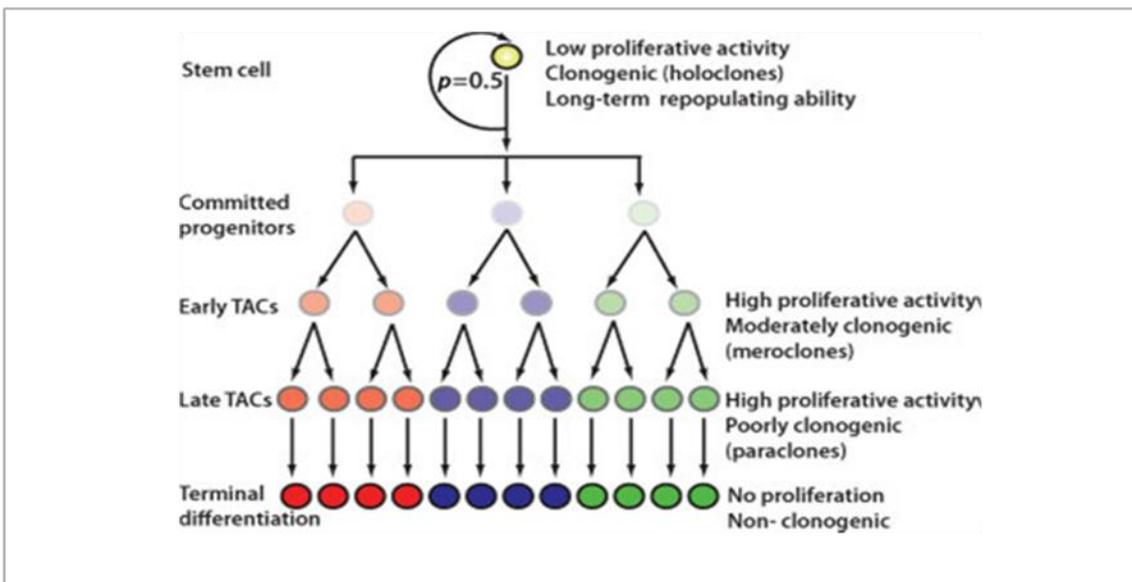


- **Adult stem cells**

Adult stem cells comprise a wide range of progenitors derived from non-embryonic, non-foetal tissue such as bone marrow, adipose tissue, and resident stem cells pools (Mimeault M *et al.*, 2006). They are leading candidate for clinical application in regenerative medicine, based on accessibility autologous status, and favorable proliferative potential. Numerous studies have revealed that, a population of adult stem cells and supporting cells reside within specific areas designated as **niches** in most of adult mammalian tissues/organs, including BM, heart, kidneys, brain, skin, eyes, gastrointestinal tract, liver, pancreas, lungs, breast, ovaries, prostate, and testis (Fig. 5) (Modlin IM *et al.*, 2003; Lavker RM *et al.*, 2004; Li L *et al.*, 2005; Griffiths MJ *et al.*, 2005). In fact, adult stem cells appear for the first time during ontogeny and persist in



symmetric division into two identical daughter stem cells. In contrast, the asymmetric division of stem cells during differentiation involves each stem cell dividing to produce one daughter stem cell and one daughter transit-amplifying (TA)/intermediate cell. The TA cells (TACs) that are generated during asymmetric division are generally characterized by a high proliferative index and migratory properties. The TACs may give rise to other intermediate cell progenitors and, ultimately, to more differentiated cells constituting tissues or organs where they originate or other, distant tissues. In fact, the migration of TACs to distant sites, as well as the changes in their local microenvironment during amplification, might be the basis of a population asymmetric division, which might result in the differentiation or trans-differentiation of TACs into different cell lineage progenitors (Fig.6) ( Alison MR *et al.*, 2009).



**Fig.6** In most renewing systems a cell hierarchy can be recognized in which a few self-renewing stem cells ( $p$  = probability of self-renewal) give rise to a limited number of committed progenitors, also called transit-amplifying cells (TACs). TACs have limited proliferation potential and eventually give rise to reproductively sterile terminally differentiated cells ( Alison MR, 2009)

In addition, many works have indicated that the adult stem cells, and more particularly BM stem cells, may be redistributed under certain physiological and pathological conditions to other distant tissues via circulation (Blau HM *et al.*, 2001).

Adult stem cells can be divided into 3 categories according to the germ layer from which they originate:

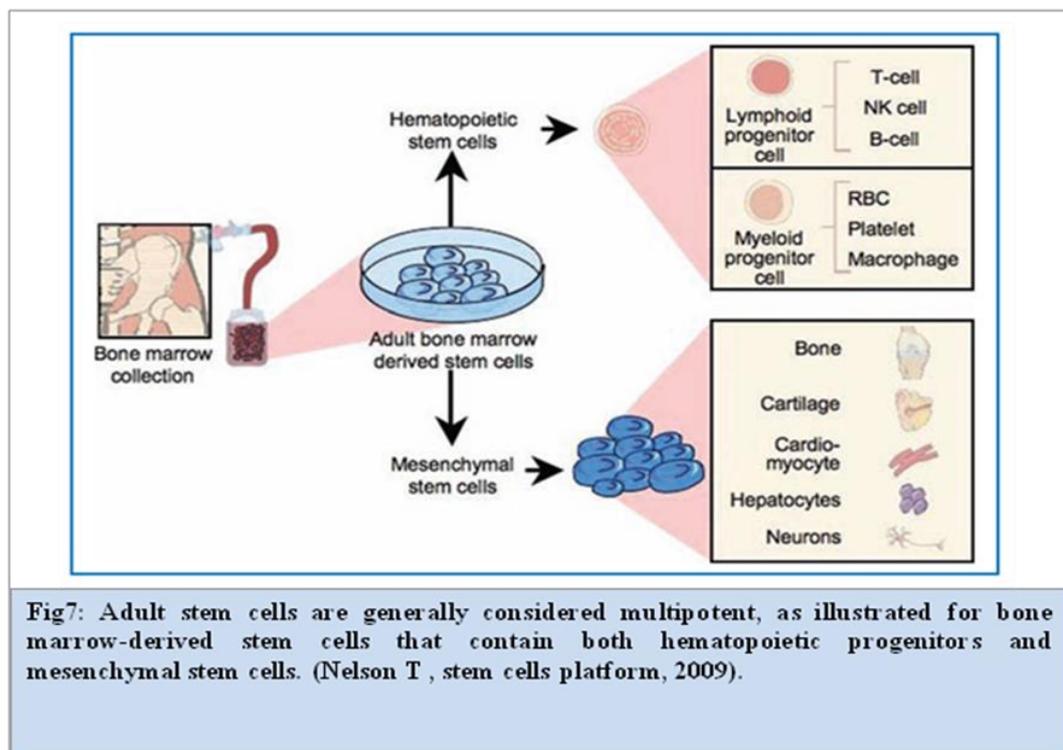
- endodermal origin (pulmonary epithelial stem cells, gastrointestinal tract stem cells, pancreatic stem cells, hepatic oval cells, mammary and prostatic gland stem cells, and ovarian and testicular stem cells),
- mesodermal origin (bone marrow hematopoietic and stromal stem cells, and cardiac stem cells),
- ectodermal (neural stem cells, skin stem cells, and ocular stem cells).

These adult stem cells are also able to trans-differentiate in different mature cell lineages in vitro and in vivo (Choumerianou DM, 2008) .

#### - **Bone Marrow and Mesenchymal Stem Cells**

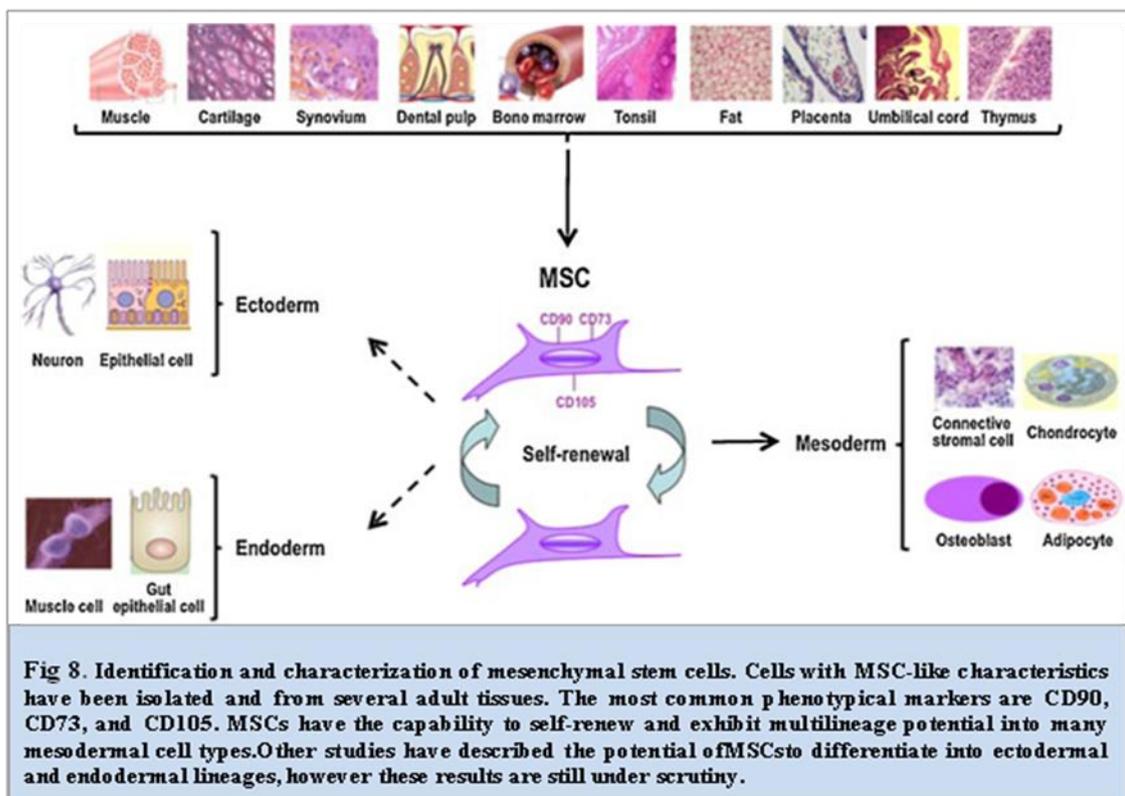
The **most common source** of adult stem cells is the bone marrow (Fig 7), a mesoderm derived well-organized tissue, composed of the basic elements from the stroma and the hematopoietic system, and located at the centre of large bones (Arai F *et al.*, 2004). For decades, it has been known that the bone marrow contains two types of stem cells: hematopoietic stem cells (HSC), which are committed to differentiate into mature blood cells, and the less-differentiated stromal mesenchymal cells (MCs). The evidence of the presence of multipotent stem cells, was first reported in 1968 by Friedenstein and his colleagues by establishing that these cells are adherent, clonogenic and fibroblastic

(Friedenstein AJ *et al* 1968, 1974). For over 40 years, the stem cells from bone marrow (BM) have been denoted as a ‘colony forming unit fibroblast’, ‘marrow stromal cells’, or ‘mesenchymal progenitor cells’ in published literature. Currently they **are called ‘mesenchymal stem cells’ (MSCs)**, which are defined as cells that can differentiate into multiple mesenchymal lineage cells (Phinney DG, 2002; Pittenger MF *et al.*, 1999), a notable exception to the tissue specificity of adult stem cell. MSCs contribute to the regeneration of mesenchymal tissues such as bone, cartilage, muscle, ligaments, tendons, adipose and stroma (Caplan AI, 2005). To date, bone marrow stem cells are the most frequently used cell source for bone tissue engineering (Seong JM *et al.*, 2010).



Ever since the emergence of the MSC, cells with MSC-like characteristics have been identified in not only mesoderm derived, but also endoderm- and ectoderm-derived

tissues (Fig. 8). 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 (mesoderm and ectoderm-derived), prenatal and perinatal tissues—umbilical cord, umbilical cord blood, and placenta (Kunh NZ *et al.*, 2009).



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. 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. The ultimate test for pluripotency is tetraploid

complementation where stem cells injected into the mouse blastocyst generate germ-line competent chimeric mice (Kunh NZ *et al.*, 2009).

- **Surface marker characterization of mesenchymal stem cells**

MSCs isolated from human bone marrow and other mesenchymal tissues must demonstrate in vitro:

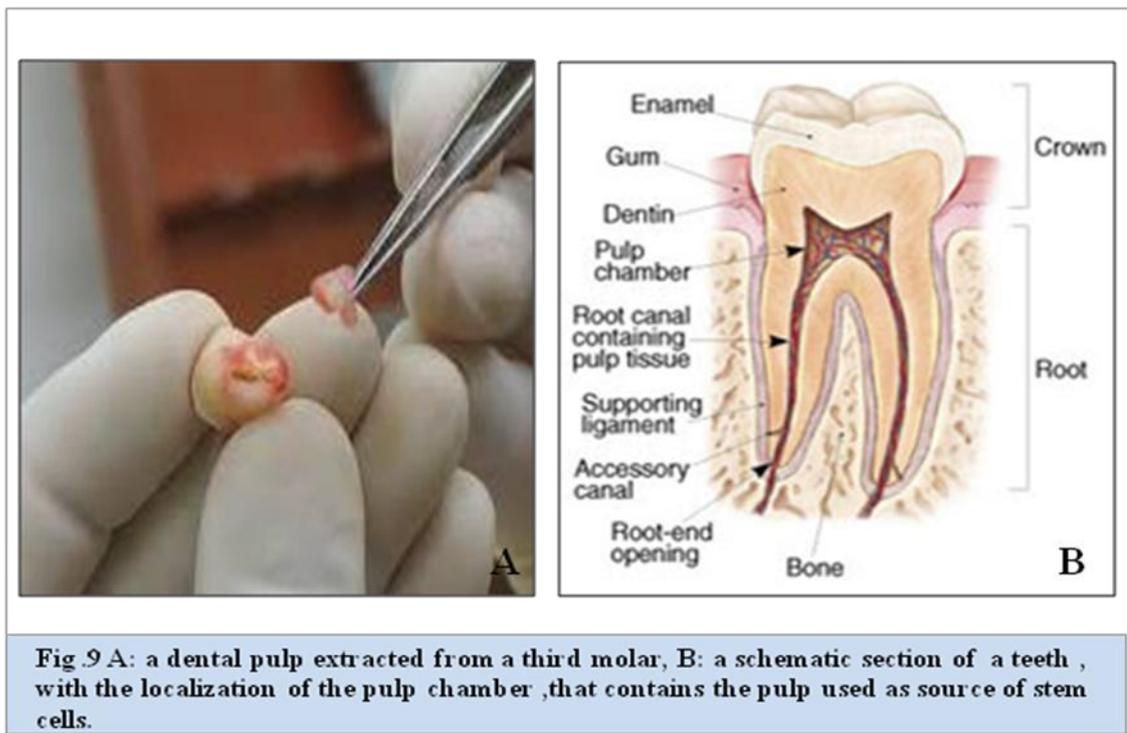
- ✓ adherence to plastic in standard culture conditions,
- ✓ specific surface antigen expression,
- ✓ multipotent differentiation potential along the osteogenic, chondrogenic, and adipogenic lineages as demonstrated by histological staining and other methods.

Identification of adult human mesenchymal stem cells as multilineage progenitors relies on their capacity to be induced to differentiate into specific phenotypes. Although the ganglioside GD2 has been proposed as a single surface marker that distinguishes mesenchymal stem cells, a panel of biomarkers has been typically employed to secure isolation. BM-MSCs have been found to be positive for a variety of markers, such as STRO-1 (Simmons and Torok-Storb, 1991; Gronthos S *et al.*, 1994), CD105 (endoglin), SH3, CD29, CD44, CD71, CD90 (Thy-1), CD106, CD120a, CD124, and other surface proteins (Pittenger MF *et al.*, 1999). The consensus of “The International Society for Cellular Therapy (ISCT)”, which is subject to future modification, is that measurements by flow cytometry show that no more than **2%** of the cells may express CD34 (hematopoietic progenitor and endothelial cell marker), CD45 (pan-leukocyte marker), CD11b or CD14 (monocyte and macrophage markers), CD19 or CD79a (B cell markers), and HLA-DR (marker of stimulated MSCs).

Gene	BM-MSCs	DPSCs
STRO-1	+ (5-10%)	+ (5-10)
CD+(selected)	CD44,CD73,CD105,CD106	CD13,CD29,CD44,CD59, CD73,CD90,CD105,CD146
CD-(selected)	CD11,CD14,CD34,CD45	CD14,CD24,CD34,CD45
Oct4	+	+
Collagen type I	+	+
ALP	+	+
Runx2	?	+
OCN	+/-	?
Osx	+	?
Nanog	+	?

**Tab 1: In vitro phenotypic characteristics of BM-MSCs and DPSCs (Huang GTJ, MSCs, 2010)**

## Dental pulp stem cells (DPSCs)



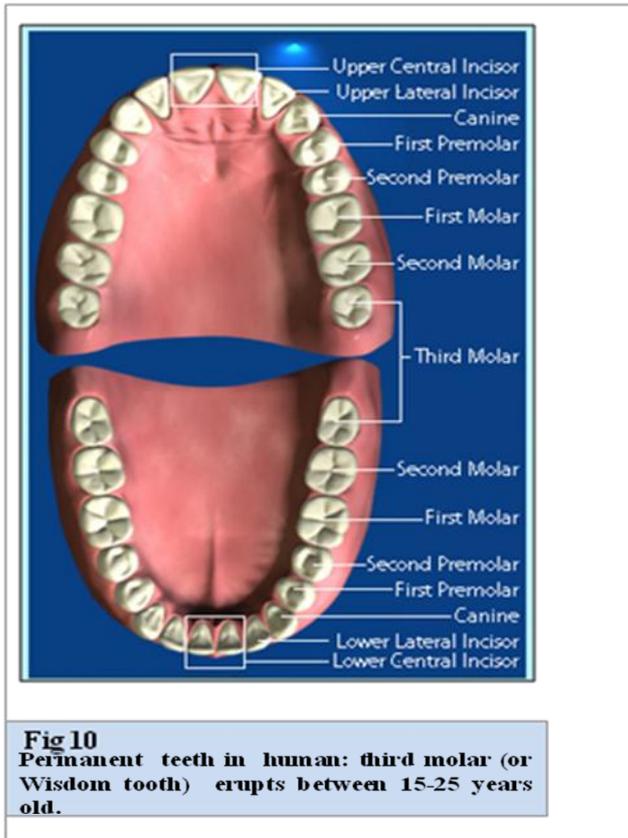
Since the discovery and characterization of multipotent mesenchymal stem cells (MSCs) from bone marrow (BM), MSC-like populations from other tissues have now been characterized based on the ‘gold standard’ criteria established for BMMSCs (Friedenstein AJ *et al.*, 1976; Caplan AI, 1991; Prockop DJ, 1997; Pittenger MF *et al.*, 1999; Gronthos S *et al.*, 2003). The search for MSC-like cells in specific tissues has led to the discovery of a variety of stem cells in every organ and tissue in the body in the past decades (Kolf C *et al.*, 2007) like human muscle and adipose tissue. However, more easily available alternative sources of cells expressing an ability to differentiate and to modulate the immune response are badly needed (Huang GTJ *et al.*, 2009). A promising source is dental pulp (Fig 9-10), a loose vascular connective tissue surrounded by

dentine and consisting of a heterogeneous population of cells: the potential preodontoblast, fibroblasts, stromal cells, endothelial and perivascular cells, neural cells and others (Pierdomenico L *et al.*, 2005). Dental-tissue-derived MSC-like populations are cells residing in this specialized tissue that have been isolated and characterized. The first type of dental stem cell was isolated from the human pulp tissue and termed 'postnatal dental pulp stem cells' (DPSCs) (Gronthos S *et al.*, 2000). Subsequently, 3 more types of dental-MSC-like populations were isolated and characterized: stem cells from exfoliated deciduous teeth (SHED) (Miura M *et al.*, 2003), periodontal ligament stem cells (PDLSCs), and stem cells from apical papilla (SCAP) (Sonoyama W *et al.*, 2006, 2008). Recent studies have identified a fifth dental tissue-derived progenitor cell population, referred to as 'dental follicle precursor cells' (DFPCs) (Morszeck C *et al.*, 2005). Recently, several groups have initiated investigation of this potential source and have begun to examine the properties of stem cells isolated from dental pulp. (Nakashima M, 2009) Stem cells extracted from the pulp of the not-erupted third molar (Fig.10) have some advantages for clinical applications compared with other mesenchymal stem cells derived from bone marrow, adipose tissue, peripheral blood, and umbilical cord blood.

- Easy surgical access and low invasiveness.
- 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.

In fact, according to the ISTAT data in 2008, 30.2 million (equal to 60.1%) Italian citizens have at least one tooth extracted, whereas people with five teeth extracted or

fallen are 1.9 million people. Therefore, the extraction, from healthy subjects, of one or



more molars seems to be quite widespread among the population.

- Minimal tooth processing may be needed for the banking of samples with no immediate plans for DPSC expansion and use, which in turn may limit costs and facilitate clinical banking of this potentially important cell type.

Stem cells obtained from any adult tissue are less, about 1-4%. Further,

their isolation, expansion, and storage is a very technique-sensitive procedure.

- Cryopreservation.

Cells expanded in culture are readily preserved and can be stored for at least six months and likely longer at  $-85^{\circ}\text{C}$  with respect to qualitative ability to differentiate in at least a tri-lineage manner. So they can be cryopreserved, retaining multi-differentiation capacity (Graziano A. *et al.*, 2008).

- They do not form teratoma in vivo.
- High differentiation ability.

They are highly clonogenic, showing a high proliferation potential and are able to self-maintain for long time (Laino G *et al.*, 2005).

- DP-MSCs inhibit the proliferation of phytohemagglutinin (PHA) stimulated T-cells and the effect being stronger than in BM-MSCs. (Pierdomenico L *et al.*, 2005)

The rapid proliferative kinetics of DP-MSCs, as compared to their bone marrow counterparts, might represent an immunosuppressive advantage when treating immune reactions, such as graft-versus-host disease, in view of their potentially greater effect. Therefore, dental pulp-MSCs may prompt future studies aimed at using these cells in the treatment or prevention of T-cell alloreactivity in hematopoietic or solid organ allogeneic transplantation.

- MSC populations are considered immunomodulatory as they lack MHC type II (major histocompatibility complex) antigen and therefore do not provoke immune reactions.

As of now, autologous stem cells are ideally suited for a patient as there is no risk of immune rejection, the process is least expensive, and avoids legal and ethical concerns: there are many *in vivo* studies which support that they are immunologically safe.

- Up to now, in trauma and reconstructive surgery, DPSCs can be used in combination with biomaterials in the treatment of critical size bone defects, which are too large to be repaired by endogenous repair mechanisms. (Wenish S *et al.*, 2006)

- **Tooth morphogenesis( origin of dental pulp)**

During the sixth week of embryogenesis, after the migration of neural crest cells into head and neck mesenchyme, the ectoderm covering the stomodeum begins to proliferate, giving rise to the **dental laminae**. From the dental lamina, following ectomesodermic interactions, ovoidal structures start to separate and then develop into tooth germs, where **neural crest** cells differentiate into the dental organ, **dental papilla** and **dental follicle**, forming the main part of the dental and periodontal structures. Therefore, dental pulp is made of both ectodermic and mesenchymal components, containing neural crest cells that display plasticity and multipotential capability.

After crown mineralization, dental pulp tooth germ remains entrapped within a hard structure that preserves it from environmental differentiation stimuli (D'Aquino R *et al.*, 2006).

The pulp is divided into four layers, from the outer to the inner part: (1) the external layer made up of odontoblast producing dentin; (2) the second layer, called "cell free zone", poor in cells and rich in extracellular matrix; (3) the third layer, called "cell rich zone", containing progenitor cells that display plasticity and pluripotential capabilities, like **DPSCs** (Sinanan AC *et al.*, 2004); 4) the inner layer, that comprises the vascular area and nervous plexus.

After the crown eruption the **dental pulp**, remains entrapped within the pulp chamber, a sort of "sealed niche" and may explain that it is possible to find, within it, a pretty large number of stem cells although in an adult tissue. In the case of the third molar, the development begins at the sixth year of life: it means that until this time, embryonic

tissues from dental lamina remain quiescent and undifferentiated within the jaws, the only organogenesis event that completely occurs after the birth.

### **-DPSCs**

DPSCs usually remain quiescent within adult dental pulps, but respond during injury to produce progenies with **high proliferative** potential, which can differentiate into terminally differentiated odontoblasts.

As a type of adult stem cells, DPSCs usually perform the **asymmetric cell division**, which gives rise to one daughter cell with stem-cell fate and another which can pursue further cell divisions to generate differentiated progenies (Gronthos S *et al.*, 2002, Huang GT-J *et al.*, 2009). Thus, the amount of DPSCs in the normal dental pulp remains relatively constant. When one DPSC divide 9 times, it will generate 9 lineage-specific progeny cells plus one unaltered daughter stem cell. These lineage-specific progenies *in vivo* may bring about different cell types, which contribute to the maintenance and homeostasis of dental pulp tissues (Yu J *et al.*, 2010).

Although dental tissues are specialized tissues that do not undergo continuous remodeling, as shown in bone tissue, many studies have demonstrated that DPSCs have the ability to undertake the self-renewal and the multi-differentiation capacity. DPSCs can be isolated from digested pulp tissue, with a colony selection or an immunomagnetic isolation method (MACS) and cultured in different inductive media (Seong JM., 2010). They display clonogenic and highly proliferative characteristics, and they can differentiate into the neurogenic, osteogenic, dentinogenic, and myogenic cell lineages (Zhang W *et al.*, 2006).

To date, there is no optimal culture medium that can allow adult stem cell amplification without differentiation (D'Aquino R. *et al.*, 2007). It is reasonable that native DPSCs in vitro can spontaneously differentiate (termed self-differentiation) into mature cell lineages via asymmetric cell division, as reported in the paper of Yu *et al.*, (2010): the differentiation capacity of these stem cells change during cell passaging and DPSCs restrict their differentiation potential to osteoblast lineages.

Previous studies have demonstrated that DPSCs are able to differentiate into CD44+/RUNX-2+ osteoblast precursors and then into osteoblasts in  $\alpha$ -MEM containing 20% FBS, ascorbic acid- 2-phosphate, and L-glutamine. For tissue engineering approach, in order to produce new –formed bone, we need to select and define a clonal subset of stem/progenitor cells particularly able to differentiate in the osteogenic lineage after culture in a specific osteogenic medium.

This sub-population of Stromal Bone Producing-DPSC, named SBP-DPSCs, was isolated from DPSCs using immunoselection of c-Kit, CD34 and STRO-1 antigen markers by flow cytometry and magnetic-activated cells sorting (Papaccio G *et al.*, 2006).

#### - **Markers**

**C-kit** is a membrane tyrosin-kinase III receptor, product of the c-Kit proto-oncogene, which specifically binds the stem cell factor (SCF) (Thomas ML, 1989). In human adult it is expressed by the cells of the so called “side populations”, resident in several tissues. Typically, SCF and c-Kit are expressed by different cells in the same microenvironment; i.e. SCF is secreted by bone marrow stromal cells and supports

hematopoietic cells survival and proliferation. Of relevance, c-Kit is expressed in neural crest-derived stem cells, like melanocyte precursors (Garcia-Pacheco JM *et al.*, 2001), and cells deriving from neural crest progenitors, like dental pulp cells. In DPSCs c-kit is highly end early expressed and its presence could be attributed to the ectomesenchymal origin of these cells.

In the craniofacial region, it has been used to identify multipotent stem cells (Sinanan, AC *et al.*, 2004) however after differentiation into lineage-committed progenitors, c-Kit is gradually lost (Simmons & Torok-Storb, 1991).

**CD34** is a single-chain transmembrane glycoprotein expressed on human haematopoietic stem and mesenchymal progenitor cells: it is a marker for primitive pluripotential stem cells both stromal and hematopoietic. Although it is currently used in clinical applications of stem cell therapy with cells of hematopoietic origin, its presence is not detectable in mesenchymal stem cells from bone marrow (Liu L *et al.*, 2006; Lee SY *et al.*, 2007) and its expression in bone marrow stem cell-derived bone progenitors has not been definitively demonstrated (Sinanan AC *et al.*, 2004). However it must be considered that in mesenchymal stem cell, CD34 expression might be detectable from different sources such as adipose tissue (Yoshimura K *et al.*, 2006), and by other cell types, such as the mesangioblasts, that can give rise to bone progenitors too (Yoshimura K *et al.*, 2006). In previous studies, it has been demonstrated that CD34, in association with CD117, can be used to select a population of dental pulp stem cells able to differentiate into pre-osteoblasts *in vitro* (Laino G.*et al.*,2005, 2006).

**STRO-1** recognizes a trypsin-resistant cell-surface antigen of perivascular cells and is thought to be a putative cell surface marker for the isolation of stem cells from human

dental pulp. This cell surface antigen is present on precursors of various stromal cell types, including marrow fibroblasts, osteoblasts, chondrocytes, adipocytes, and smooth muscle cells isolated from adult and foetal bone marrow (Simmons TJ Torok-Storb 1991; Gronthos S *et al.*, 1994). Yu *et al.*, (2010) suggest that STRO-1<sup>+</sup>DPSCs consist of at least three interrelated subpopulations including the progenitors of odontoblasts, osteoblasts, and chondrocytes, which can form dentin, bone, and cartilage tissues respectively.

Previous studies have implicated STRO-1 as a marker of preosteogenic populations, where its expression is progressively lost after cell proliferation and differentiation into mature osteoblasts in vitro (Stewart K *et al.*, 1999). The STRO-1 antigen was also found to be present on the outer cell walls of human bone marrow and dental pulp blood vessels, in agreement with previous studies that localized STRO-1 on large blood vessels, but not capillaries, in different adult tissues such as brain, gut, heart, kidney, liver, lung, lymph node, muscle, and thymus. (Bianco P *et al.*, 2001). Therefore, STRO-1 seems to be an early marker of different mesenchymal stem cell populations and infers a possible perivascular niche for these stem cell populations in situ.

For all of these reasons, CD34, STRO-1 and c-Kit expression, identify a population of mesenchymal stem cells of neural crest origin (Laino G *et al.*, 2006).

#### - **DPSCs osteogenic differentiations**

Both osteogenic and angiogenic cytotypes can be found in the human dental pulp stem cell (CD34<sup>+</sup>/c-Kit<sup>+</sup>/STRO-1<sup>+</sup>) population (D'Aquino R *et al.*, 2007); however to

promote and to enhance the osteoblastic-differentiation *in vitro* an osteogenic medium is necessary.

The osteogenic differentiations is induced by Dexamethasone, Ascorbic acid and  $\beta$ -Glycerophosphate in culture media (Jager M *et al.*, 2005), also called DAG osteoblastic-supplements.

**Ascorbic Acid (AA; reduced vitamin C)** is an essential cofactor for osteoblast differentiation both *in vivo* and *in vitro* and it is necessary for expression of the osteoblast phenotype (Franceschi RT, 1992). Actions of AA on osteoblast differentiation require the synthesis of a collagenous extracellular matrix (ECM) and may be mediated by integrin: matrix interactions. Before it can function, this vitamin must be transported into cells via a specific  $\text{Na}^+$ -dependent AA transporter (Wilson and Dixon, 1989). Osteoblasts contain a specific  $\text{Na}^+$ -dependent AA transport system that is essential for the intracellular accumulation of vitamin C and cellular responsiveness (Pandipati S *et al.*, 1998). In primary cultures of osteoblast-like cells or no transformed osteoblast-like cell lines (such as murine MC3T3-E1 cells) (Franceschi RT, 1994), AA stimulates pro-collagen hydroxylation, processing, and fibril assembly followed by a dramatic induction of specific genes associated with the osteoblast phenotype including those encoding osteocalcin, alkaline phosphatase, bone sialoprotein, and the PTH/PTH-related protein receptor. Furthermore, induction of gene expression by AA is specifically and reversibly blocked by inhibitors of collagen matrix formation or purified bacterial collagenase. Thus, studies with AA strongly support a model in which expression of the osteoblast phenotype is profoundly affected by the extracellular matrix (ECM).

To date AA is necessary for osteoblastic production of collagen and enhances alkaline-phosphatase (AP) activity and it has been shown to decrease cellular proliferation, possibly acting as one of the signals postulated to be necessary for progression from proliferation phase to the matrix maturation phase (Lian JB *et al.*, 1997).

Glucocorticoids, i.e. **dexamethasone**, are potent stimulators of in vitro osteoblastic differentiation in bone marrow stromal osteoprogenitors (Bellows CG *et al.*, 1987), in mesenchymal stem cells and other cells types (Jager M *et al.*, 2005). Dexamethasone, a synthetic glucocorticoids, acts by stimulating the expansion, the differentiation, the gene expression and the synthesis of ECM of osteoprogenitors presents in these mixed population (IshidaY *et al.*,1996). The glucocorticoid-dependent-up-regulation of AA transport represents an appropriate response to allow maximal induction of osteoblast differentiation, because, AA-dependent collagen matrix synthesis is necessary, for subsequent expression of osteoblast-related genes (Franceschi RT, 1992) and osteoblast precursors must have an active Na<sup>+</sup>-dependent AA transporter to respond to vitamin C.

The extracellular matrix (ECM) of bone contains important structural and soluble factors required for its development and morphogenesis, i.e. type I collagen (Mizuno H *et al.*, 2002) and the interactions of collagen with integrins enhance the gene expression of the osteoblastic markers alkaline phosphatase (AP), osteocalcin (OC), and bone sialoprotein (BSP). Dexamethasone promotes osteoblast differentiation and development of bone tissue like organization in cultured osteoblasts by accelerating the developmental sequence of cell growth and bone related genes expression resulting in increased formation of mineralized bone nodules.

Moreover, dexamethasone modulates the osteoblastic phenotype *in vitro* and influences the migration and the proliferation of MSCs. Several authors empathize the proliferative effects of dexamethasone in bone precursor cells and osteoblast, but Walsh S *et al.*, (2001) reported dose-dependent inhibitory effect on bone marrow cells *in vitro*. They showed that supra-physiological dexamethasone concentrations above  $10^{-9}$  M are associated with a decrease in the median diameter of CFU, but promote the differentiation or further maturation of human osteoblast precursors in human DPSCs and bone marrow cells cultures.

**Beta-glycerophosphate** ( $\beta$ GP) is a disodium salt hydrate. It has been shown in several laboratories that addition of  $\beta$ GP, as a phosphate source and substrate for alkaline phosphatase (AP), to cultured osteoblast-like cells induces deposition of orthophosphate (Pi) and  $\text{Ca}^{2+}$  (Deliloglu Gurhan SI *et al.*, 2005). Using cultures continuously exposed to  $\beta$ GP during the bone formation stage, Tenenbaum HC *et al.*, (McCulloch CAG *et al.*, 1989) also reported an increase in alkaline phosphatase activity coincident with or just before the onset of mineralization.

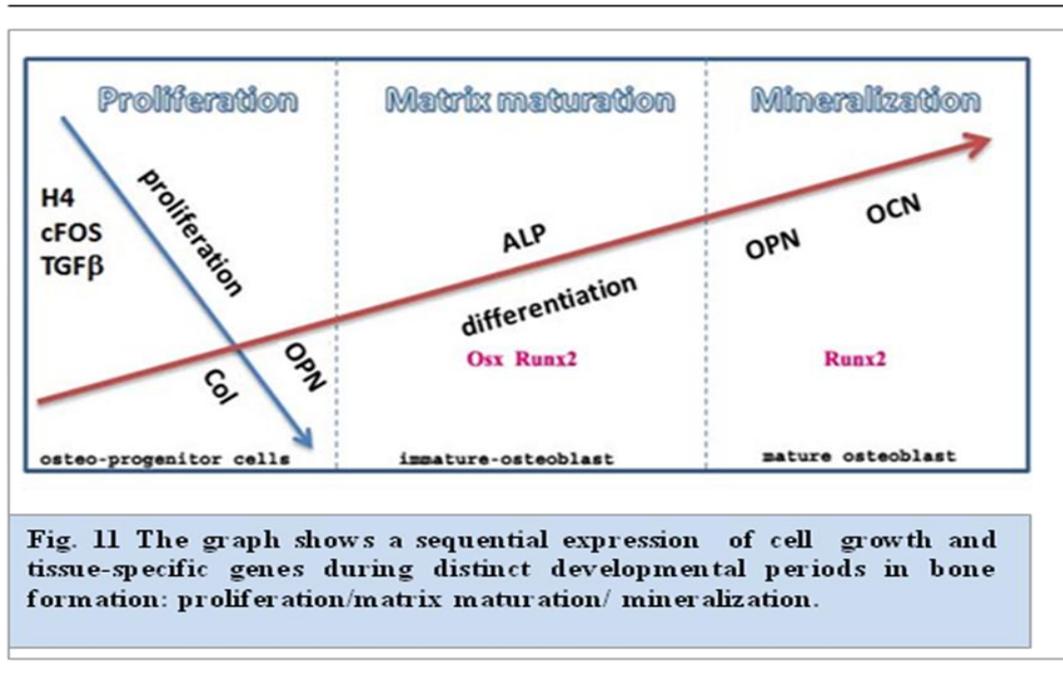
- **Osteogenic differentiation : physiological context**

A brief introduction about osteoblast differentiation and maturation occurring in physiological context is helpful to better understand how DPSCs can differentiate into osteoblast precursors and then into mature osteoblasts *in vitro* and *in vivo*, under specific stimuli.

Bone formation is a tightly regulated process that involves four distinct phases:

1. Migration of mesenchymal cells with osteogenic potential to the site of future skeletogenesis,
2. Mesenchymal-epithelial interactions,
3. Condensation (or aggregation) of mesenchymal cells,
4. Differentiation into the osteogenic lineage (Heng BC *et al.*, 2004).

After the recruitment of mesenchymal cells, it is characterized by a sequence of events starting with the commitment of osteoprogenitor cells, their differentiation into pre-osteoblasts and in mature osteoblasts whose function is to synthesize the bone matrix that become progressively mineralized. Osteoblast (polyhedral mesenchymal cell responsible for synthesizing new bone matrix) commitment, differentiation and function are all governed by several transcription factors, resulting in expression of phenotypic genes and acquisition of the osteoblast phenotype (Marie PJ *et al.*, 2008). All the steps of the lineage determinations, are a multiple-step series of events modulated by an integrated cascade of gene expression that initially supports a proliferation phase, then the sequential expression of genes associated with the biosynthesis, organization and



mineralization of the bone extracellular matrix , that characterize the matrix maturation phase and the mineralization phase. In 1986, Bellows and his colleagues developed a method to culture normal diploid calvarial-derived osteoblast under conditions that support development of a tissue like organization similar to embryonic bone and provided a variable *in vitro* model system for experimentally addressing the osteoblast proliferation/differentiation relationship within the context of physiological regulation. The tissue-like organization *in vitro* is reflected by the progressive development of nodules of multilayered cells and mineralized extracellular matrix with organized type I collagen fibrils. The ordered deposit of minerals within the collagen fibrils initiates and is primarily associated with the nodular area. The sequential expression of cell growth and tissue-specific gene defines three distinct developmental periods as shown in Figure 11.

The growth (proliferation) period is reflected by increase of osteoblast cell population with expression of cell cycle (e.g. histones, cyclines) and cell growth regulated genes (e.g. c-myc, c-fos, c-jun). At the same time several genes associated with formation of extracellular matrix [type I collagen (Col I), fibronectin and transforming growth factor- $\beta$  (TGF- $\beta$ )] are actively expressed.

The second period initiates following the down regulation of proliferation where expression of the genes associated with maturation and organization of bone extracellular matrix are up-regulated. These later genes contribute to rendering the ECM competent for mineralization.

The third developmental period reflects mineralized cultures in which other bone related genes are induced, e.g. bone sialoprotein (Nagata, T., *et al.*, 1991), Osteopontin (OPN) and Osteocalcin (OCN) levels are increased, paralleling the accumulation of mineral. OCN levels increase 100-fold when maturation of bone-tissue like organization is ongoing: the maximal level of expression observed with the mineralization of extracellular matrix is similar both *in vivo* and in *in vitro*.

The high expression of OCN during the later stage of osteoblast development sequence makes it a marker for mature osteoblast phenotype. As the mineralization proceeds, alkaline phosphatase mRNA declines and OCN mRNA levels become down regulated in heavily mineralized cultures.

The mineralization period is followed by the appearance of apoptotic cells along with the mineralized nodule; during this period, collagenase and type I collagen gene expression increase and may be involved in the collagen turnover associated with reorganization of the collagen matrix. These events have suggested to serve a

remodeling function for modifying the bone extracellular matrix to maintain the structural and functional properties of the fully differentiated bone cell

In the bone, as observed with other cells and tissues, the relationship between growth and differentiation must be maintained and strictly regulated, both during embryonic development and throughout the life of the organism to support tissue remodeling. Understanding the factors regulating bone growth, the continue remodeling of bone throughout life and the regeneration of injured tissue are basic biological questions and concerns of today's clinician for the treatment of bone related disorders.

- **Transcription factors and bone related protein: osteoblastogenesis control**

In osteoblast (bone forming cells), the proliferation/differentiation relationship is controlled by a number of genes, some of which are specifically expressed in bone. Previous studies have demonstrated that, like osteoblasts, pulp cells express bone markers such as bone sialoprotein, ALP, Col I, and OCN (Kuo MY *et al.*, 1992).

During in vitro osteogenesis, the molecular and cellular mechanisms underlying the sequential differentiation of mesenchymal stem cells into osteogenic lineages have been the object of numerous studies. Several factors have been indentified which influence these processes (Banerjee C *et al.*, 2001).

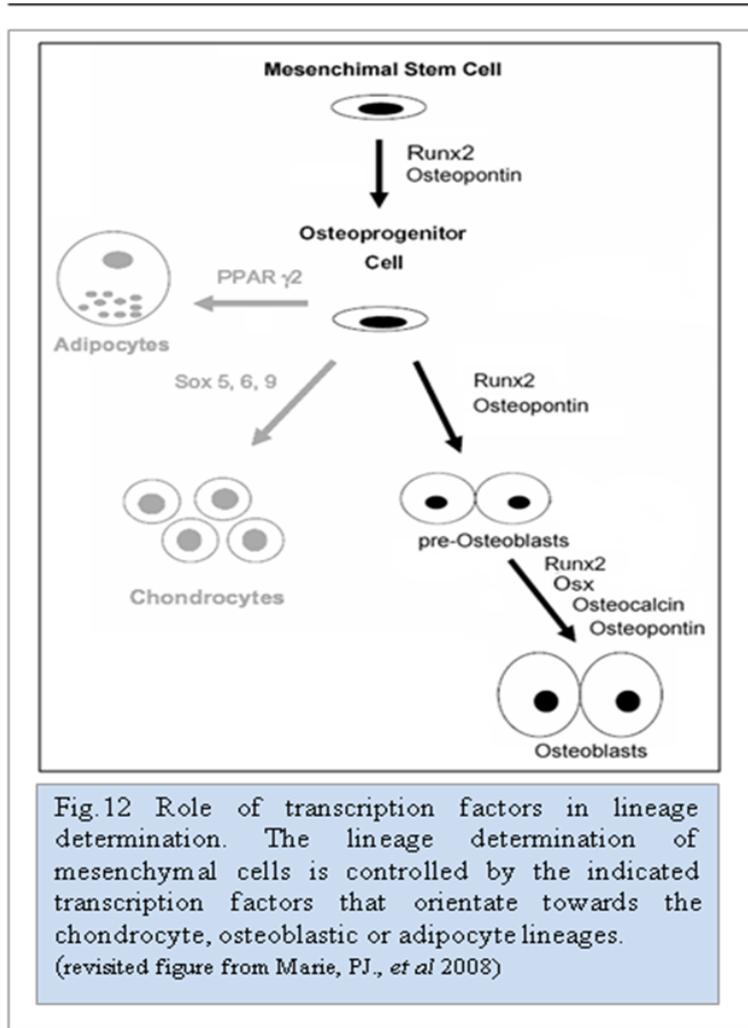
**OCN** is the major bone-specific protein, produced by the mature osteoblast, which is expressed only post-proliferative during differentiation of normal diploid osteoblasts, when mineralization of extracellular matrix occurs. OCN is the most abundant non-collagenous protein that constitutes 1-2% of 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. It is a small protein, Vitamin K-dependent, that contains three residues of calcium-binding amino acid g-carboxyglutamic acid (Gla), although the human protein contains only two Gla residues (Aronow MT *et al.*, 1990). Gla residues are formed by a post-translational enzymatic carboxylation of glutamic acid, and allow specific conformational changes in presence of calcium and promote OCN binding to hydroxyapatite and subsequent accumulation in bone matrix. 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.

Bone sialoprotein is a highly sulfated, phosphorylated, and glycosylated protein that mediates cell attachment through a RGD motif to extracellular matrices (Ganss, B., *et al.*, 1999). Due to its highly negatively charged characteristics, bone sialoprotein can sequester calcium ions while conserving polyglutamate regions, which have hydroxyapatite- crystal nucleation potential (Hunter CK *et al.*, 1993). In the absence of osteocalcin, bone sialoprotein could contribute to an overall metabolic shift toward new bone formation.

A decade ago, Runx2 (Cbfa1) has been identified as the major transcription factor controlling osteoblast commitment and differentiation. It is a member of the Runt homology domain family of transcription factors, expressed in early mesenchymal cells at the onset of skeletal development, as well as in all osteoblast throughout their differentiation (Marie, PJ., 2008). Although Runx2 is the most abundant factor in

mesenchymal stem cells, Runx2 and Runx3 are also present in the osteoblast lineage. Runx2 was found to control bone formation during both skeletal development and post-natal



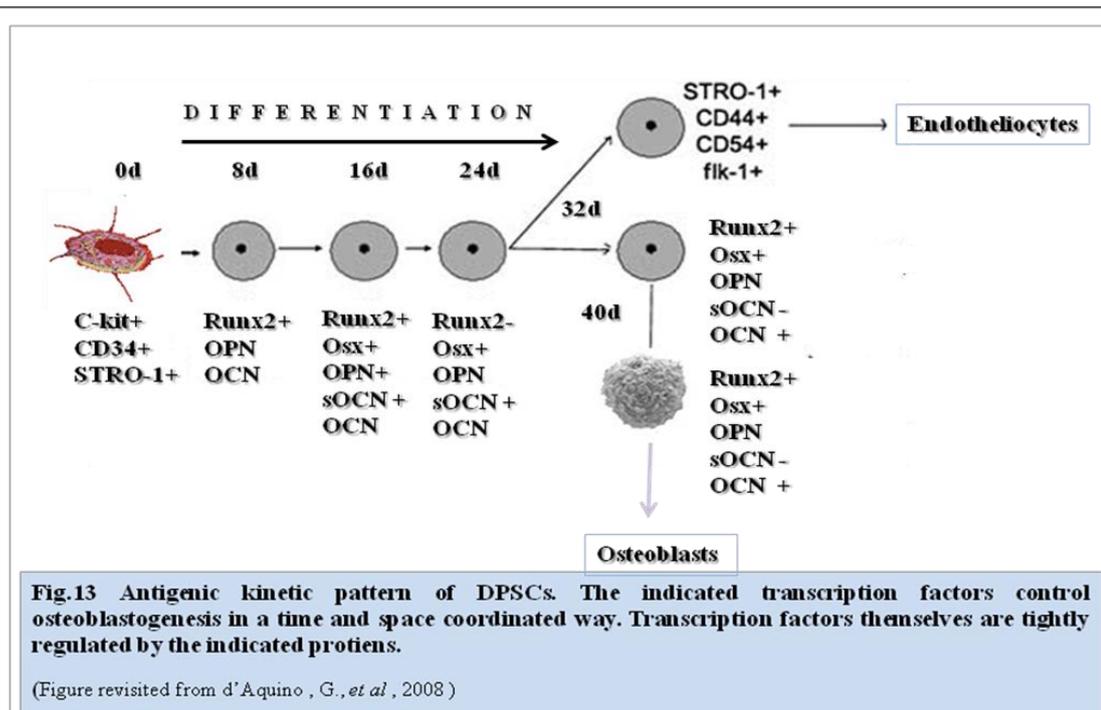
life (Karsenty G, 2002). Molecular studies and genetic manipulation of Runx2 in vivo indicated that the expression of Runx2 is both necessary and sufficient for mesenchymal cell differentiation towards the osteoblast lineage (Komori T et al., 2006) (Fig.12).

Its invalidation inhibits the differentiation of mesenchymal cells into osteoblasts and Runx2 haplo-insufficiency in humans' results in cleidocranial dysplasia, a disease characterized by defective bone formation. 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 (OSE2); its regulatory elements can be found in the promoter of all major osteoblast genes controlling their expression, including type I collagen and sialoproteins, such as

osteocalcin and osteopontin. In addition to control osteoblast differentiation, Runx2 was found to negatively control osteoblast proliferation, by acting on the cell cycle (Pratap J *et al.*, 2003): it can modulate the expression of kinases (such as p85 PI3K) that controls osteoblast differentiation and survival, and with several regulatory proteins within the nuclear architecture. Although its important role in osteoblast commitment, Runx2 is not essential for the maintenance of the expression of major bone matrix protein genes in mature osteoblasts. In fact, mice over-expressing Runx2 exhibit osteopenia, because of reduced number of mature osteoblast, indicating that Runx2 negatively controls osteoblasts terminal differentiation and maintains osteoblastic cell in an immature stage (Liu W *et al.*, 2001). Molecular and genetic studies revealed that **Osterix** (Osx) is a zinc finger transcription factor indispensable for osteoblast differentiation and specifically expressed by osteoblasts: Osx deficient mice show absence of osteoblast and defective bone formation (Nakashima K *et al.*, 2002). Osx transcription is positively governed by Runx2 and acts by directing pre-osteoblast to immature osteoblasts: Osx acts downstream of Runx2 (Runx2 is expressed in Osx-deficient mice). Although little is known about 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. The regulation of Osx is still poorly known: p53 was found to repress Osx transcription and thereby down-regulates osteoblastogenesis (Wang X *et al.*, 2006). 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 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 (Gerike 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 MD *et al.*, 1995b), and then it is progressive down-regulated according to its role of mineralization inhibitor during bone development (Hunter GK *et al.*, 1996).

The similarity of the gene expression profiles and the expression of specific markers between DPSCs and precursors of osteoblasts has recently been reported (Fig.13) (Shi *et al.*, 2001).



## Foetal stem cells

In recent years, foetal stem cells (FSCs) and stem cells isolated from cord blood or extraembryonic tissues have emerged as a potential 'half way house' between ES cells and adult stem cells. FSCs can be found in foetal tissues such as blood, liver, bone marrow, pancreas, spleen and kidney, and stem cells are found in cord blood and extra-embryonic tissues such as amniotic fluid, placenta and amnion (Marcus & Woodbury, 2008). Their primitive properties, expansion potential and lack of tumorigenicity, make them an attractive option for regenerative medicine in cell therapy and tissue engineering settings. While extra-embryonic tissues could be used with few ethical reservations, the isolation of FSCs is subject to significant public unease (Abdulrazzak H *et al.*, 2010

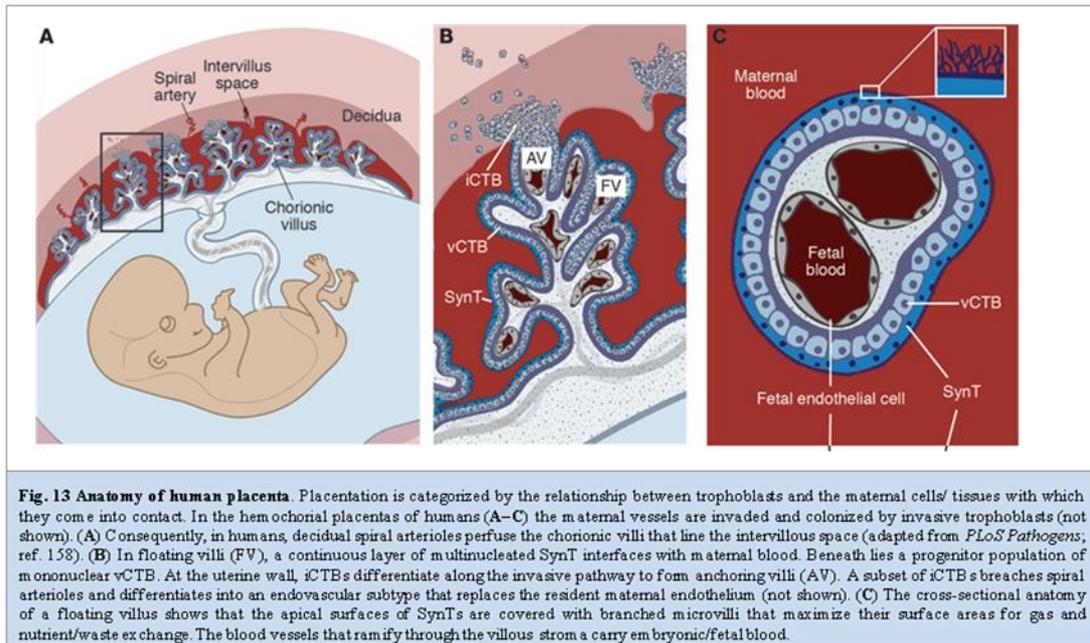
**Foetal stem** cells are advantageous for research for at least five reasons (Spitalieri P *et al.*,2009):

- ✓ First, they are obtained **routinely** during gestation, using minimally invasive techniques, for prenatal diagnosis of a variety of developmental and genetic disorders. Several foetal stem cell types have been characterized at the molecular and cellular levels, including those cells harvested from amniotic fluid and umbilical cord blood, together with their differentiation capacity (De Coppi P *et al.*, 2007; Fauza D, 2004; Guillot PV *et al.*, 2007; O'Donoghue K *et al.*, 2004).
- ✓ Second, foetal stem cells have a **higher potential for expansion** than cells taken from adults. Investigators have discovered that mesenchymal cells from umbilical cord blood can be induced to form a variety of tissues when cultured in vitro, including bone, cartilage, myocardial muscle, and neural tissue (Bieback K *et al.*, 2004). Foetal stem cells of mesenchymal origin are capable of self-renewal, as well as to differentiating into multiple tissue types (Tsai M *et al.*, 2004)
- ✓ Third, the ability to isolate pluripotent autogenic progenitor cells during gestation may be advantageous for the timely treatment of congenital malformations or genetic diseases in newborns.
- ✓ Fourth, foetal cells do not form teratomas when injected into adults and are either non immunogenic or less immunogenic, making them particularly suitable for transplantation. (Kakishita K *et al.*,2003).

- ✓ Fifth, their use is devoid of the ethical issues associated with embryonic stem cells (Weiss and Troyer, 2006).

Recent interest in stem cell biology and its therapeutic potential have led to the search for foetal stem cells in foetal organs obtained at termination of pregnancy, as well as for accessible sources of foetal stem cells that might be collected for autologous use in ongoing pregnancies. Until recently, foetal stem cells were thought to be multi-potent, but this picture is now changing as evidence is mounting, regarding the existence of pluripotent subpopulations in some foetal and extraembryonic tissues, such as placenta. (Cananzi M *et al.*, 2009).

- **Extraembryonic tissue: Placenta**



The placenta is a remarkable organ. Short lived by design, its brief existence enables the mammalian embryo/fetus to survive within the confines of the intrauterine environment. The diversity of functions performed by the placenta is impressive, ranging from anchoring the conceptus and preventing its rejection by the maternal immune system to enabling the transport of nutrients and wastes between the mother and the embryo/fetus. As with all organs, it performs these functions via multiple specialized cell types derived from lineage-committed precursors that either proliferate or differentiate. This process depends on a coordinated interaction among genetic, epigenetic, and physiological cues that are differentially interpreted as a function of gestational age. Each developmental event leading to the formation of a mature placenta is contingent upon the conditional transformation of prior structures (Maltepe E *et al.*, 2010).

Developmental information, therefore, has its own history, i.e., an “ontogeny of information,” that includes changes in the environment over time as an important variable (Oyama S, 2000). Human placenta, besides playing a fundamental and essential role in foetal development, nutrition, and tolerance, may also represent a reserve of progenitor/stem cells.

### - Placental Compartments

The foetal adnexa is composed of the placenta, foetal membranes, and umbilical cord. The **term placenta** is discoid in shape with a diameter of 15–20 cm and a thickness of 2–3 cm. From the margins of the chorionic disc extend the foetal membranes, amnion and chorion, which enclose the fetus in the amniotic cavity, and the endometrial decidua. The chorionic plate (Fig. 13) is a multilayered structure that faces the amniotic cavity. It consists of two different structures: the amniotic membrane (composed of epithelium, compact layer, amniotic mesoderm, and spongy layer) and the chorion

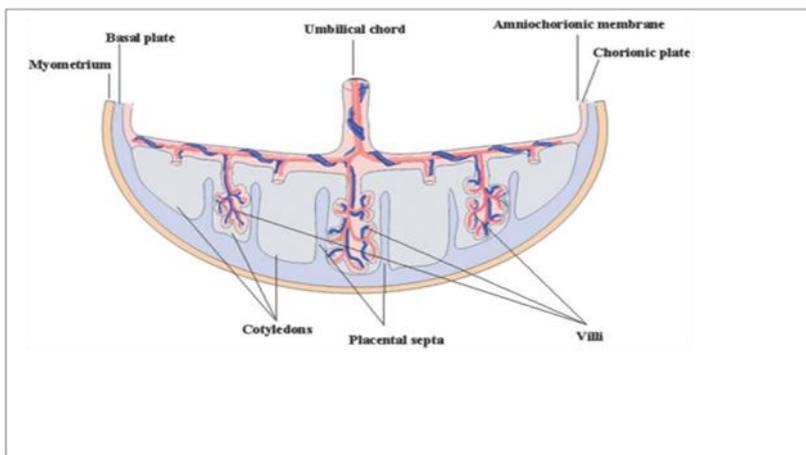
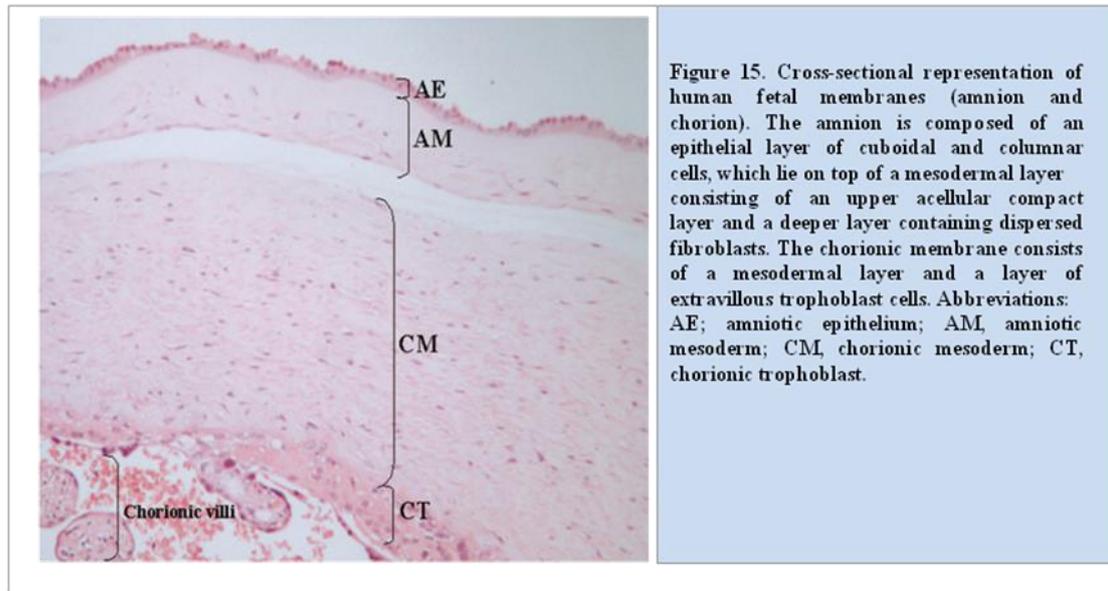


Figure 14. Schematic section of the human term placenta. During placenta formation, from the maternal side, protrusions of the basal plate within the chorionic villi produce the placental septa, which divide the parenchyma into irregular cotyledons. Each cotyledon contains several villi, which originate from the chorionic

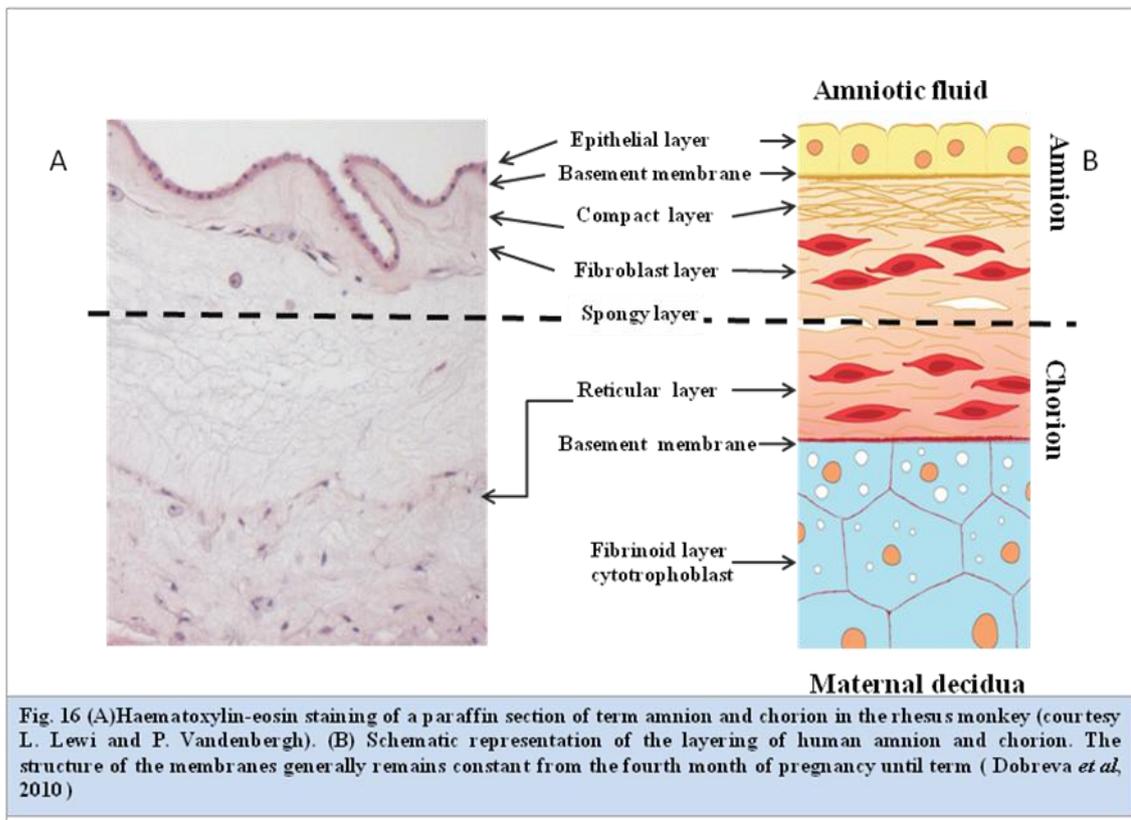
(composed of mesenchyme and a region of extravillous proliferating trophoblast cells interposed in varying amounts of Langhans fibrinoid, either covered or not by

syncytiotrophoblast). Villi originate from the chorionic plate and anchor the placenta through the trophoblast of the basal plate and maternal endometrium. From the maternal side, protrusions of the basal plate within the chorionic villi produce the placental septa, which divide the parenchyma into irregular cotyledons (Fig. 14). Some villi anchor the placenta to the basal plate, whereas others terminate freely in the intervillous space. Chorionic villi present different functions and structure. In the term placenta, the stem



villi show an inner core of foetal vessels with a distinct muscular wall and connective tissue consisting of fibroblasts, myofibroblasts, and dispersed tissue macrophages (Hofbauer cells). Mature intermediate villi and term villi are composed of capillary vessels and thin mesenchyme. A basement membrane separates the stromal core from an uninterrupted multinucleated layer, called syncytiotrophoblast. Between the syncytiotrophoblast and its basement membrane there are single or aggregated Langhans cytotrophoblastic cells, commonly called cytotrophoblast cells (Parolini O *et al.*, 2010) Foetal membranes continue from the edge of the placenta and enclose the amniotic fluid and the fetus. The amnion is a thin, a vascular membrane composed of an

epithelial layer and an outer layer of connective tissue, and is contiguous, over the umbilical cord, with the foetal skin. The amniotic epithelium (AE) is an uninterrupted, single layer of flat, cuboidal and columnar epithelial cells in contact with amniotic fluid. It is attached to a distinct basal lamina that is, in turn, connected to the amniotic mesoderm (AM) (Fig. 15). In the amniotic mesoderm closest to the epithelium, an acellular compact layer is distinguishable, composed of collagens I and III and fibronectin. Deeper in the AM, a network of dispersed fibroblast-like mesenchymal cells and rare macrophages is observed. Very recently, it has been reported that the mesenchymal layer of amnion indeed contains two subfractions, one having a mesenchymal phenotype, which is referred to throughout as amniotic mesenchymal stromal cells, and the second containing monocyte-like cells (Magatti M *et al.*, 2007). A spongy layer of loosely arranged collagen fibers separates the amniotic and chorionic mesoderm. The chorionic membrane (chorion leave) consists of mesodermal and trophoblastic regions (Fig.15). Chorionic and amniotic mesoderm is similar in composition. A large and incomplete basal lamina separates the chorionic mesoderm from the extravillous trophoblast cells. The latter, similar to trophoblast cells present in the basal plate, are dispersed within the fibrinoid layer and express immunohistochemical markers of proliferation. The Langhans fibrinoid layer usually increases during pregnancy and is composed of two different types: a matrix type on the inner side (more compact) and a fibrin type on the outer side (more reticulate). At the edge of the placenta and in the basal plate, the trophoblast interdigitates extensively with the deciduas (Cunninghan FG *et al.*, 1997; Benirschke K *et al.*, 2000).



## - Embryological Development

In humans, by days 6–7 after fertilization (during the implantation window), the blastocyst implants and placenta development begins. At this stage, the blastocyst is flattened and composed of an outer wall (**trophoblast**) that surrounds the blastocystic cavity. A small group of larger cells, the inner cell mass, is opposed to the inner surface of the trophoblastic vesicle. The embryo, umbilical cord, and amniotic epithelium are derived from the inner cell mass. As the blastocyst adheres to the endometrial, invading trophoblasts erode the deciduas, facilitating implantation of the blastocyst. By 8–9 days

after fertilization, trophoblastic cells at the implanting pole of the blastocyst proliferate robustly, forming a bilayered trophoblast. The outer of the two layers become the **syncytiotrophoblast** by fusion of neighboring trophoblast cells, whereas the inner cells (**cytotrophoblast**) remain temporally unfused. The proliferating cytotrophoblasts and the syncytiotrophoblasts give rise to a system of trabeculae intermingled with hematic lacunae. From these trabeculae are generated the primordial villi that are distributed over the entire periphery of the chorionic membrane. Villi in contact with the decidua basalis proliferate to form the leafy chorion or chorion frondosum, whereas villi in contact with the decidua capsularis degenerate into the chorion leave. At day 8–9 after fertilization, the inner cell mass differentiates into two layers: the epiblast and the hypoblast. From the epiblast, small cells that later constitute the amniotic epithelium appear between the trophoblast and the embryonic disc and enclose a space that will become the amniotic cavity. On the opposite side, between the hypoblast and cytotrophoblast, the exocoelomic membrane and its cavity modify to form the yolk sac. The extraembryonic mesoderm arranges into a connective tissue that surrounds the yolk sac and amniotic cavity, giving rise to amniotic and chorionic mesoderm (**Fig.16**).

- **Placenta-derived progenitor/ stem cells:**

The placenta is a fetomaternal organ involved in maintaining foetal tolerance and allows nutrient uptake and gas exchange with the mother, but also contains a high number of progenitor cells or stem cells (Parolini O *et al.*, 2010). It has two sides: one foetal (amnion and chorion) and one maternal (deciduas). The availability, phenotypic plasticity and immunomodulatory properties of placenta-derived progenitor/ stem cells

are useful characteristics for cell therapy and tissue engineering (Abdulrazzak H *et al.*, 2010) .Cells can be isolated:

1. during ongoing pregnancy using minimally invasive techniques such as chorionic villus sampling (CVS) and placental tissues are readily available at delivery for allogeneic or autologous use;
2. from the term placenta : placenta is a readily available tissue that is normally disposed off after delivery of the baby (Brooke G *et al.*, 2009).

Cells that have been isolated from placenta include:

- a) hAECs: human amniotic epithelial cells (from the amniotic membrane),
- b) hAMSCs: human amnion mesenchymal stromal stem cells (both from amnion and chorion throughout gestation from first trimester to delivery) ,
- c) **hCMSCs**: human chorionic mesenchymal/stromal stem cells ( isolated during pregnancy CVS or from the term placenta at delivery),
- d) hCTC: human chorionic trophoblastic cells,
- e) HSCs: human hematopoietic stem cells (Parolini O *et al.*, 2008).

- **Chorion derived stem cells : Chorion Mesenchimal Stem Cells (CMSCs)**

Extensive phenotypical and functional characterization is available on hAMSC, whereas there are few reports of investigation on hCMSC (Soncini M *et al.*, 2007). CMSCs have

been isolated during pregnancy (chorionic villus sampling, CVS, 9<sup>th</sup>-12<sup>th</sup> week of gestation), or from placenta at delivery. The immunophenotype of term placental cells are similar to that of adult bone marrow MSCs, although renin and flt-1 have been shown to be expressed uniquely in the placenta MSC (Fukuchi Y *et al.*, 2004). Mesenchymal placental cells are plastic adherent, share a similar immunophenotype and have lineage differentiation potential. They express stromal markers such as CD166, CD105, CD73, CD90 and others, while they are negative for the hematopoietic markers CD14, CD34 and CD45 (Sudo K *et al.*, 2007). Additionally, they express pluripotency markers such as SSEA3, SSEA4, Oct-4, Nanog, Tra-1-60 and Tra-1-81 (Battula VL *et al.*, 2007).

Of relevance, MSCs from chorion, similarly to human amnion, successfully engraft in neonatal swine and rats (Bailo M *et al.*, 2004), without a xenogenic response, indicating that these cells may have an immunoprivileged status consistent with their low level of HLA I and absence of HLA II expression (Kubo M *et al.*, 2001). Of relevance for prenatal autologous and allogeneic therapy, chorion MSCs can be successfully isolated from chorionic villi during first-trimester gestation. The chorionic villi do not express typical MSC cell surface antigens and have the capacity to differentiate into lineages of the three germ layers, with a subset of cells expressing the pluripotency markers Oct-4, ALP, Nanog and Sox2 (Spitalieri P *et al.*, 2009).

## **Bone tissue surgery**

Large bone defects that occur through trauma, loss of large bone areas after cancer surgery or bone loss through metabolic and degenerative diseases connected with weakening of the whole bone structure and the intrinsic regeneration mechanism, have a limited capacity for self-repair. Therefore, such bone defects could benefit from the development of novel treatments that take advantage of readily available and implantable bone grafts (Csaki C *et al.*, 2009). Surgical methods such as autologous and/or allogenic osteochondral grafts have been established in surgical medicine and have been used for several decades to fill osteochondrotic defects. Patients with large defects, such as large bone defects after tumor resection, osteonecrosis, extensive trauma or broad focal osteoarthritis (OA) have especially benefited from these grafts (Marco P *et al.*, 1993). However, the risk of donor site morbidity, infections from allografts or the sheer size of the implant are important limitations in reconstructive medicine and necessitate development of novel and innovative approaches to overcome these problems. These limitations have resulted in the development of new strategies to provide bone substitutes from in vitro production through tissue engineered bone constructs. Most of these evolve around bio-compatible and bio-degradable scaffold construction, seeding of scaffolds in vitro with adequate cells such as primary osteoblasts or mesenchymal stem cells (MSCs) or DPSCs and various combinations of growth factors.

The use of adult **MSC-like populations** provides an alternative and ethically acceptable source of multipotent cells instead of using embryonic stem cells, which have proved to be less practical and more controversial.

Several studies have demonstrated that re-implanted MSC-like populations *in vivo* undergo site-specific differentiation and are capable of bone formation. This suggests that tissue specific cells may promote and enhance cells to differentiate towards their fully differentiated cell type by promoting dynamic and intensive cell–cell interactions.

### **-3D Scaffolds**

Tissue engineering is a developing branch of science that merges the fields of cell biology, molecular biology, bioengineering, material science, and surgery to provide new functional tissue using living cells, biomatrices and signaling molecules. Using this technology, tissue loss or organ failure can be treated by implantation of an engineered biological substitute, that is either functional at the time of implantation or has the potential to integrate and form the expected functional tissue at a later stage.

Three dimensional (3-D) cell cultures on a bio-degradable cell scaffold is the basis of tissue engineering, where the specific cells can grow and multiply into a structure similar to tissue or organs in the living body (George J *et al.*, 2006).

Bone formation requires a three-dimensional scaffold to drive cellular growth and differentiation (Donzelli E *et al.*, 2007). An ongoing increasing numbers of biomaterials have been proposed as scaffold for tissue regeneration, whit the aim of reproducing the setting (or milieu) where the complex interaction between cells and their matrix.

To achieve this goal, an ideal scaffold:

- should act as a biomimetic platform allowing cells to properly accommodate and rebuild the target tissue;
- it should feature physical and chemical cues (like *bioactive* signals) that stimulate cell commitment, differentiation and regeneration of target tissue ;
- It must be easy to shape it onto the bone defect;
- It can also provides mechanical support against *in vivo* forces (Atala A ,2009);
- It must have a reabsorbition time compatible with the time require for bone formation, but not so long to interferes with the bone substitution of the scaffold (Donzelli E *et al.*, 2007).

Generally, three classes of biomaterials have been used for tissue engineering: naturally derived, such as Collagen and Silk fibroin; extracellular matrices, like Matrigel™ and synthetic polymers, such as poly-lactic acid (PLA) or P(d,l)LA.

## Matrigel™

Matrigel™ basement membrane is composed by thin extracellular matrices underlying cells *in vivo*. It is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins. Its major component is laminin, followed by



collagen IV, heparan sulfate proteoglycans, entactin/nidogen. Matrigel™ also contains

TGF-beta, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, tissue plasminogen activator, and other growth factors that occur naturally in the EHS tumor. It is effective for the attachment and differentiation of both normal and transformed anchorage dependent epithelioid and other cell types: neurons, hepatocytes, Sertoli cells, chick lens, vascular endothelial cells and MSC.

Since its discovery in the 1980s, Matrigel has been used in numerous studies as an extracellular matrix for the *in vitro* and *in vivo* analysis of cell differentiation, invasiveness of tumor cells, and angiogenesis (Laschke MW *et al.*, 2008). Because

Matrigel contains a combination of different angiogenic growth factors, it ideally promotes the development of new blood vessels (Kleinman HK and Martin GR, 2005).



Matrigel matrix also supports *in vivo* propagation of human tumors in immunosuppressed mice.

(See BD datasheet PRODUCT: BD Matrigel™ Basement Membrane Matrix Phenol Red Free, 10 ml vial catalog number: 356237).

### **-Collagen Condress**

Condress is extracted from bovine Achilles' tendon through a non-denaturing procedure in the absence of proteolytic enzymes. It has not been submitted to a chemical cross-linking process before lyophilization. Chemical identification of



Condress as type-I acid-insoluble collagen has been carried out by evaluation of total nitrogen and hydroxyproline contents and by chromatographic examination. Electrophoretic analysis and morphological examination by electron microscopy confirm that the procedure employed to extract collagen does not alter the polypeptidic composition of the molecule and its structure. A gamma-ray dose between 0.5 and 1.5 Mrad is quite adequate to sterilize the final product and certainly devoid of degradative effect. The finished product has a special (peculiar) absorbing capacity, immersion time, strain resistance, wrinkling temperature and enzymatic digestion time. It is a nonallergenic product suitable for clinical use. Condress can be easily adapted to the application surface. When it has been applied in chronic leg ulcers, pressure sores, or reconstructive surgery, Condress seems to substantially improve wound repair: in these situations, Condress promotes the development and proliferation of granulation tissue .

### **-Poly (d,l-lactide): P(d,l) LA**

Poly(lactic acid) or polylactide (PLA) is a biodegradable, thermoplastic, aliphatic polyester derived from renewable resources, such as corn starch (in the United States), tapioca products (roots, chips or starch mostly in Asia) or sugarcanes (in the rest of

world). Although PLA has been known for more than a century, it has only been of commercial interest in recent years, in light of its biodegradability.

Polymerization of a racemic mixture of L- and D-lactides usually leads to the synthesis of poly-DL-lactide (PDLLA) which is amorphous. Use of stereospecific catalysts can lead to heterotactic PLA, which has been found to show crystallinity. The degree of crystallinity, and hence many important properties, is largely controlled by the ratio of D to L enantiomers used, and to a lesser extent on the type of catalyst used.

The addition of hydrophilic polymers into the PLA material, has been suggested as a method of increasing both the water uptake and degradation rate of polymers, thus enhancing the dissolution of drugs. Several methods have been used to achieve these polymers: the high temperature plasticisation of PLA with poly(ethylene glycol) (PEG), co-polymerisation of poly(L-lactide) (PLLA) and PEG and chemical modification through photo-grafting of the hydrophilic component to the PLA material. PDLLA are hydrophobic, slowing the rate of water ingress and taking up to 6 months to degrade slowing the release of any entrapped (Ginty PJ *et al.*, 2008).

### **-Silk fibroin**

Silks are naturally occurring protein polymers produced by a wide variety of insects and spiders that have been used clinically as sutures for centuries. When naturally extruded from insects or worms, silk is composed of a filament core protein, termed fibroin, and a glue-like coating consisting of sericin proteins. In recent years, silk fibroin has been increasingly studied for new biomedical applications due to the

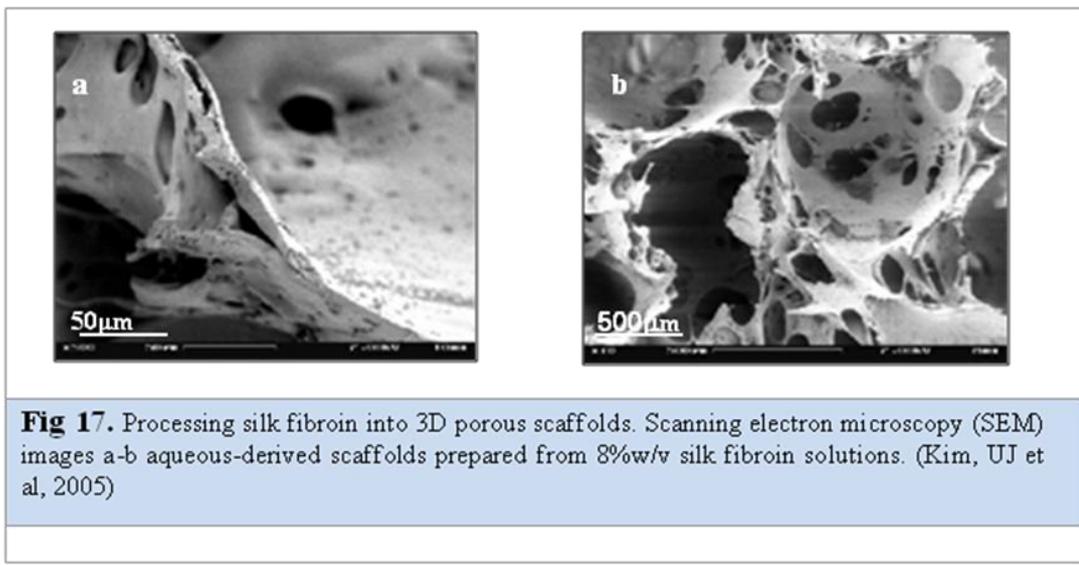
- biocompatibility,
- slow degradability ,

- remarkable mechanical properties of the material (Wanga Y *et al.*, 2006) that resemble those of several structural tissues in the body, and has been successfully explored for the tissue engineering of bone, cartilage and ligaments.

When appropriately purified, silk fibroin (SF) is non-toxic, non-immunogenic and has been demonstrated to support cell and tissue growth (Altman GH *et al.*, 2003).

The diverse functions of silks range from web construction and prey capture (spider webs), safety line (draglines) to reproduction (cocoons), and numerous studies have explored the potential of native and regenerated silk fibroin-based biomaterials in various forms, including films/membranes, micro/nano-fiber mats/nets, hydrogels, and porous sponges.

Silks provide an excellent combination of lightweight (1.3 g/cm<sup>3</sup>), high strength (up to 4.8 GPa as the strongest fiber known in nature), and remarkable toughness and elasticity (up to 35%). In summary, SF is a well-established biomaterial for tissue scaffolding mainly owing to its amenability to form tissue conductive microstructures (Fig.17).



## MATERIALS AND METHODS

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## **Dental pulp extraction and digestion**

Cells were isolated from dental pulp as described in a previous study (Laino G *et al.*, 2006). Human dental pulp was extracted from third molar or permanent teeth of adult subjects (18 and 35 years of age) after informed consent of patients undergoing routine tooth extraction (only disease-free patients).

Dental pulp was removed from the teeth with a dentinal excavator or a Gracey curette, and then immersed in a digestive solution (3 mg/mL type I collagenase plus 4 mg/mL dispase in  $\alpha$ -MEM) for 1 hour at 37°C.

Once digested, the pulp tissue was chopped, dissociated, and then filtered onto 100 $\mu$ m Falcon Cell strainers to obtain a cell suspension.

## **Cell culture**

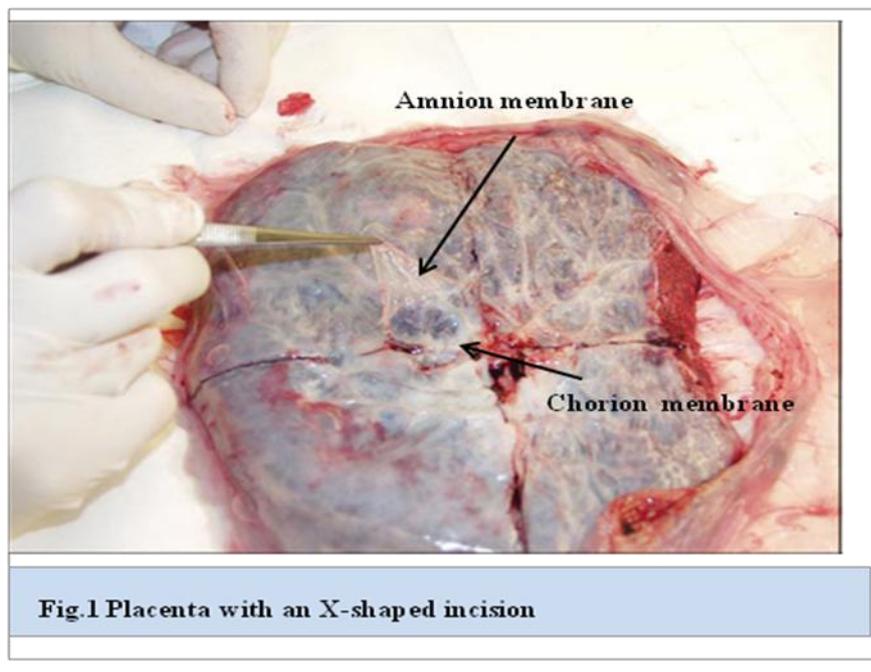
Cells were then plated and cultured in culture medium ( $\alpha$ -MEM with 20% FBS, 100  $\mu$ M 2P-ascorbic acid, 2 mM L-glutamine, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin), in a humidified atmosphere of 95% air with 5% CO<sub>2</sub> at 37°C. The medium was changed twice a week. Just before cells become confluent, they were subdivided into new flasks by trypsinization (0.05% trypsin/EDTA solution).

Cells obtained from a single dental pulp were subcultured or plated at clonally density (1.6 cell/cm<sup>2</sup>) to obtain a single clone. After 6 days of culture eight cell populations were isolated from nodules originated by single cells. One of the expanded cell cultures from each sample/experiment was storage in vials of 1 million cells per vial: the vials were plunged into LN<sub>2</sub> (Liquid Nitrogen) at -196°C.

## Term placental sample collection

The term human placentae were collected, for this study, with an average patient gestation of  $38 \pm 1$  weeks (mean $\pm$ SD). Delivery mode was elective Caesarean section. The study was performed with informed consent according to Italian law and ethical committee guidelines that approved the use of term placenta tissue for this work.

Following collection, the umbilical cord was trimmed, close to the placental surface, and an X-shaped incision were cut into, but not through, the placental tissue (Fig.1). The amnion membrane was peeled from the underlying chorion layer of the placenta body and discarded. 2 cm cubes of chorionic placental tissue were then cut and the samples were rinsed in physiological solution, to wash the blood in excesses.



### -Cell culture: placenta

The sample was washed 3 times in PBS solution, than enzymatically dissociated in a solution of Collagenase type IA (Sigma) 1%(w/v) in water diluting in  $\alpha$ -MEM with 1%

of penicillin: streptomycin (pen:strep) solution for 90 min at 37°C. Before the incubation with collagenase type IA, the sample were mechanically disrupted with a 5-ml syringe and a 16-G needle. After the incubation, the cell solution was passed three times through a 20-G needle, filtered through a 50 µm cell strainer and centrifuged 5min at 300xg.

The supernatant was removed and the pellet was washed by adding 5ml of medium, and then resuspended in the culture medium and transferred in a flask. The medium was change twice a week, and the cells were cultured for 10 days.

### **Magnetic-activated cell sorting (MACS®)**

DPSC were obtained by magnetic cell sorting using MACS® separation Kit (Miltenyi biotech, Germany), according to the manufacturer instructions. Three successive sorting were performed by using specific antibodies:

- ✓ Mouse anti-CD34 (Millipore Corporation), a marker of stromal and hematopoietic pluripotent stem cells (Simmonson, PJ., 1991);
- ✓ Rabbit anti-c-Kit (Santa Cruz Biotechnology), the tirosin-kinase receptor of stem cells factor (Barclay, AN., *et al.*, 1988);
- ✓ Mouse IgM anti- STRO-1 (Santa Cruz Biotechnology), an antigen present in a stromal cell population containing osteogenic precursor (Gronthos, S., *et al.*, 1994).

These primary antibodies were detected by magnetically labeled secondary Abs (anti-mouse IgG, anti-rabbit IgG and anti-mouse IgM, all from Miltenyi Biotech, Germany).

For each selection approximately  $7 \times 10^6$  cells were used. Firstly, pulp cell expanded in culture, was sorted by anti-CD34 Ab. CD34<sup>+</sup> cells were expanded and then sorted by using anti-c-Kit Ab to obtain a CD34<sup>+</sup>/c-Kit<sup>+</sup> population. In the same way the CD34<sup>+</sup>/c-Kit<sup>+</sup> population was sorted by anti-STRO-1 Ab to obtain the CD34<sup>+</sup>/c-Kit<sup>+</sup>/STRO-1<sup>+</sup>, that represents a selected subpopulation of DPSC called Stromal Bone Producing( SBP-DPSC) as described by Laino *et al.*, (2005). Human term placental cells were sorted only for c-Kit: we obtained, as previously described, c-Kit<sup>+</sup> placental cells.

### **Flow Cytometry analysis (FACS)**

The expression of CD34, c-Kit and STRO-1 antigens was analyzed by indirect staining, using mouse anti CD34 IgG, rabbit anti c-kit, and mouse anti STRO-1 IgM, followed by sheep anti-mouse FITC, goat anti rabbit-FITC and goat anti-mouse IgM- FITC (Jackson Immuno Research, USA).

Non-specific fluorescence was assessed by using normal mouse IgG or IgM followed by secondary antibody as described above. Analysis were performed with a EPICS XL flow cytometer (Beckman Coulter, Brea, CA, USA). All experiments were performed in independent triplicates.

### **Osteogenic differentiation *in vitro*:2D**

In order to obtain a differentiation into osteoblast on 2D surface, DPSCs were seeded at 3000 cells/cm<sup>2</sup> on culture dishes in the osteogenic medium ( $\alpha$ -MEM, supplemented with

10% FBS, 100  $\mu$ M 2P-ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin, 100nM dexamethasone, 10mM  $\beta$ -glycerophosphate). The medium was changed twice a week. Control DPSCs were cultured in the same medium without dexamethasone,  $\beta$ -glycerophosphate and ascorbic acid. For immunofluorescence experiments DPSCs were differentiated on Thermanox<sup>®</sup> plastic cover-slips. Cell counting was performed in control and in dDPSC of three independent experiments by a Nikon TE2000 inverted Microscope using a 10x objective and differential interference contrast (DIC). For each experimental point, the mean of cell number were calculated and cell density was expressed as cells/cm<sup>2</sup>.

### **-Scaffolds**

Silk fibroin scaffolds were prepared and sterilized for cell cultures according to previous established protocols (Bondar B *et al.*, 2008). Degummed silk fibers were soaked into 98–100% formic acid (Riedel-deHaen, Seelze, Germany), at room temperature, and the acid solution was evaporated under atmospheric conditions. The average fiber diameter of the scaffolds was between 100 and 200 nm and the thickness of the matrices was measured in the dry state and in the wet state as well. Nets were repeatedly washed with double distilled water in order to remove any residual acid and kept in wet conditions and sterilized by gamma rays. To prepare the fibroin nets for cell culture, the material was cut into disc-like shape with a height of 1.5 mm, diameter of 5.2 mm and pore size of 250–420  $\mu$ m that allows cells to migrate into the nets.

The numbers of scaffolds used were indicated above.

## **Osteogenic differentiation in vitro:3D**

Matrigel™ (Becton, Dickinson) Collagen Sponge (Condress, ABIOTEN PHARMA s.p.a.), P(d,l)LA, and silk-fibroin were used as 3D scaffolds in this study. Cells were seeded in each scaffolds in an adequate volume of medium to obtain a starting density of 1000 cell for mm<sup>3</sup>. For every samples, cells were added to Matrigel™ at 4°C when it appears as liquid. A total volume of 400 µl of DPSCs-Matrigel™ was placed in a 12 multiwell plate in order to form a 1 mm thick layer, which was polymerized at 37°C.

DPSCs were injected in a 500 mm<sup>3</sup> sample collagen sponge by a micropipette tip in different points to obtain a homogenous cell distribution. After 8 hours from cells seeding, 2 ml of osteogenic medium was added to each sample. The medium was changes twice at week.

For the differentiation studies on 3D scaffolds, the P(d,l)LA scaffolds (slightly hydrophobic) were soaked in ethanol for 30 min and then exchanged with phosphate-buffered saline (PBS) for three times (30 min each).

The scaffolds were then washed twice with αMEM medium containing 20% FBS (2 h for each rinse). 1000cells/mm<sup>3</sup> were seeded on each scaffold and transferred 24 h later into 12-well tissue culture plates containing 3 mL of αMEM medium per well supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin 50 mg/mL supplemented with the differentiating elements: 10 mM β-glycerophosphate, 100nM dexamethasone and 100 µM 2P-ascorbic acid, for up to 4 weeks in an incubator at 37 °C with 5% CO<sub>2</sub>. Collagen Sponge and Fibroin were rinse, before seeding, with medium without the differentiating supplements. The rinse were performed to prepare

the scaffolds for the successive culture-conditions. The same amount of cells was seeded in each scaffold following the previously described.

### **Western Blotting**

Whole cell lysates were obtained from undifferentiated and differentiating DPSCs at different times of culture. Cells lysates were obtained by addition of a Lysis Buffer (20 mM Tris-Cl, pH 7.0, 1% NP-40, 150 mM NaCl, 10% Glycerol, 10 mM EDTA, 20 mM NaF, 5 mM Sodium Pyrophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and freshly added Sigma-Aldrich Protease Inhibitor Cocktail) at 4°C for 10 min. After sonication, lysates were cleared by centrifugation for 15 min at 14,000g in a refrigerated centrifuge. The protein lysate were then added to SDS (Sodium Dodecyl Sulphate) sample buffer and 20% of 10mM β-Mercaptoethanol, and immediately boiled.

50µg of protein extract, quantified by a Bradford Protein Assay (Biorad; Bradford, 1976) from each sample, underwent 10% or 15% SDS-polyacrylamide gel electrophoresis and then blotted onto a PVDF membrane. The protocols of the western blot were performed as described by Sambrook et al (Sambrook, J., 1989). The membrane was blocked with 3% dry milk and 2% bovine serum albumin (BSA) in phosphate-buffered saline-Tween 20 (0.1%) for 30min. Blots were incubated overnight with one of the primary Abs (diluted 1:1000 in blocking buffer: TBS-T + 2% BSA and 3% milk): anti-CD34, anti-c-Kit, anti-STRO-1, anti-Runx2, anti-OCN, anti-OPN and anti-Osx. After washing three times in blocking buffer, membranes were further incubated with peroxidase-labelled anti-rabbit, anti-mouse or anti-goat secondary abs diluted 1:5000, for 30 min at room temperature. Following further washing in TBS-T, all membranes were detected using ECL (Enhanced ChemioLuminescence,

Amersham). Anti-actin Ab was used as control of protein loading in timing experiments.

To detect secreted OCN (sOCN) each sample was cultured with 2.5 ml of medium. 2 ml of medium were collected for each sample at the same time-point and then precipitated in 10% Trichloroacetic acid (TCA). Precipitated proteins were re-suspended in 0.1N NaOH in H<sub>2</sub>O and then in Sample Buffer. The whole protein amount obtained for each sample was loaded in the SDS-PAGE in order to have as a unique variable proteins secreted from dDPSCs.

Densitometry was performed on WB from three independent experiments by NIS software (Nikon). An equal area (AOI) 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.

The same protocol was used to detect the expression of Oct4 in the whole cells lysates of placental cells after MACS® selection. Whole cell lysates were obtained from not selected, c-Kit<sup>+</sup> and c-Kit<sup>-</sup> placental cells after 10 days of culture.

### **RT-PCR of c-Kit<sup>+</sup> cultured placenta-cells.**

Total RNA isolation from c-Kit<sup>+</sup> cells (placenta-sample), first-strand cDNA synthesis, and PCR were performed by using a TaqMan Gold RT-PCR Reagents kit (Applied BioSystems, Bedford, MA) with an oligo dT primer. First-strand cDNA (2 µL) was diluted in a 20 µL reaction mixture containing of GeneAmp Fast PCR Master Mix (2X) (Applied BioSystems)/500 nM of each human-specific primer sets (see Tab.1). The

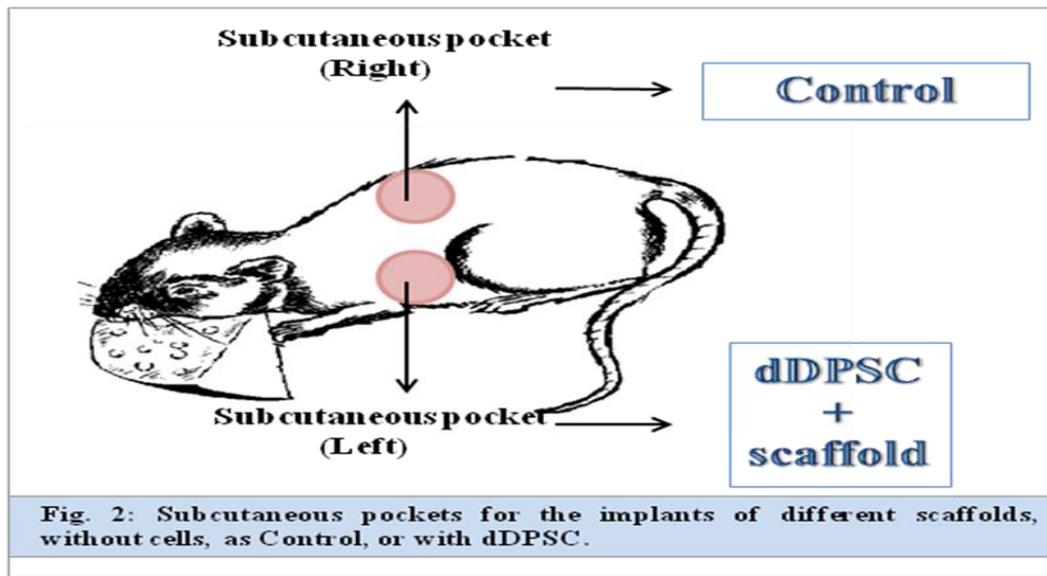
reactions were incubated in a GeneAmp PCR system 9700 (Applied BioSystems) at 94°C for 2 minutes for 1 cycle and then 94°C/(45seconds), 64°C/(45 seconds), 72°C/(60 seconds) for 35 cycles, with a final 7-minute extension at 72°C. After amplification, 10 µL of each reaction was analyzed by 2% agarose gel electrophoresis, and visualized by ethidium bromide staining.

<b>Gene</b>		<b>Sequence</b>	<b>pb</b>
<b>Oct4</b>	sense	5'-GTACTCCTCGGTCCCTTTCC-3'	167
	a-sense	5'-CAAAAACCCTGGCACAACACT-3'	
<b>hGADPH</b>	sense	5'- AGCCGCATCTTCTTTTGCGTC-3'	239
	a-sense	5'-TCATATTTGGCAGGTTTTTCT-3'	

**Tab.1: Primer sequences for RT-PCR analysis**

## Osteogenic Differentiation on 3D surface for ectopic implant: subcutaneous implantation

hDPSC-scaffold constructs were cultured in osteogenic medium-for 1 week (medium



changed twice) before implantation. For implantation, in the study male outbred rats with age ranging between 8 and 12 weeks (Charles River Laboratories) were used. The rat back was shaved, washed and disinfected with povidoneiodine. One mid-sagittal incision was made on the dorsa and two subcutaneous pockets were created using blunt dissection. One scaffold with a volume of  $600 \text{ mm}^3$  ( $15 \text{ mm } \varnothing \times 3.5 \text{ mm}$  thickness) was implanted subcutaneously into each pocket. Each animal received consequently 2 constructs. Three samples were implanted for each scaffold or scaffold/cell construct. The scaffolds were placed alternatively at different sites in each rat. Afterwards, the incisions were sutured with prolene 4-0 suture (Ethicon). Animals were immunocompromised using Cyclosporine A at a dosage of  $15 \text{ mg/Kg b. wt.}$ , administered 4 h before transplantation and then daily for 2 weeks. During the last 2 weeks the daily dosage was reduced gradually up to  $6 \text{ mg/Kg b. wt.}$

4 weeks later rats were sacrificed and the cell-scaffold constructs were rapidly fixed in 4% Paraformaldehyde in phosphate buffered saline (PBS). The 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**

Samples of 2D or 3D cultures were fixed in 4% Paraformaldehyde in PBS at pH: 7.4 for 15-60 min at room temperature. Samples were then washed twice with PBS and then processed for successive steps. Cells differentiated on Termanox coverslides were processed for immunofluorescence or histological staining. Matrigel samples were *in toto* processed. Collagen samples were firstly cryo-protected using a solution of 20% of sucrose in PBS for 1 day. The samples were placed on a support, embedded in Tissue-Tek O.C.T (Sakura Finetek Europe) followed by a rapidly freezing in -75°C isopentane and then freezed in N<sub>2</sub>-liquid.

Blocks of frozen collagen were cut into 10µm thick cryo-sections, using a cryomicrotome (Zeiss, Germany), and mounted on Superfrost plus slides. Scaffold samples from *in vivo* ectopic implants, composed by Matrigel™, Collagen sponge, P(d,l)LA and Fibroin plus dDPSCs, were embedded in paraffin and cut in ten-micrometer thick histological sections.

Routine haematoxylin & eosin staining was performed on some samples to analyze morphological details. Fixed cells or ten-micrometer sections were stained with Alizarin Red S staining: the samples were incubated for 30 min at room temperature in a solution

containing 0.1% alizarin red and 1% ammonium hydroxide. Counterstaining with fast green was performed to visualize cell morphology.

To demonstrate calcium deposition, sections were also stained with Von Koss Staining: the slides were incubated with 5% silver nitrate/H<sub>2</sub>O then exposed to UV lamp 20 mins. Rinsed in distilled water, slides were then treated with 2% sodium thiosulphate for 2 mins, washed in running tap water and in distilled water, rapidly dehydrated, cleared and mounted. Images of histological samples were obtained by a Zeiss Axiophot microscope equipped with a Nikon DS-5Mc CCD colour camera. The percentage of mineralized area of every specimen was measured using ImageJ software (NIH, Bethesda, MD).

Term placental tissues were embedded in paraffin blocks, using a common protocol.

### **Immunofluorescence and Confocal microscopy**

Fixed monolayer cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min. Permeabilized samples and histological sections were washed three times with 0.1M PBS. Samples from ectopic implants were incubated with 1 mM EDTA pH8 and exposed with microwaves (2 times at 750W), to unmask antigens.

Then, all the slides, were blocked with 3% BSA in PBS for 30 min at room temperature. After washing, samples were incubated for 1 h at room temperature (RT) with the primary antibodies diluted 1:50 in PBS containing 3% BSA: rabbit anti-c-Kit, mouse anti-CD34, mouse IgM anti-STRO-1; rabbit anti-Runx2 (Abcam, UK); mouse anti-OPN (Santa Cruz Biotechnology); rabbit anti-Osx (GeneTex, USA); mouse anti-OCN (GeneTex, USA); mouse anti-Human Mitochondrial protein (Millipore Corporation),

mouse anti-Stella(Millipore Corporation), mouse anti-Oct4(Chemicon USA), mouse anti  $\beta$ -actin( Millipore Corporation).

The samples were washed in PBS containing 3% BSA, and incubated for 1 h at room temperature with the secondary Abs diluted 1:200 in PBS containing 3% BSA (donkey anti-rabbit-AMCA; sheep anti-mouse-FITC, and goat anti-mouseIgM-Cy5<sup>TM</sup>; donkey anti rabbit-Cy3<sup>TM</sup>). After washing in PBS, samples were stained with 1  $\mu$ g/ml DAPI in H<sub>2</sub>O for 1 min (not performed in samples treated with donkey anti-rabbit-AMCA Ab) and then mounted with anti-fading medium (0.21 M DABCO and 90% glycerol in 0.02 M Tris, pH 8.0). Samples not incubated were considered negative controls with the primary antibody.

The multi-labelling immunofluorescence experiments were carried out avoiding cross-reactions between primary and secondary antibodies.

Fluorescent samples were observed by a Nikon TE2000 microscope equipped with a CCD camera Hamamatsu ORCA 285. Images were captured and processed by NIS software (Nikon). Confocal imaging was performed on a Leica TCS SP2 AOBS confocal laser scanning microscope. The confocal serial sections were processed with the Leica LCS software to obtain three-dimensional projections. The image rendering was performed by Adobe Photoshop software.

### **Immunohistochemistry (DAB)**

Paraffin term-placenta slides were deparaffined and washed two times in PBS; the endogenous peroxidase activity was blocked with freshly made 0.3% H<sub>2</sub>O<sub>2</sub> in methanol, for 20 minutes. After washing with PBS, to unmask the antigen epitope, we performed

as antigen retrieval, the citrate buffer method. The slides were arranged in a staining container, and were incubated for 15 minutes at 95-100°C with 10mM citrate buffer, pH 6.0. Rinsed the slides with PBS, the sections were then blocked with 3% BSA in PBS for 30 min at room temperature. Then samples were incubated for 1 h at RT with the primary antibodies diluted 1:50 in PBS containing 3% BSA: rabbit anti-c-Kit, mouse anti-Oct4, and mouse anti-Stella. The samples were washed in PBS containing 3% BSA, and incubated for 1 h at room temperature with the peroxidase labelled-secondary Abs diluted 1:50 in PBS + 3% BSA (anti-rabbit-HRP, anti-mouse-HRP). After washing in PBS, samples were stained with DAB substrate solution (freshly made just before use: 0.05%DAB - 0.015% H<sub>2</sub>O<sub>2</sub> in PBS) to reveal the colour of antibody staining (<5 min until the desired colour intensity).

The slides were washed in PBS, counterstained with Haematoxylin for 1-2 minutes, and rinsed in running tap water for < 15min. The slides were dehydrated before to be mounted with anti-fading medium (0.21 M DABCO and 90% glycerol in 0.02 M Tris, pH 8.0).

## **Electron microscopy**

Samples processed for morphology observations were fixed in 2.5% glutaraldehyde in Phosphate buffer (pH: 7.4) for 1 h and post-fixed in 1% OsO<sub>4</sub> for 1 h. Ultrathin sections, obtained from Durcupan embedded samples, were stained with uranyl acetate–lead citrate and then observed with a Zeiss EM109 Transmission Electron Microscope. Immunogold experiments were performed as previously described (Riccio M *et al.*, 2001) Briefly, samples were fixed in 1% glutaraldehyde in Phosphate buffer (pH: 7.4)

for 1 h, dehydrated in ethyl alcohol (50%, 70%, 90%, 95% and 100%), diaphanized in propylene oxide and embedded in Durcupan resin. Following a preincubation in 0.5 M Tris-HCl, pH 7.6, 1% 0.14M NaCl, 0.1 % BSA (TBS buffer I), the samples sections were incubated for 1 h at room temperature with the primary Ab (Human collagen type I) diluted 1:50 in TBS buffer I. After being washed in TBS buffer I and then in TBS buffer II (0.2M Tris-HCl, pH 7.6, 1% 0.14M NaCl, 0.1 % BSA), human collagen was detected, in ultrathin sections, by a mouse anti-human type I collagen Ab, diluted 1:20 in TBS buffer II. After 1h of incubation, the primary Ab was revealed by a 10 nm gold labelled anti-mouse secondary Ab. Incubation of the grids with gold-conjugated secondary antibodies only was used as control.

### **Statistical analysis**

Quantitative or semi-quantitative data were expressed as mean  $\pm$  standard deviation (SD). Differences between experimental points were evaluated by Student's *t*-test. In all analyses, *p* values  $< 0.05$  were considered a significant statistical difference.

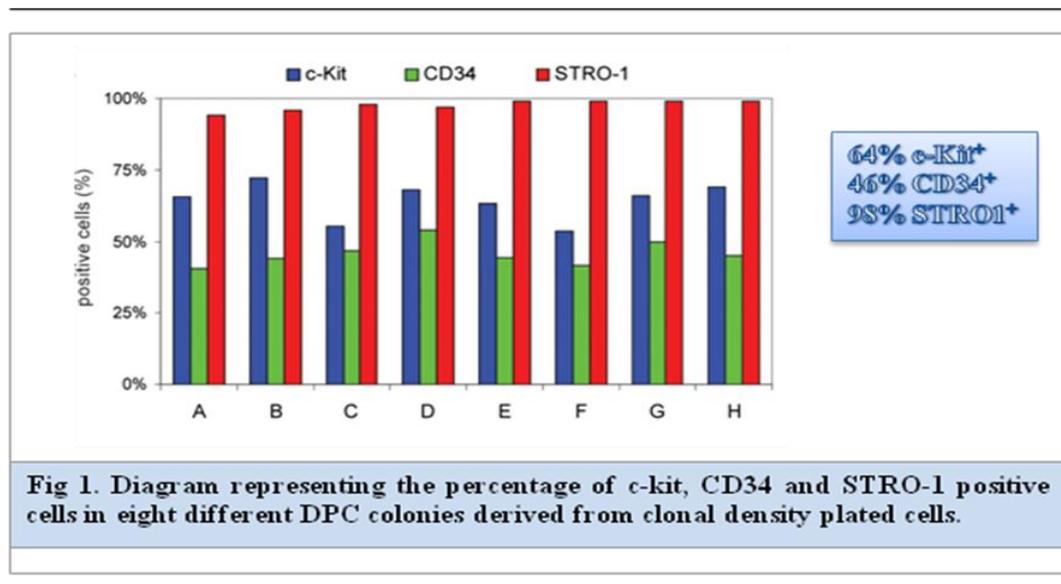
## Results

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## DPSC: isolation and characterization

It is generally accepted that MSCs can be recognized as adherent cells, capable of extensive proliferation, with a fibroblastic profile and with the ability to differentiate into mesenchymal lineage (in our experiments into osteoblastic lineage). The cells obtained from dental pulp by enzymatic digestion were cultured, as described in the *Materials and Methods*, for several days to isolate DPSCs. After two days of culture, cells were adherent, showed a spindle-like phenotype and often aggregated in groups.



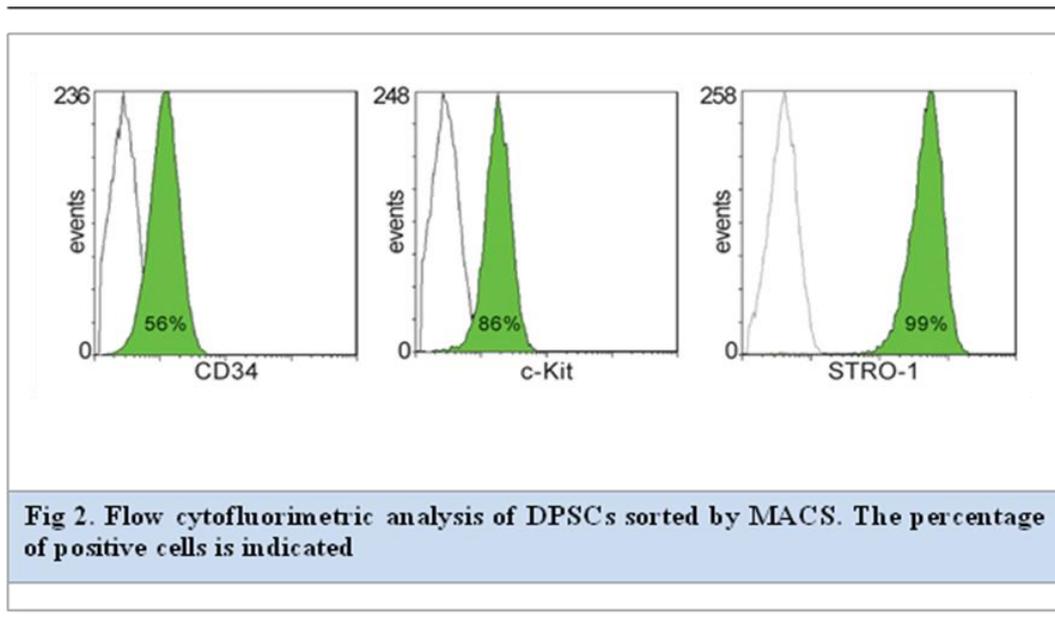
## Cell sorting

Flow cytometric analysis (Fig. 1) of each of eight populations isolated from nodules, after 6 days of culture, originated from 1 single cell clone, showed that the percentage of c-Kit<sup>+</sup>, CD34<sup>+</sup> and STRO-1<sup>+</sup> cells were 64%, 46% and 98% respectively (mean values).

The majority of nodule constituting cells expressed c-Kit, CD34 and STRO-1, and could be considered the SBP-DPSCs subpopulation isolated by Laino *et al.*(2005).

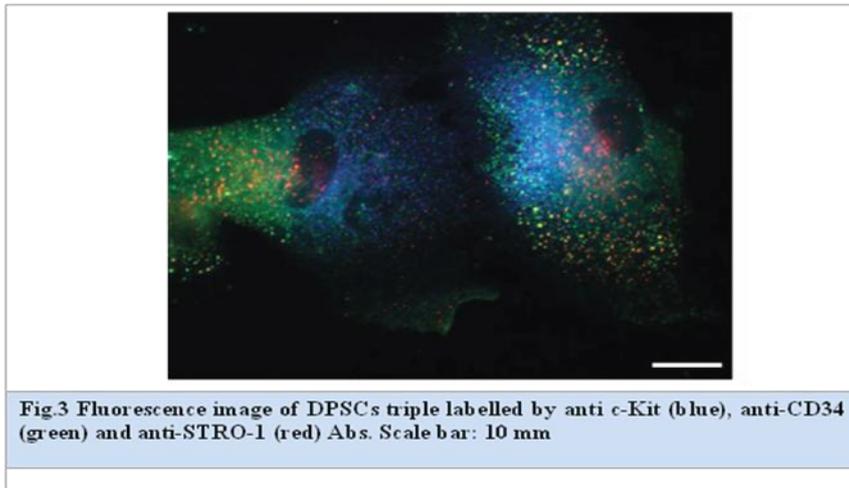
To improve the percentage of cells expressing c-Kit, CD34 and STRO-1 markers, immunoselection protocol was subsequently used. The use of MACS® technology to further characterize DPSCs, was restrictive because of the rarity of these cells, obtained after processing (approximately  $10^5$  cells per pulp sample). To verify if positive-sorted cells were c-Kit<sup>+</sup>, CD34<sup>+</sup> and STRO-1<sup>+</sup>, flow citometry and triple immunofluorescence experiments were performed by using specific antibodies.

Flow citometry demonstrated that MACS® sorting improves the percentage of CD34<sup>+</sup> (plus 20%) and c-Kit<sup>+</sup> ( plus 34%) cells (Fig. 2).



### Immunofluorescence analysis

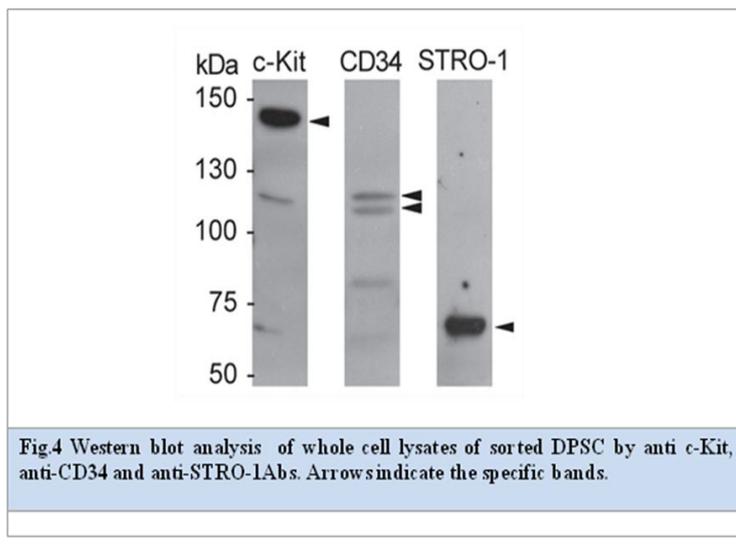
Immunofluorescence profile of the sorted cells was done, detecting specific antigens, to show that the most of the sorted cells were simultaneously positive to the three antigens



used for the sorting (Fig. 3). Each positive cell showed a diffuse labeling on the plasma membrane, with the three signals

appearing mostly as separate pixels and without overlap or co-localization: blue for c-Kit, green for cD34 and red for STRO-1. Only a small number of spots showed the co-presence of two signals probably due to clusterization of receptors (Fig. 3 the yellow/orange signal).

### Western blot analysis



To confirm the Ab specificity, whole cell lysate of sorted cells were probed for c-Kit, CD34 and STRO-1. The immunological results described, more precisely, the identity of the selected cells,

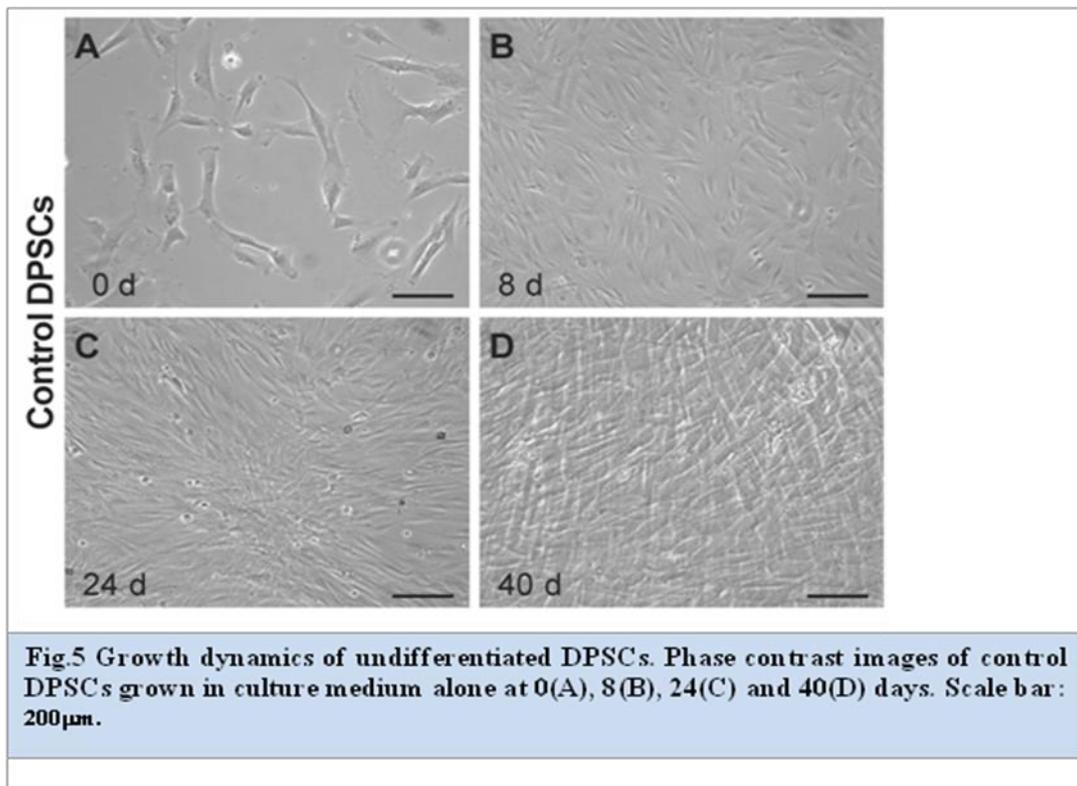
and was confirmed by WB experiments (Fig. 4).

### **Osteogenic differentiation on 2D surface**

In order to obtain osteogenic differentiation, DPSCs were plated on a plastic 2D surface, at the density of 3000 cell/cm<sup>2</sup> and then cultured in osteogenic medium for up to 40 days. Osteogenic differentiation was evaluated by morphological, biochemical and immunocytochemical methods. DPSCs treated with culture medium alone represented control cells. The differentiating cultures and control cells were observed by phase contrast microscopy to evaluate the morphological changes and the proliferation rate (Fig. 5).

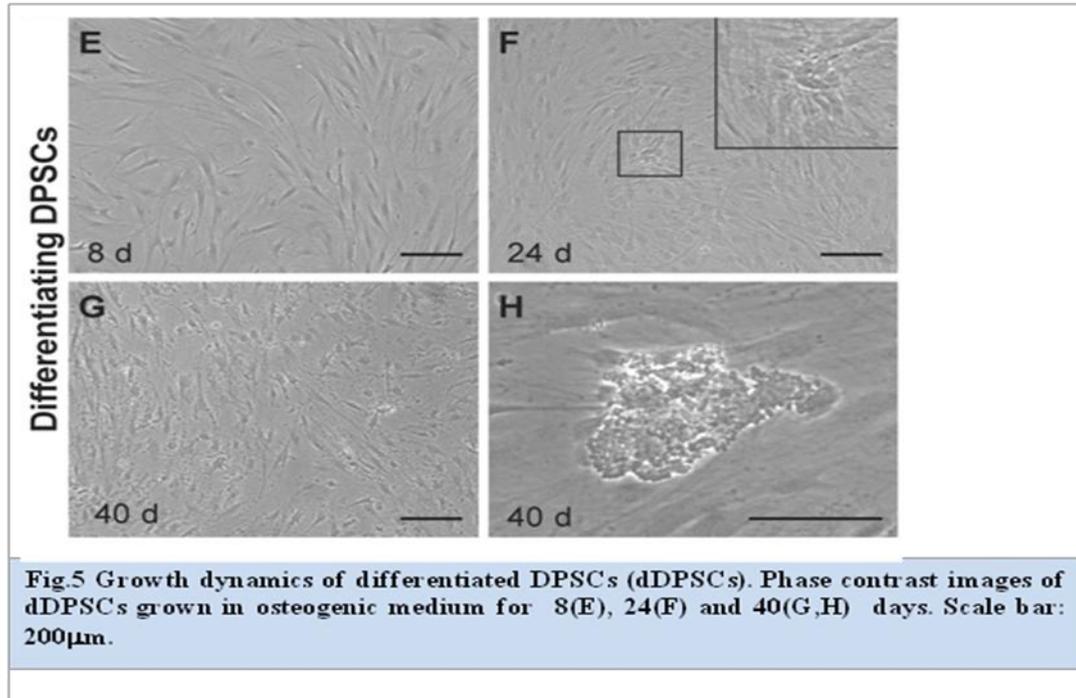
In monolayer culture, seeded cells showed a fibroblast-like morphology (fig.5A).

The cells proliferated and reached the confluence at day 8 in both differentiating and



control cultures (Fig. 5 E, B).

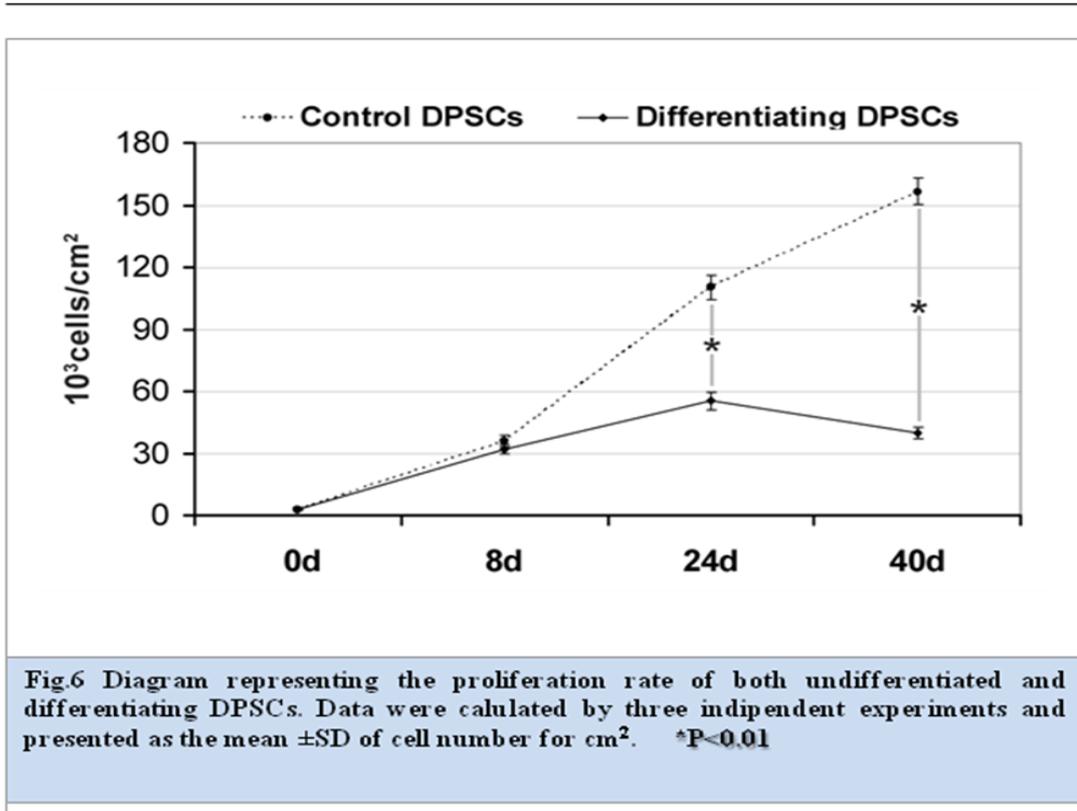
At this time, dDPSCs showed a lower cell density and presented a cytoplasm more extended (Fig. 5E). After day 8 control cells proliferated actively and formed



overlapped layers that become evident at day 24 and 40 (Fig. 5 C, D). On the contrary, differentiated cells were characterized by a lower proliferation activity and started to form nodular aggregates at day 24 (Fig. 5 F and inset). At day 40, in differentiated cultures, mineral deposits appeared in the extracellular space and in nodular aggregates (Fig. 5 G, H).

Cell counting indicated that differentiating cells presented the same high levels of proliferation, similar to that of control DPSCs, until day 8. Then undifferentiated DPSCs continued to grow exponentially and at day 40 reached a density of over 150.000 cells/cm<sup>2</sup>. On the contrary, differentiating DPSCs showed a lower proliferation

activity until day 24. After this time the number of dDPSCs were slightly decreased (Fig. 6).



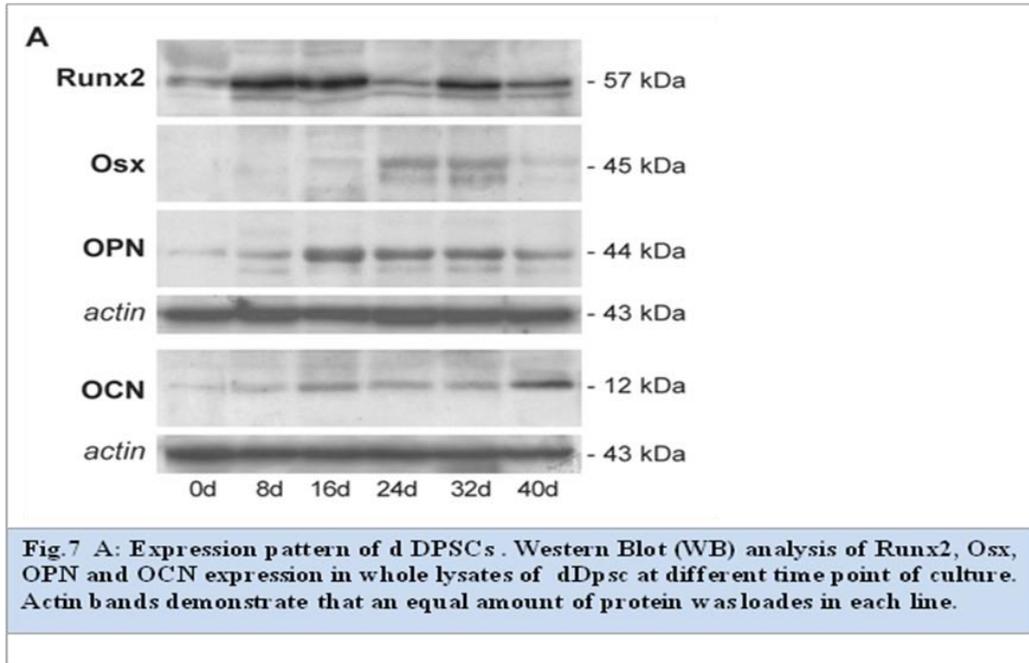
### Western blot analysis

To verify the commitment and differentiation of DPSCs into osteoblast-like cells, the presence of specific markers, such as Runx2, Osx, OPN and OCN, was analyzed by WB in whole lysates of dDPSCs, at different differentiation-time points (Fig 7 A).

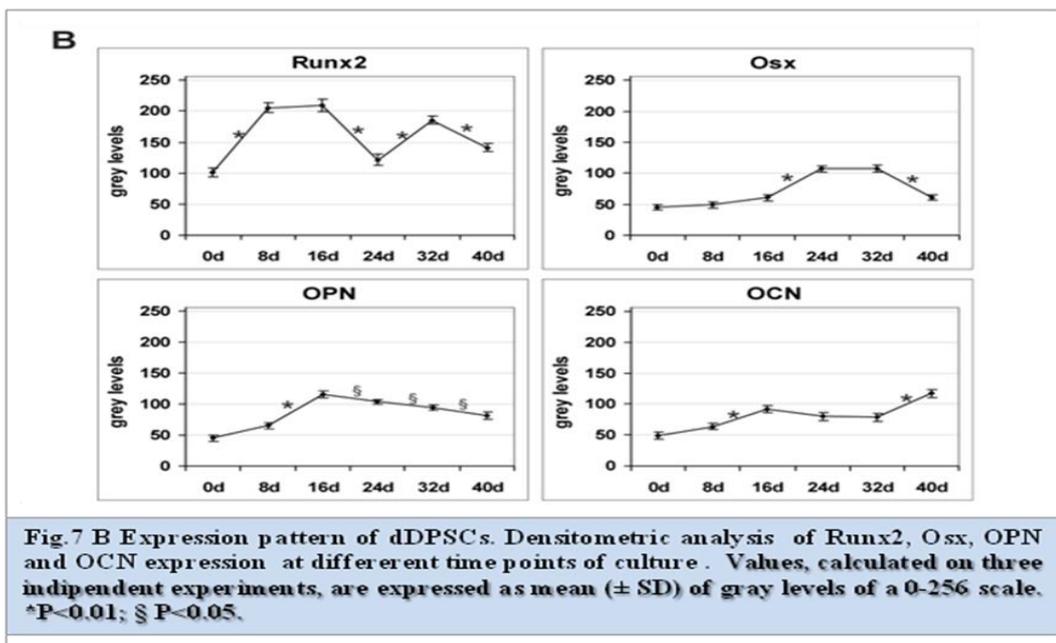
A densitometric analysis was carried out on the WB bands to obtain a semi-quantitative analysis of the protein amount (Fig. 7 B).

**Runx2** was detectable in all the experimental time points, but presented significant expression peaks. The two major peaks occurred at days 8-16, indicating the starting of DPSCs differentiation, and the third peak was evident at day 32. Runx2 amount

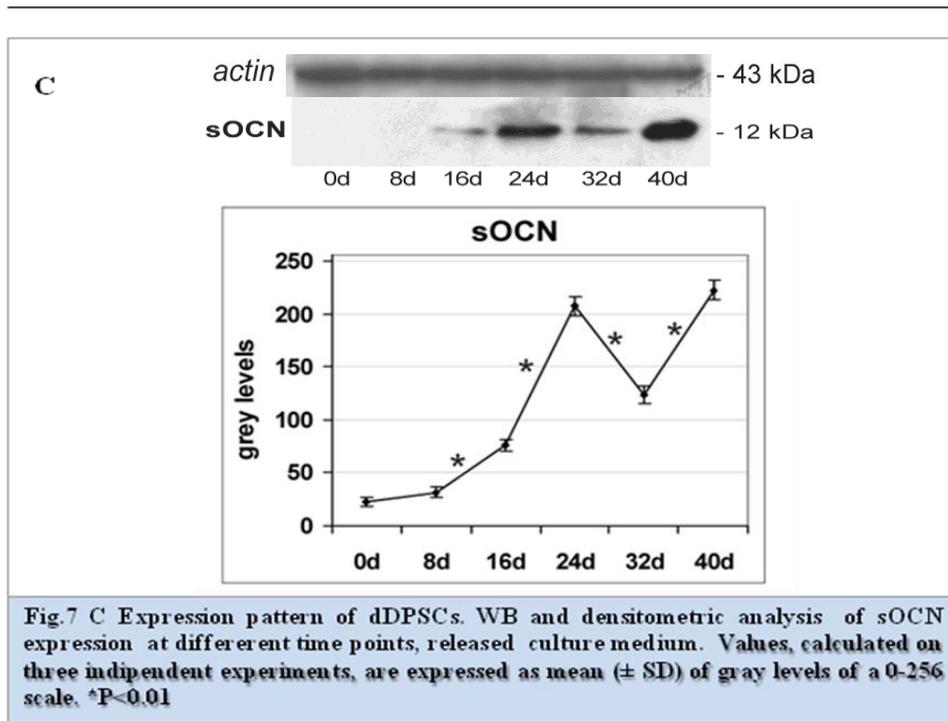
decreased at days 24 and 40 suggesting a down-regulation of this protein. **Osx** was



weakly present at day 16, then it increased, showing two expression peaks at day 24-32, while at days 40 the protein level slightly decreased. The expression of **OPN** was evident from day 8 to the end of the experiment, reaching an expression peak at day 16



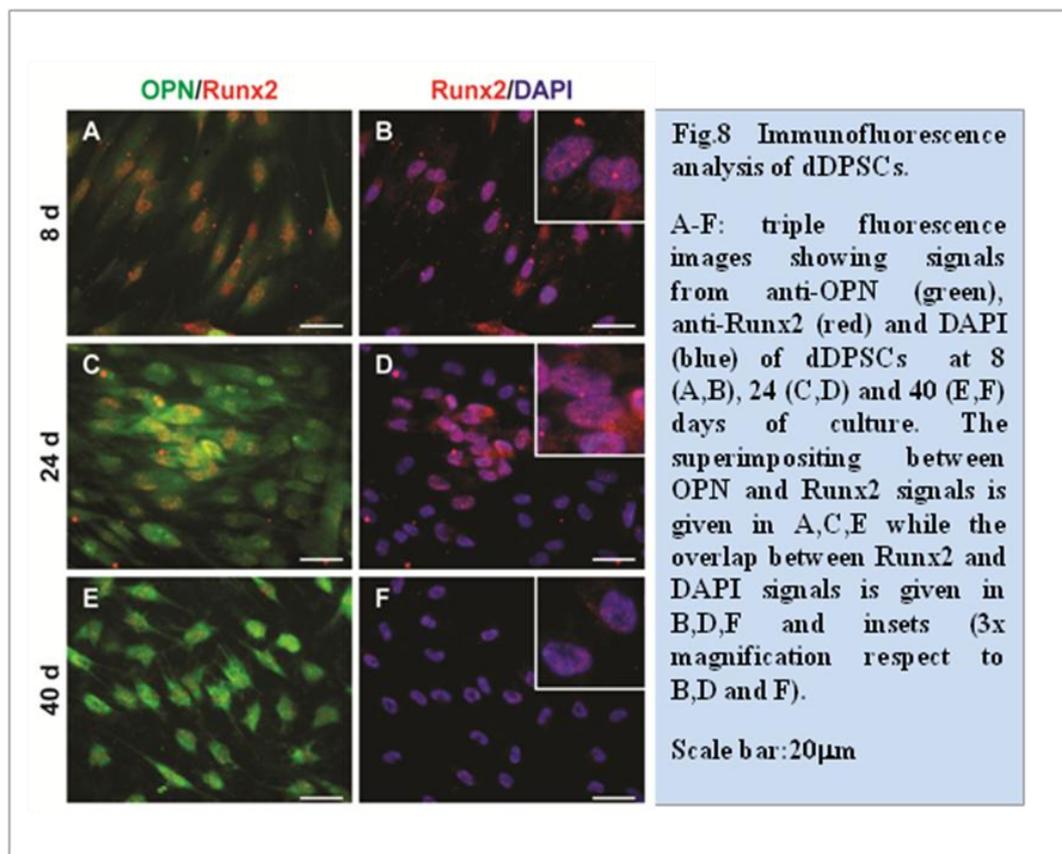
and decreasing progressively until day 40. **OCN** was detectable during DPSC osteogenic differentiation, both in cell lysates and in culture medium, as secreted protein (**sOCN**). This protein was significantly expressed in whole lysates from day 16, increased at day 24, then decreased at day 32 and reached the highest level at day 40 (Fig. 7C).



### Immunofluorescence analysis

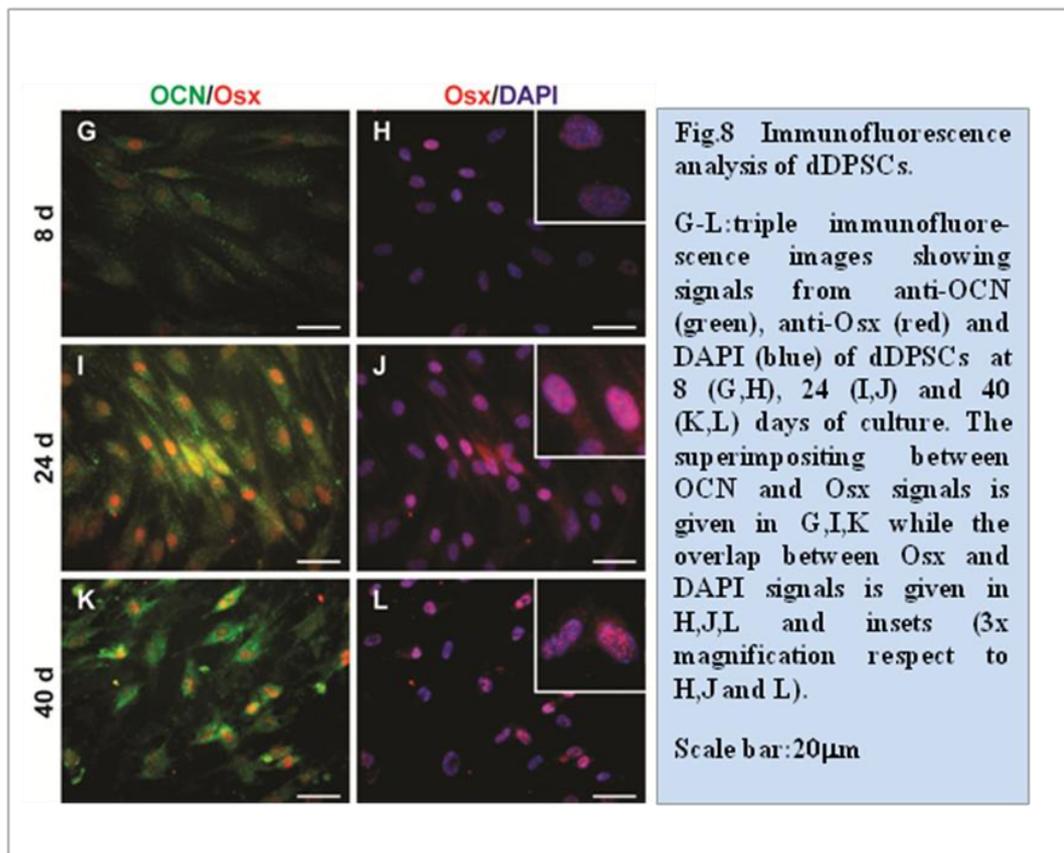
Western blot results were confirmed by immunofluorescence analysis. Double immunofluorescence labeling was carried out to analyze simultaneously the localization of OPN and Runx2 in dDPSC (Fig. 8 A, F). The distribution of OCN and Osx (Fig. 8 G, L) was observed in the same manner. During DPSCs osteogenic differentiation, OPN was localized in the cytoplasm, above all in the perinuclear region that normally contains the RER (Fig. 8 A, C, E; green signal). Runx2 was localized in the cell

nucleus, as demonstrated by the overlapping with DAPI signal, and the nucleoplasmatic distribution typical of transcription factors could be observed (Fig. 8 B, D, F and insets; DAPI, blue signal; Runx2, red signal). At day 8 it was present approximately in all nuclei, while at day 24, when a strong decrease was observed by WB, it was mainly expressed in nodular aggregates, and nuclei of manolayered cells showed a lower labeling. At day 40 Runx2 appeared heterogeneously expressed at low levels only by some cells (Fig. 8 A, F; red signal). Immunofluorescence analysis using anti-OCN and



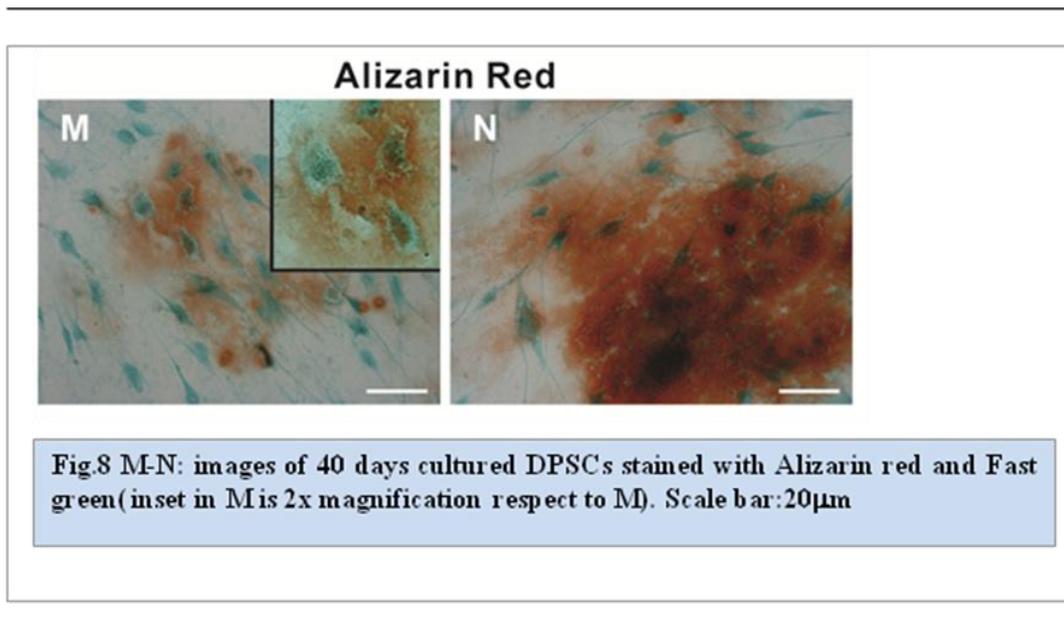
anti-Osx also confirmed WB data. OCN showed typical cytoplasmatic localization with a pattern compatible with the distribution of RER (Fig. 8 G, I, K, green signal) and, as well as Runx2, showed a nucleoplasmatic localization (Fig. 8 H, J, L). It was interesting to note that the signals from the two proteins increased significantly at days 24 and 40

(Fig. 8 I, L) and that nodular aggregates were more labeled than cells of surrounding monolayer (Fig. 8 I,J ). At day 40 we observed a significant change of cell morphology with evident cellular processes typical of differentiating osteoblasts (Fig. 8 E, K).



### Histochemical analysis

In order to verify if dDPSCs were able to produce calcified matrix, cells were stained with Alizarin red and counterstained with fast green. The presence of calcium deposits became evident after 40 days of culture (Fig. 8 M, N), when nodular aggregates appeared stained in red/orange. It was interesting to note that cells entrapped in calcium deposits showed an osteocyte-like morphology (Fig. 8 M and inset).

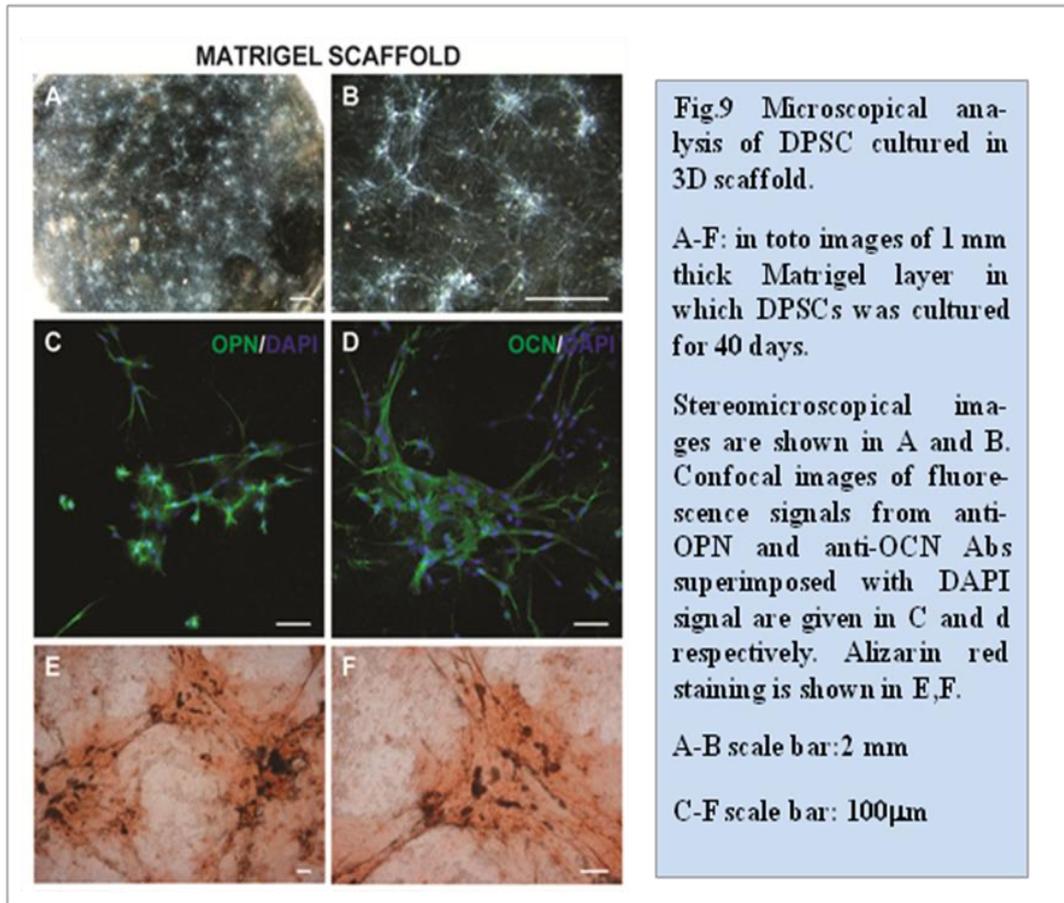


### **Osteogenic differentiation on 3D scaffolds**

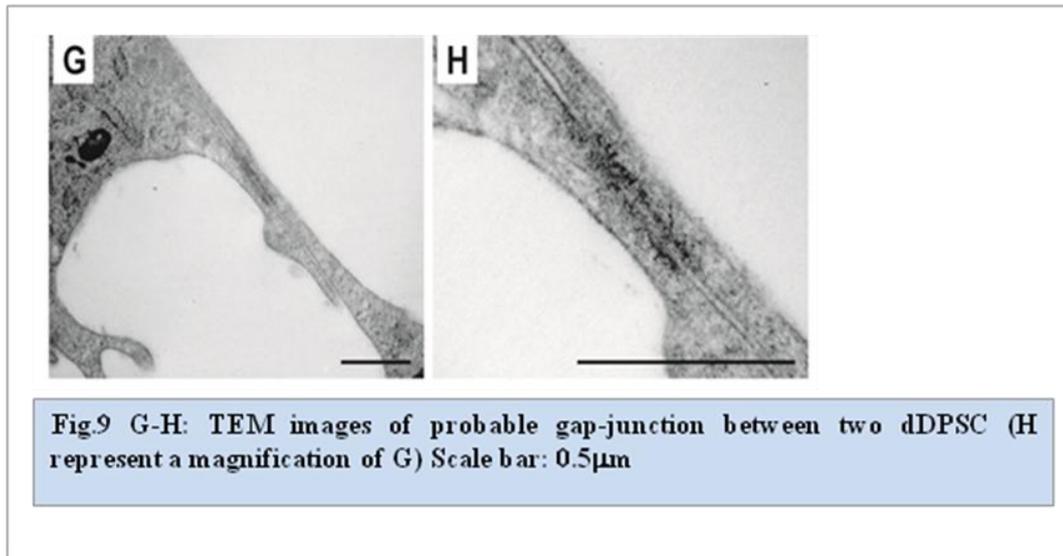
To evaluate DPSCs differentiation in 3D scaffolds, cells were cultured and differentiated in two commercial devices: i) BD Matrigel™, a solubilized extracellular matrix preparation extracted from Engelbreth-Holm-Swarm (EHS) mouse sarcoma; ii) Collagen Sponge, a biomaterial made of insoluble equine type I collagen, already used in clinical practice as scaffold to support bone regeneration. Cells were seeded in both scaffolds at the density of 1000 cell/mm<sup>3</sup> and treated for osteogenic differentiation for up to 40 days. Cell growth and osteogenic differentiation were evaluated at day 40 by histological, immunocytochemical and ultra-structural methods.

## Matrigel™

DPSCs seeded into Matrigel appeared distributed in all the volume of the sample. They



grew actively during the first week and formed numerous cellular aggregates distributed homogenously throughout the culture plate (Fig. 9 A). Each cellular aggregate, composed of cells with spindly-type morphology, showed a core with a higher cell density than the marginal area. The peripheral cells were in contact with cells of the near aggregates and probably interacted, forming a cellular network (Fig. 9 B).

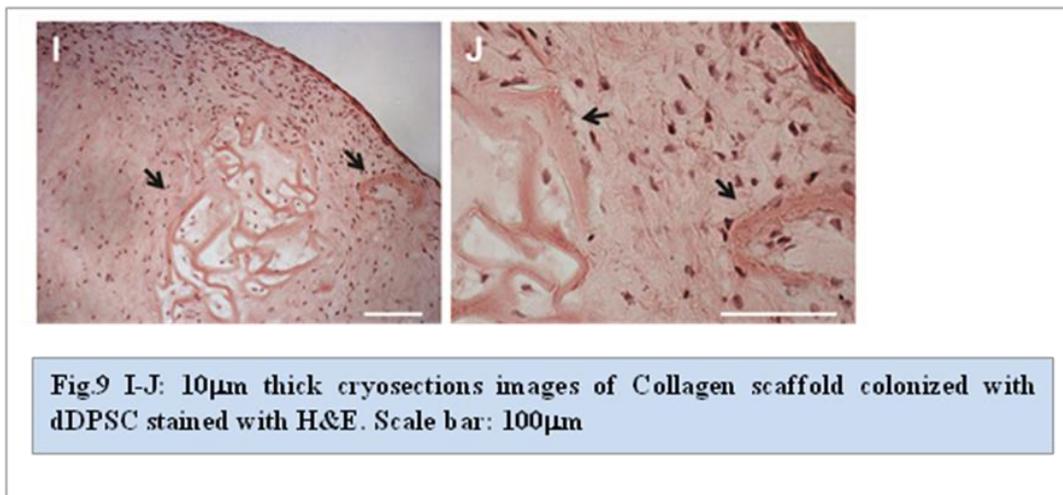


Confocal analysis of immunofluorescence experiments with anti-OPN and anti-OCN Abs showed that cells of nodular aggregates expressed the two proteins after 40 days of culture (Fig. 9 C, D) indicating that DPSCs were differentiating in osteogenic lineage. The Alizarin red staining of 40-day samples showed that nodular aggregates contain calcium deposits localized in the matrix close to the cells (Fig. 9 G, H).

TEM analysis of dDPSCs, cultured in Matrigel™, demonstrated that peripheral cells of nodular aggregates were connected by junctions probably classifiable as gap-type (Fig. 9 G, H).

## Collagen Sponge

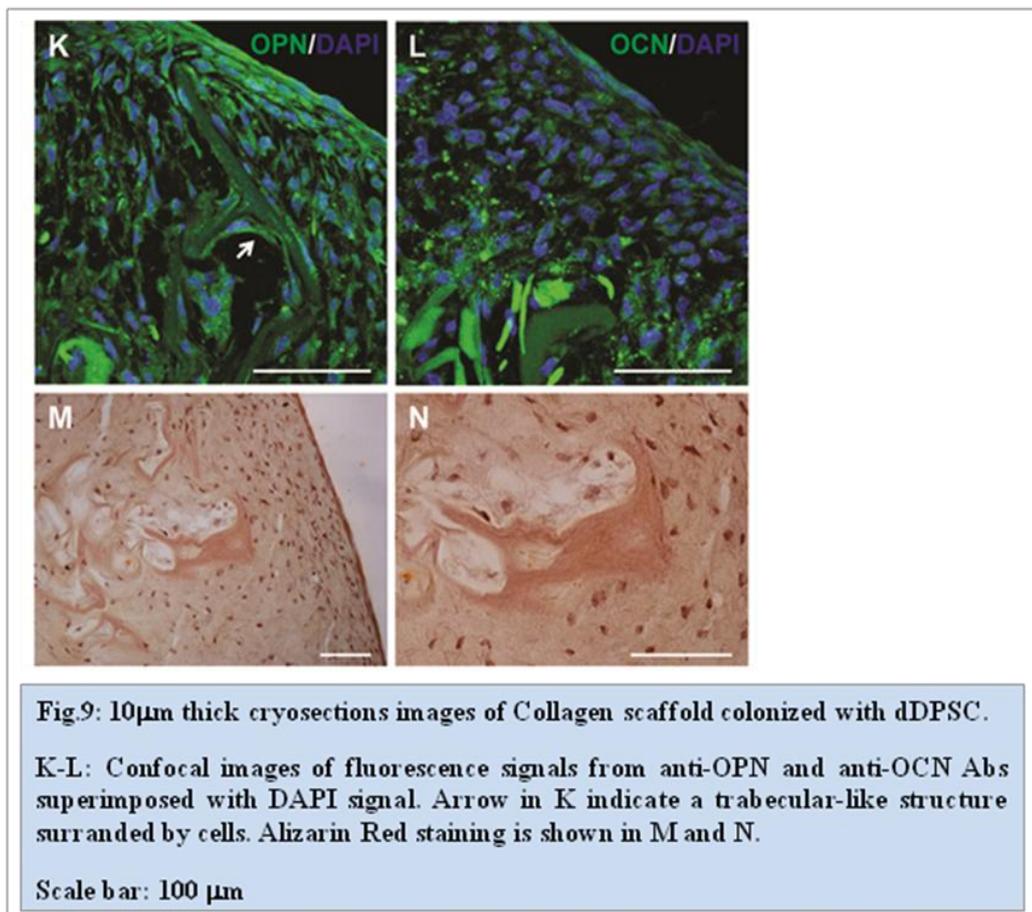
H&E staining showed that DPSCs colonized collagen scaffold, penetrating into the sponge and proliferating three-dimensionally. Cells were not homogeneously distributed in the sponge thickness, showing a higher cell density in the superficial layer, that decreased progressively towards the centre of the sponge (Fig. 9 I). Superficial cells had a flattened morphology and formed a thin compact layer of 4-5 cells. Cell morphology changed from the surface to the inner center of the sponge and became progressively spindle-type with cellular process typical of differentiating osteoblasts (Fig. 9 J). In the



sponge aggregates of collagen fibers were visible, surrounded by cells resembling trabecular-like structures (Fig. 9 I, J arrows).

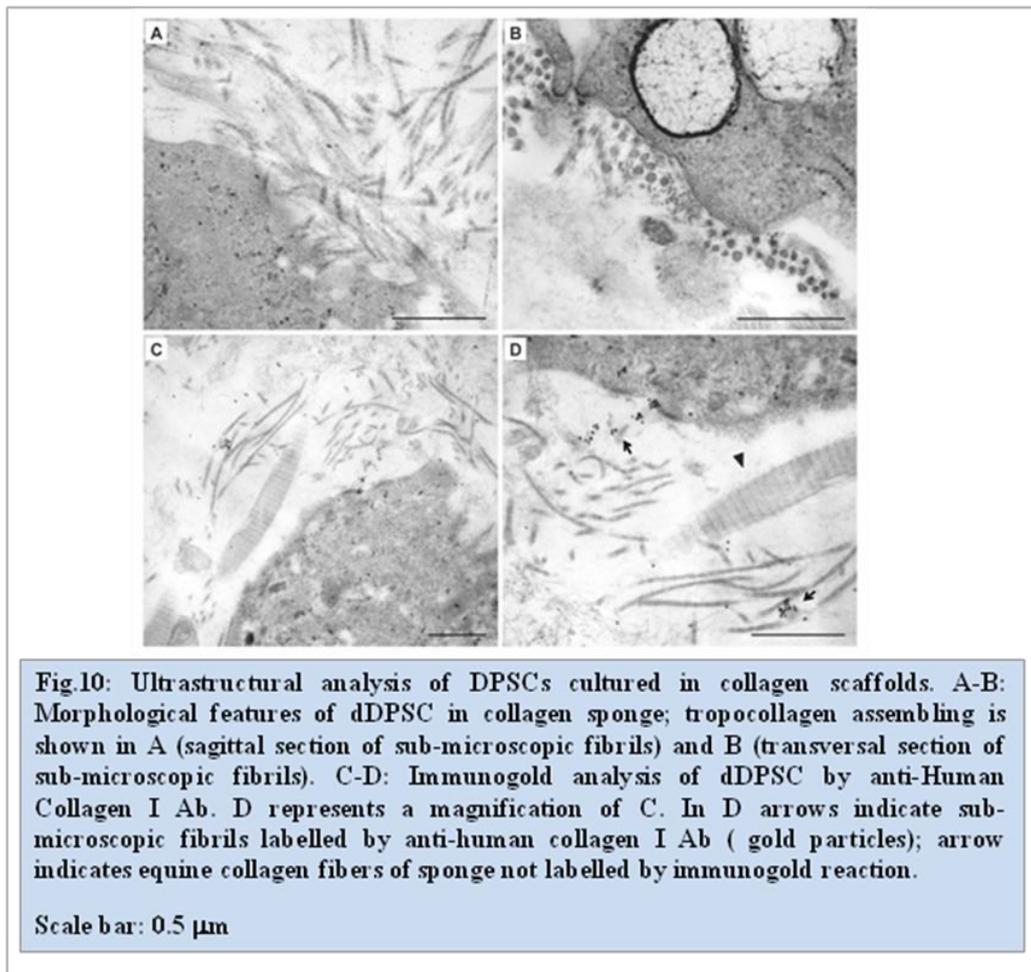
Immunocytochemical evaluation of osteogenic differentiation showed that dDPSCs growing in collagen sponge expressed both OPN and OCN osteogenic markers. OPN was strongly expressed by DPSCs localized in superficial layers and was less present in the inner ones (Fig. 9 K). Cells positive to OPN Ab were strictly adherent to collagen fibers associated in trabecular-like structures, as also observed by H&E staining

(arrows). DPSCs, localized in inner layers or near the trabecular-like structure, presented an osteoblast-like morphology and expressed higher levels of OCN, with respect to superficial cells (Fig. 9 L). Alizarin red staining showed that calcium deposits were present in the whole sponge thickness and much more in trabecular-like structures (Fig. 9 M, N).



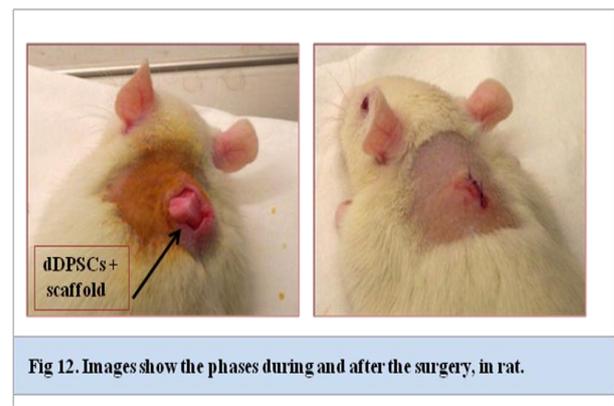
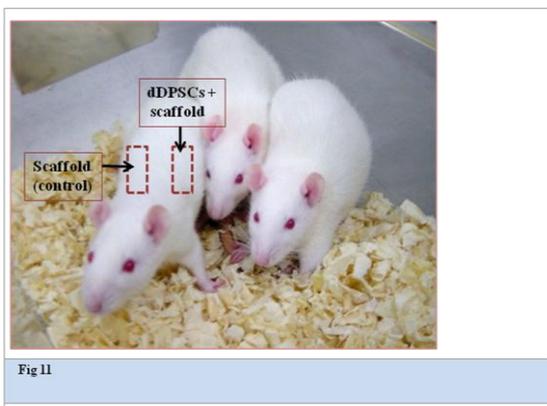
TEM analysis of dDPSC, cultured in the collagen sponge, demonstrated that cells were inter-connected by gap-junctions as described for Matrigel (data not shown). The assembling of neo-synthesized tropo-collagen molecules in collagen sub-microscopic

fibrils, was well visible both in sagittal and in transversal sections (Fig.10 A, B). To verify if sub-microscopic fibrils were effectively secreted by dDPSC, immunogold experiments with anti Human Collagen type I Ab were carried out. This Ab recognize collagen I of human origin but it does not react with the equine type I collagen of the sponge (Fig.10 C, D). Immunogold experiments demonstrated that sub-microscopic fibrils were clearly labeled by anti-Human collagen I Ab (Fig.10 D arrows). On the opposite, equine collagen fibers of the sponge were not labeled by anti Human collagen I Ab (Fig.10 D, arrowhead).

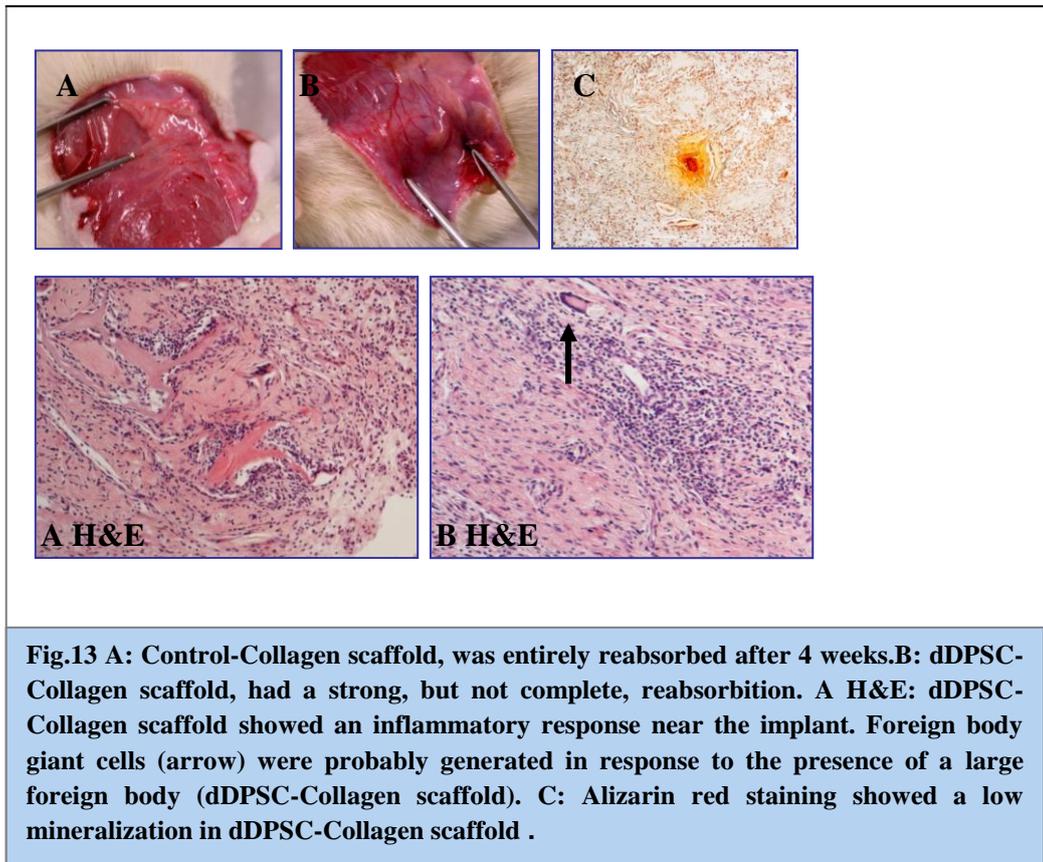


## Osteogenic Differentiation on 3D surface for ectopic implant

In order to study the bone forming ability of hDPSCs on collagen, fibroin and P(d,l)LA scaffolds, ectopic implants were performed following the protocol previously described. Each scaffold (without cells, as control, and with dDPSCs), was tested in three different animals (Fig. 11). Stem cells-scaffolds were induced in osteogenic medium for 7 days and were subcutaneously implanted into rats treated with *immune-suppressing agent* (Fig. 12).

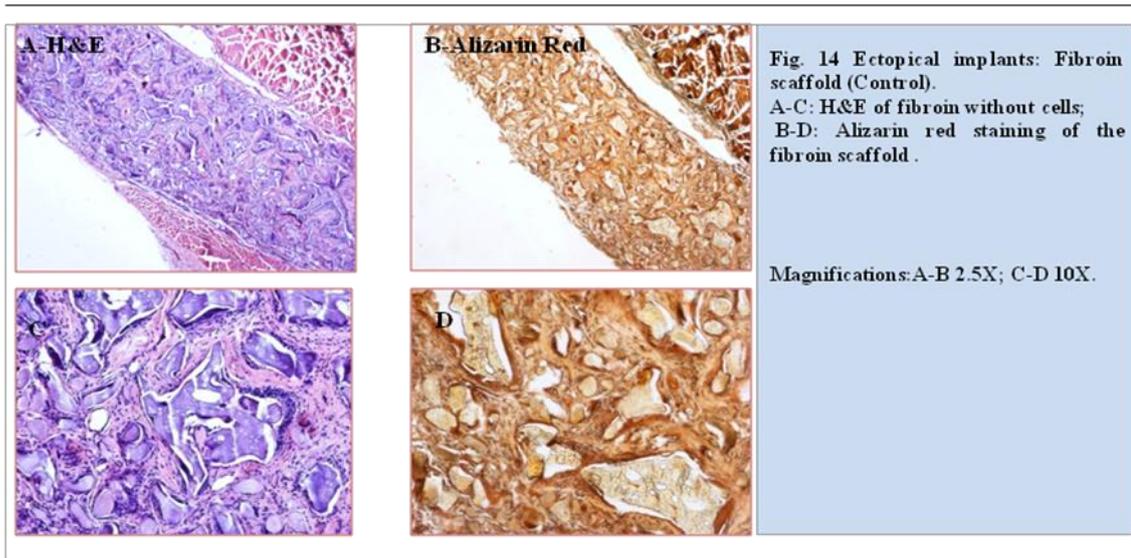


Four weeks after subcutaneous implant, control-collagen was completely and rapidly reabsorbed (Fig. 13 A), while dDPSCs-collagen showed a strong reabsorption, with a inflammatory response near the implant (Fig. 13 B, A H&E).

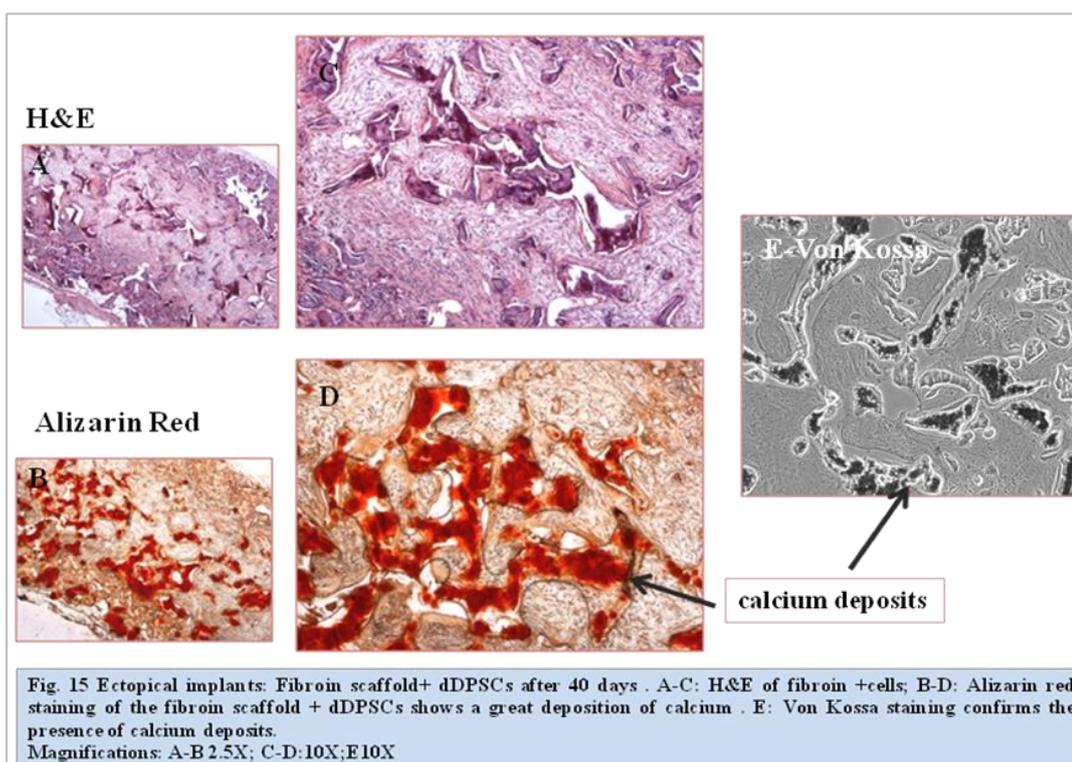


The Alizarin red staining scaffold showed a low mineralization (Fig. 13 C) of the subcutaneous implant. The inflammatory response against the dDPSC-collagen construct was also characterized by the presence of foreign-body giant cells, as shown in Fig. 13 B ( H&E).

After 40days, the implant of fibroin scaffold alone (control sample), did not present mineralized matrix (Fig. 14 B-D), while a significant presence of cells was found near the scaffold (Fig.14 A –C).

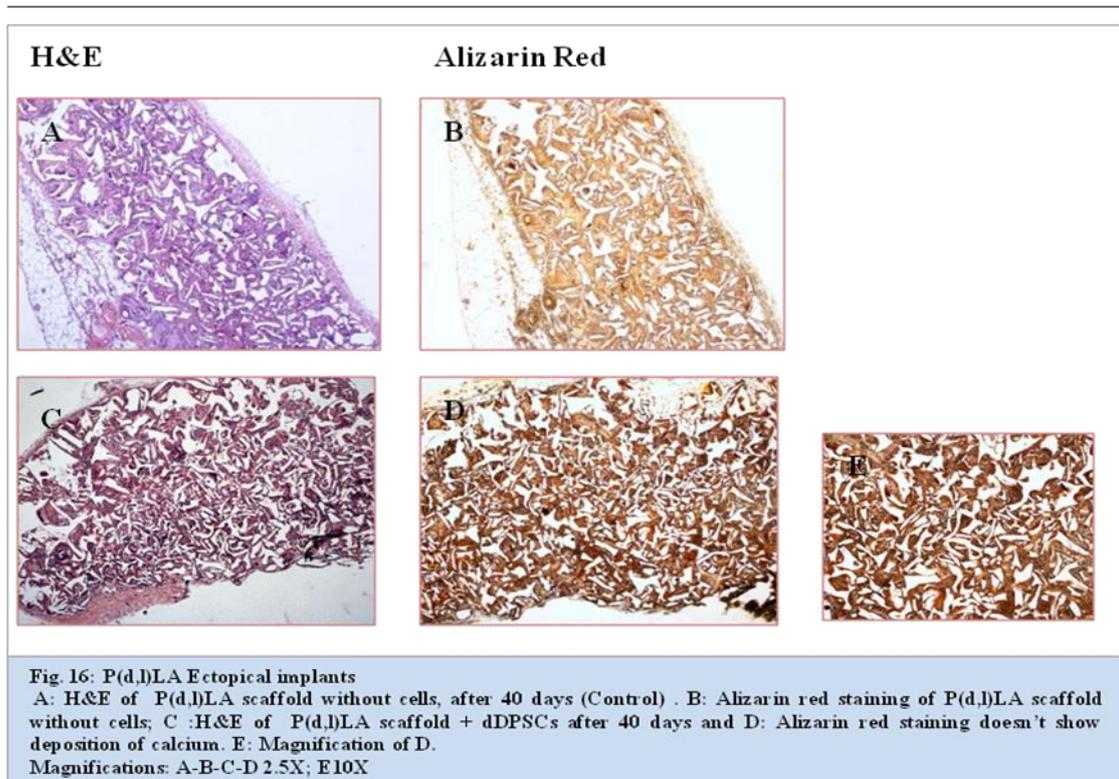


A significant ectopic calcium deposition was found in dDPSC/fibroin construct, as showed by Alizarin staining (Fig. 15 B, D) and by Von Kossa staining (Fig. 15 E). Ematossilin/eosin (H&E) staining showed mineral deposition inside the scaffold area (Fig. 15 A, C), indicating that fibroin was a suitable scaffold for bone regeneration by stem cells.



On the other hand, mineralization in P(d,l)LA scaffolds did not occur (Fig. 16), which was confirmed by Alizarin staining for the determination of calcium contents in the scaffolds, both in the control-scaffold as in the hDPSC/P(d,l)LA scaffold after 40 days of implant ( Fig. 16 B, D, E).

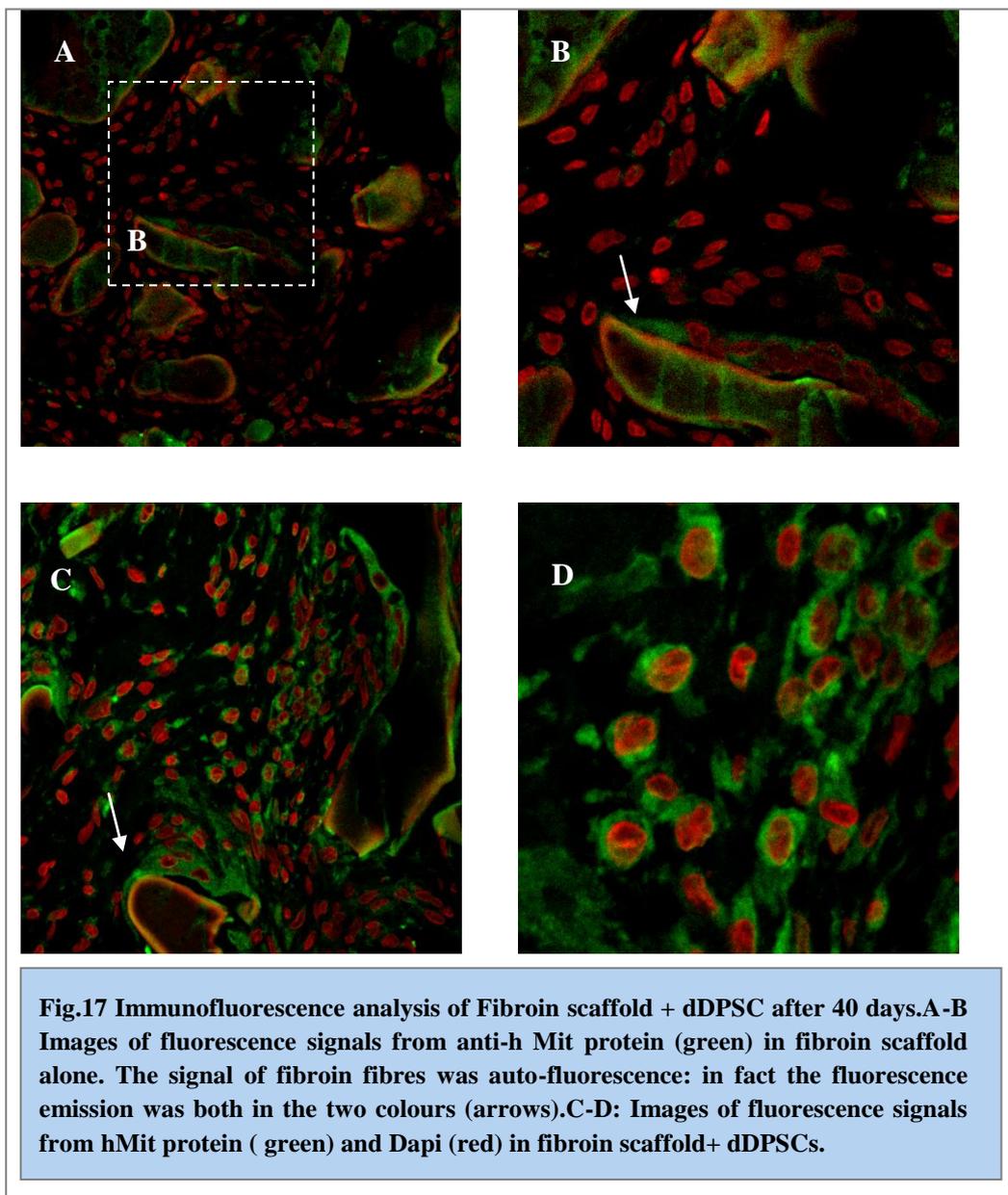
Therefore, the *in vivo* data showed that fibroin scaffolds provided dDPSCs with a more favourable microenvironment for their osteogenic differentiation and bone formation than P(d,l)LA scaffolds did.



To check if cells inside the scaffold were still from human origin and not from the host, immunofluorescence experiments with anti Human mitochondria Ab were carried out. This Ab only recognizes mitochondrial protein from human origin. Data demonstrated that most of osteoblast-like cells were clearly labelled by anti Human mitochondria Ab

(Fig. 17 C, D), indicating that most of the stem cells remain inside fibroin construct subcutaneously implanted up to the end of the experiment.

Cells not positive for the Human mitochondria Ab were host cells that probably participated to the osteogenic-process, creating a network between dDPSCS/scaffold and the regeneration of the bone defect.



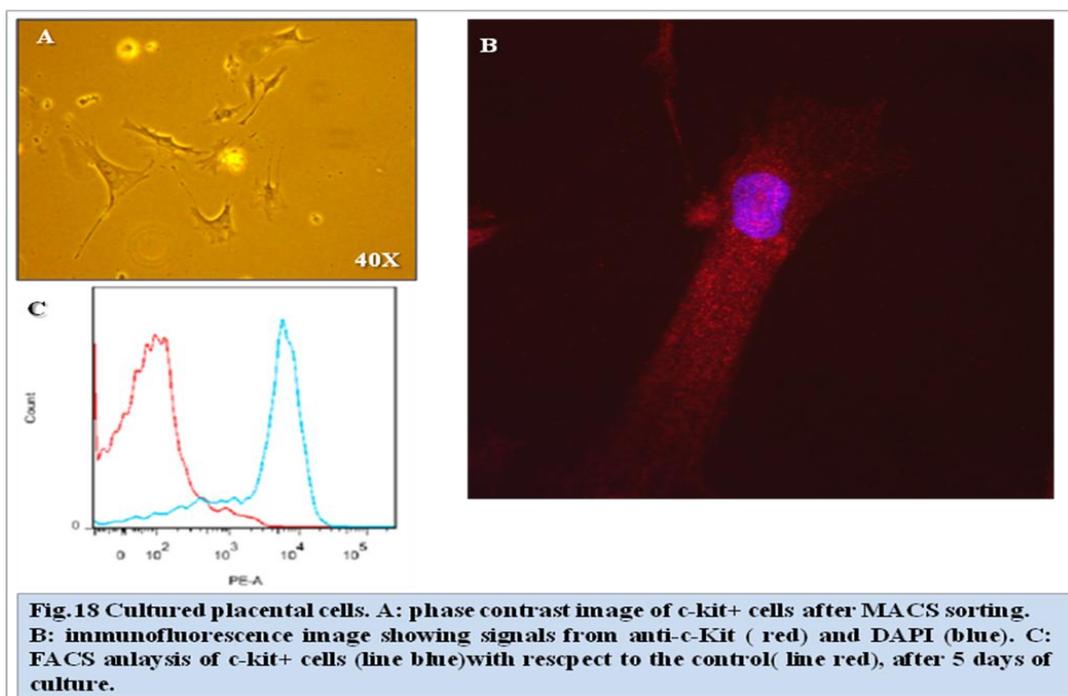
## Placenta

### Placenta: c-Kit<sup>+</sup> cells isolation

The cells, obtained from term placenta by enzymatic digestion, were cultured for about 10 days in culture medium, as described in M&M, to reach an appropriate amount of cells for the next steps. Cells expressing c-Kit were selected by MACS™ technology, to isolate c-Kit<sup>+</sup> cells from the whole population.

To verify if sorted cells were c-Kit<sup>+</sup>, FACS analysis and immunofluorescence experiments were performed on selected cells (Fig. 18).

Sorted cells were observed by phase contrast microscopy to evaluate their morphology: seeded cells showed a fibroblast-like morphology, as previously described for DPSC. Immunofluorescence analysis showed that most of the sorted cells were c-Kit<sup>+</sup> (Fig.18 B red signal, DAPI: blue signal). Each positive cell showed diffuse labeling on the plasma

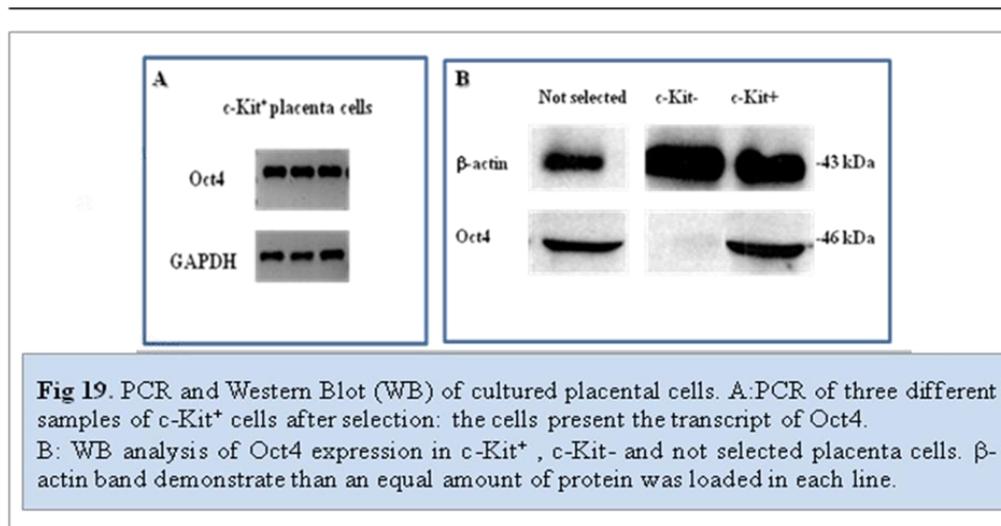


membrane. FACS analysis demonstrated that almost 96.3% of the cells analyzed were

positive for c-Kit (Fig. 18 C blue line), with respect to the isotype-control (Fig. 18 C red line).

### Western Blot and PCR

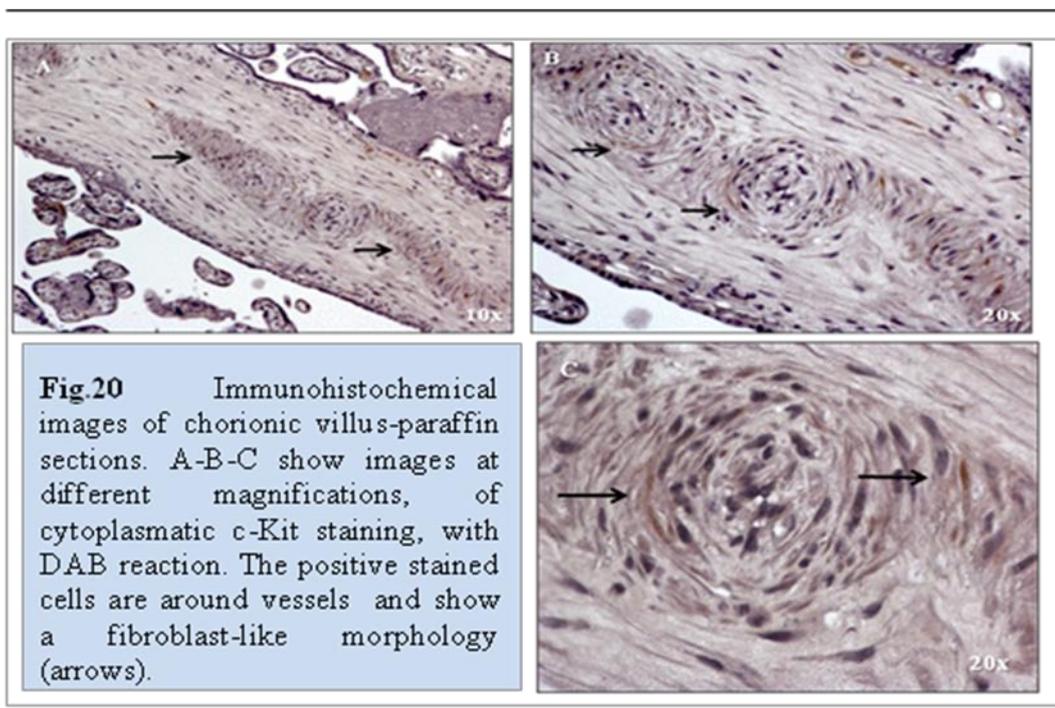
To verify the expression of the pluripotent marker Oct4 in cultured placenta-cells after MACS® selection, PCR and WB analysis were performed. The selected population, c-Kit<sup>+</sup> placenta-cells expressed Oct4 specific transcripts as determined by PCR analysis (Fig. 19 A). WB experiments (Fig.19 B) were carried out in whole cell lysates of placenta-cells not selected (no MACS® selection), c-Kit<sup>+</sup> (positive selection) and c-Kit<sup>-</sup>



(negative cells fraction after c-Kit selection). Oct4 was detectable in c-Kit<sup>+</sup> and in not-selected lysates of placenta-cells, and there was no band in c-Kit<sup>-</sup> cell-lysates. Western blot experiments confirmed the expression of Oct4 protein in placenta-cells, showing a higher presence in c-Kit<sup>+</sup> samples than in the not-selected cells.

## Immunohistochemistry analysis on paraffin sections

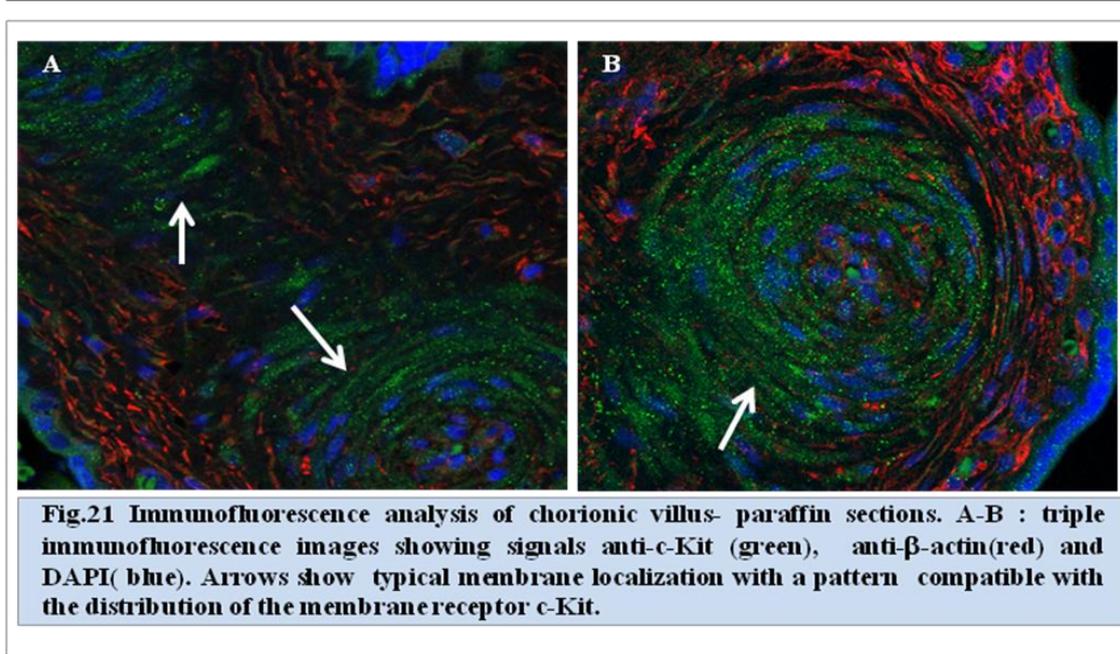
In serial sections of a placental sample, we first immune-localized the presence of c-Kit by an immunoperoxidase reaction: the antibodies were visualized via a peroxidase-catalyzed reaction, in which Peroxide/DAB were the substrate and horseradish peroxidase (HRP). C-Kit staining was cytoplasmatic with an high intensity and a specific localization in the endoplasmic reticulum (ER). C-kit positive cells were localized around the vessels, probably in the “avventizia” (Fig. 20 A, B), but no



immunoreactivity was detected into the mesenchymal cells forming the undifferentiated connective tissue in which these fetal blood vessels course through.

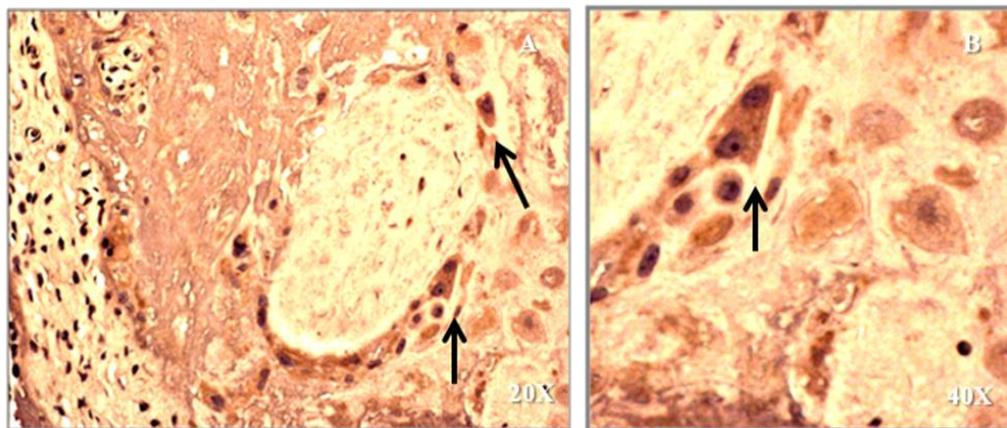
A better resolution was given by Fig. 20 C: the strong cytoplasmatic staining, that confirms the protein localization, was detected in fibroblast-like cells around vessels, in the perivascular region.

Immunohistochemical results were confirmed by immunofluorescence analysis (Fig. 21 A). Triple immunofluorescence labeling was carried out to analyze the



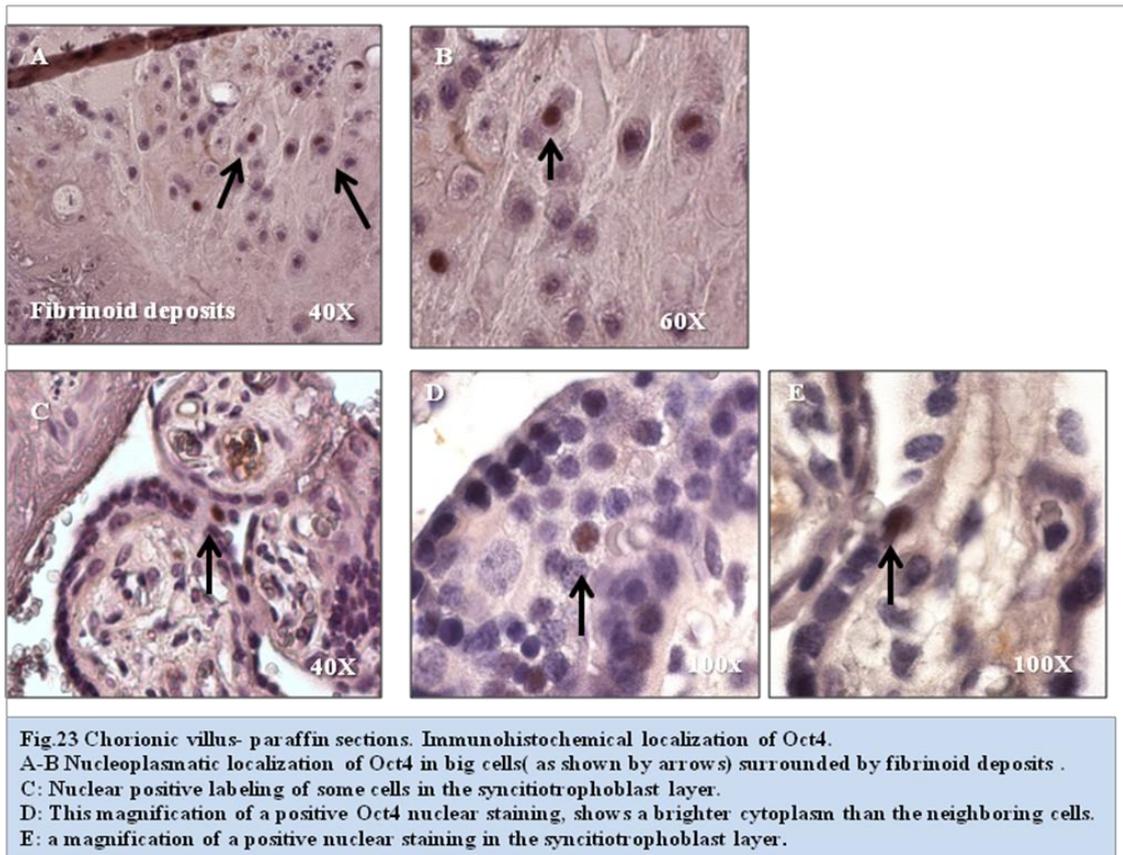
localization of c-Kit (green signal) and β-actin (red signal) in the placental slides. C-Kit<sup>+</sup> cells showed a diffuse labeling on plasma membrane and cells were localized mostly around the vessels (Fig. 21 B).

In Figures 22 A and B, the cytoplasmatic localization of c-Kit was also detected with immunohistochemical staining in big cells, characterized by a stained cytoplasm, a round morphology and a specific “peri-chorionic” localization, between the wall of an anchoring villus (cut in longitudinal section) and the fibrinoid deposits .

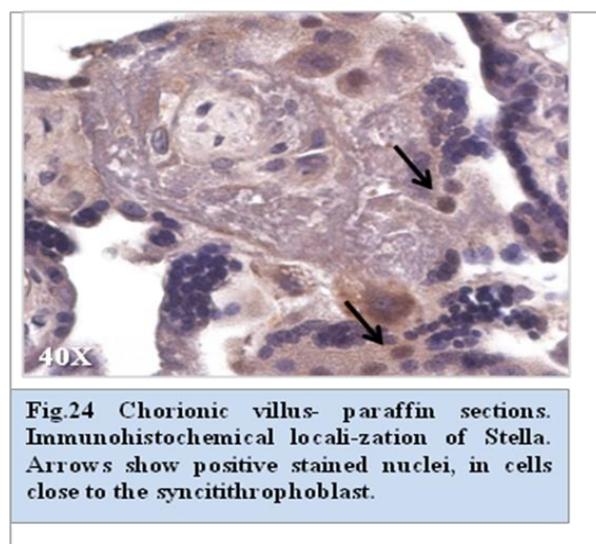


**Fig.22 Paraffin sections of chorionic villi. Immunohistochemical localization of c-Kit. A: Cytoplasmatic staining, in the RE, expressed by cells located in the interface between fibrinoid deposits and connective tissue. B: a magnification of A.**

To identify the distribution of cells positive for stem cell markers, as nuclear markers Oct4 and Stella, further immunohistochemistry analysis were performed (Fig. 23). Oct4 showed a typical nucleoplasmic localization in big cells surrounded by fibrinoid matrix: the cytoplasm of these cells was not labeled and was more extended than the cytoplasm of the neighboring cells (Fig. 23 A, B). The immunoperoxidase detection of Oct4 showed positive nuclei also in syncytiotrophoblast cells, in the floating villi (Fig. 23 C-E). The cytoplasm of Oct4<sup>+</sup> cells in the syncytiotrophoblast was brighter than the cytoplasm of the other cells (Fig. 23 D): this is a characteristic of undifferentiated cells, due to their quiescent state.



Within fetal villi, Stella positive cells were immersed in an undifferentiated connective tissue, with fetal blood vessels coursing through this region and fibrinoid deposits around the cells. Stella, a marker of pluripotent cells, was immunodetected in the nucleus of these cells located close to the syncytiotrophoblast layer (Fig. 24).



## **DISCUSSION: DPSC**

In this thesis, we analyzed the osteogenic differentiation features of DPSCs cultured on 2D surface and on 3D scaffolds *in vitro*, and the ectopic implants of the 3D scaffolds, *in vivo*. DPSCs population, isolated from dental pulp of adults' teeth that presents the c-Kit, CD34 and STRO-1 antigens, coincides with the DPSC sub-population (SBP-DPSCs) described by Laino *et al.*, (2005). According to methods previously described, osteogenic differentiation of DPSCs was obtained by treatment with dexamethasone, to stimulate cell differentiation, ascorbic acid, to stimulate the extracellular matrix synthesis and  $\beta$ -glycerolphosphate to promote ECM mineralization (Zhang W *et al.*, 2006; Karner E *et al.*, 2008). DPSCs responded to this osteogenic condition with a strong decrease of proliferation after day 8 of culture, when the confluence was reached. Afterword this, cells proliferate actively, forming overlapped layers, while dDPSCs grew as a monolayer showing typical mineral deposits and nodules as described by other authors (Laino G *et al.*, 2005). After 24 days, the density of dDPSCs showed a slightly reduction, probably due to the increase of cell death that normally occurs during late cell differentiation.

To verify if morphological observation really indicates an osteogenic differentiation, we investigated the expression of bone related proteins in dDPSCs. Recently Yu and co-workers demonstrated that STRO-1<sup>+</sup> DPSCs, after 9<sup>th</sup> passage, can only differentiate in osteogenic lineage, expressing specific markers (Yu J *et al.*, 2010). Different transcription factors and proteins typical of bone ECM were expressed during osteoblast differentiation. Only a small number of these can be used as stringent markers because they are clearly expressed in bone tissue or show typical expression peaks during

osteoblast differentiation. The DPSCs osteogenic differentiation observed in our experiments can be distinguished in two phases on the basis of expression of specific markers as RUNX2, *Osx*, OPN and OCN. The first phase, from day 0 to day 16, was characterized by a strong expression of Runx2 coupled with a greater presence of OPN. Runx2, actually expressed in undifferentiated cells, showed a considerable increase between 8 and 16 days, that coincided with the proliferation decrease and presumably with the starting of a late differentiation phases. OPN showed its maximum expression level at day 16 in coincidence with Runx2 peak and then decreased progressively to the end of culture. These observations indicated that this phase represents an early stage of osteogenic differentiation according to data that describe high levels of Runx2 and OPN in osteoblast proliferation (Pratap J *et al.*, 2003). In the second phase, from day 16 to day 40, dDPSCs showed a low proliferation level and expressed a higher amount of *Osx* maintained until day 32. The increase of *Osx* occurring after Runx2 peak is consistent with data describing *Osx* positively governed by Runx2 during osteoblast differentiation (Marie PJ, 2008 ; Komori T , 2006). In this phase, the regular increase of intracellular OCN levels and the strong presence of sOCN in culture medium indicated that dDPSC underwent a late differentiation phase characterized by an ECM maturation process. Previous data indicated that OCN is the most specific marker of late osteoblast differentiation because it is synthesized only by mature osteoblasts at the end of matrix maturation process (Sila-Asna M *et al.*, 2007; Valenti MT *et al.*, 2008). The OPN decrease observed in this phase was supported by observation demonstrating the presence of OPN during osteoblast differentiation and its progressive down regulation according to its role of mineralization inhibitor (Karner E *et al.*, 2008; Hunter GK *et al.*,

1996). In our model, after the induction started with the first Runx2 peak, others osteogenic inductions could be promoted by following Runx2 peaks in order to maintain an immature sub-population of dDPSCs that underwent further terminal differentiation events. On the other hand, immunofluorescence data indicated that the decrease of Runx2, observed by WB at day 24, occurred in the majority of dDPSCs while its expression was maintained in cells of nodular aggregates. On the contrary, *Osx* and *OCN* at day 24 were expressed both in nodular aggregates and in monolayer cells. Successively their presence characterized almost the entire cell population: this data suggested that nodular aggregates were contemporaneously composed of a sub-population of immature dDPSC and by a dDPSC population that progressively underwent to terminal differentiation. The change in cell morphology at day 40, characterized by thin and long cellular processes, indicated that dDPSCs were in a very late differentiation phase. Furthermore, Alizarin red staining demonstrated that calcified ECM was produced and dDPSCs with an osteocyte-like morphology were entrapped by this.

An optimal bone formation requires that osteoblasts grow and differentiate in this three-dimensional (3D) environment, allowing the formation of an intercellular network. Previous studies indicated that osteogenic differentiation is improved by culture in 3D scaffolds that mime the physiological condition (Boukhechba F. *et al.*, 2009; Ferrera D. *et al.*, 2002). Biomaterials play central roles as designable biophysical and biochemical milieus that direct cellular behaviour and function in regenerative medicine and tissue engineering (Langer and Vacanti, 1993; Lutolf and Hubbel, 2005). Calcified tissue can be produced *in vitro* using bone marrow MSC cultured on collagen sponge or similar

bio-scaffolds, already utilized in surgery to support and improve bone regeneration (Gigante A *et al.*, 2008; Zavan B *et al.*, 2007; Donzelli E *et al.*, 2007; Jager M *et al.*, 2005). In this study we compared Matrigel™ and collagen sponge with new bioscaffolds, fibroin and P(d,l)LA, seeded with dDPSCs. Our data, obtained by culturing DPSCs on Matrigel™ and collagen sponge, indicated that both scaffolds optimize DPSCs growth and differentiation. The gelatinous structure of Matrigel™ mimics the amorphous extracellular matrix components (proteoglycans and GAG) allowing three-dimensional nodules formation. TEM analysis demonstrated that peripheral cells of these nodules were interconnected by gap junctions analogously to osteocyte network observed in bone tissue (Civitelli M, 2008; Palumbo C *et al.*, 2004). A better result was obtained with collagen sponge because DPSCs colonized this scaffold with a distribution that mimed the bone histology: cells of superficial layers were less differentiated, as demonstrates by the clear expression of OPN that decreases in the inner layer. Moreover these cells present a very flat morphology and form a thin superficial layer that can be compared with the bone periosteum. Cells with osteoblast-like morphology that express high levels of OCN (late differentiation marker) and actively neo-synthesized human collagen, were present in the inner layers, unlike superficial cells. In this region structures containing dense collagen fibres and calcified matrix that resemble bone trabeculae were observed (Riccio M *et al.*, 2010).

An *ex vivo* engagement in a specific pathway prior to transplantation may be needed to induce the differentiation into a desired cell lineage before cell transplantation therapy. As specifically described with bone marrow-derived stem cells (Dezawa M *et al.*, 2005), a good approach may be to initiate differentiation *in vitro* for a lineage-specific

pathway, allowing these cells to complete their development and acquisition of specific function *in vivo*. In our experiments, we cultured DPSCs/scaffold constructs in differentiating medium, for 7 days, before subcutaneous implant in rat. We demonstrated that mineralization of dDPSCs was more present on the fibroin scaffolds compared to collagen and P(d,l)LA scaffolds. The Alizarin Red staining results on sections of DPSCs/scaffold, after 1-month implant *in vivo*, provided more convincing evidence that fibroin scaffolds were more favourable for the osteogenic differentiation of dDPSC. The present study confirmed that the biomimetic microenvironment, provided by the fibroin scaffolds, is an important element besides the morphogenetic factors for the differentiation of the stem cells (Sun H *et al.*, 2010).

On the other hand, collagen and Matrigel scaffold, as ectopic implant, were completely absorbed, therefore this experimental approach did not give information about the employ of these scaffolds for engineering osseous grafts. P(d,l)LA is not a good support for osteogenic differentiation, as shown in the results; in fact there was no calcium deposition in the ectopic implant, probably due to its biophysical characteristics.

### **-Conclusions**

In our studies, we demonstrated that DPSCs are able to differentiate in osteogenic lineage in both 2D and 3D surfaces, creating osteoblast-like cells that express specific osteogenic markers and produce mineralized ECM. The combination with 3D bioscaffolds was performed to produce a dDPSC-bioscaffold device that can be easily inserted by surgical intervention in order to repair bone tissue deficiency.

This tissue engineering approach could be a promising tool to restore bone defects that currently are treated surgically through the application of artificial permanent implants.

In particular, the *in vitro* bone production from 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 even today resist any medical or surgical solution. In addition, DPSCs can also be used to colonize prosthesis and orthodontic implants in order to optimize the osteo-integration and the efficiency of the implant itself. To produce a large pool of cells and derived- progeny, it may be preferable to culture and expand autologous mesenchymal stem cells prior to implantation in an effort to personalize cell-based therapy and differentiate toward the necessary lineage (Nelson TJ *et al.*, 2009).

In addition, it is important to note that adult human mesenchymal stem cells, as DPSC, are characteristically devoid of human leukocyte antigen (HLA) class II antigens (MHC-II) on the cell surface, and do not express the co-stimulatory molecules CD80, CD86 or CD40 (Nelson TJ *et al.*, 2009). The expressed major histocompatibility complex (MHC) class I antigens may activate T cells, but with the absence of co-stimulatory molecules, a secondary signal would not engage, leaving T cells anergic. Moreover, DPSC exhibit immunosuppressive properties, modulating T-cell functions including cell activation, and display immunomodulatory features impairing maturation and function of dendritic cells and inhibiting human B-cell proliferation, differentiation, and chemotaxis.

Understanding the interactions between DPSCs and the immune system may be important to individualize the full potential of these cells. DPSCs immune-profile is regarded as non-immunogenic and accordingly transplantation into an allogenic host may not require immunosuppression. Otherwise, traditional heterologous

transplantation requires lifelong immunosuppression to avoid rejection. The application of DPSCs in tissue engineering may be a new strategy to limitate immunosuppressive therapy in stem cell heterologous transplantation.

#### Future prospects

- The dosage reduction of the immunosuppressive treatments necessary to prevent rejection against the DPSC/scaffold construct, due to DPSCs immunosuppressive effects.

If necessary, the immunosuppressive therapy may be limited to the period required for a resumption of normal osteogenic activity in the host cells. Autologous or heterologous DPSC transplantation may promote transplant tolerance, even without immunosuppressive treatment.

- The interactions between DPSC/scaffold and the new bone products after surgical intervention. The application of stem-colonized implant promotes tissue regeneration, also involving host cells: it may be possible that DPSCs lose their functions when specialized cells from the host start to repair the bone mass defect.

In conclusion, these aspects suggest that DPSC/scaffold construct may have an expanded clinical applicability in the future and may represent a viable therapeutic option for those who would benefit from tissue replacement or repair.

## **DISCUSSION: PLACENTA**

Human placenta, besides playing a fundamental and essential role in fetal development, nutrition and tolerance, may also represent a reserve of progenitor/stem cells. Considering the complexity of the structure of the placenta, we focused our attention on fetal membranes, which extend from the margins of the chorionic disc and enclose the fetus in the amniotic cavity with the amniotic fluid (Parolini O *et al.*, 2008). The chorionic membrane (chorion leave) consists of mesodermal and throphoblastic region: chorionic and amniotic mesoderm are similar in composition. A large and incomplete basal lamina separates chorionic mesoderm from the extravillus trophoblast cells that are dispersed within the fibrinoid layer. The Langhans fibrinoid layer usually increase during pregnancy and is composed of two different types of fibrinoid substance: a matrix type on the inner side (more compact) and a fibrin type in the outer side (more reticulate).

De Coppi (2007) recently reported that amniotic fluid stem (AFS) cells can be obtained routinely from human amniotic fluid, using backup cells from amniocentesis specimens that would otherwise be discarded. AFS cells are pluripotent stem cells capable of giving rise to multiple lineages including representatives of all three embryonic germ layers, with an hold potential for a variety of therapeutic applications.

These results did not revealed where in the amniotic membrane they were located or whether similar cells might be present in other placental tissue (i.e. chorionic membrane). Miki (2007) restricted the presence of stem cell marker-positive cells to the amniotic epithelia and concluded that they are not present in other parts of the placenta.

Our preliminary studies, to identify the microenvironment, or niche, of stem/progenitor cells in the chorionic membrane, suggested different conclusions.

The stem cells niche is a highly balanced microenvironment that allows stem cells to survive and remain quiescent and then respond by replicating, migrating and differentiating to replace or repair tissue, when needed (Castrechini NM *et al.*, 2010). The identification of the anatomic location of a stem cell niche within the chorionic membrane, and then within the chorionic villi, not only provides clues as to the nature of internal/external stimuli that regulated intracellular signals, but can also reveal additional roles for stem cells in the placental development and function. Immunohistochemical and immunofluorescence analysis on term placental sections were used to identify the stem cells localization.

#### -Characterization on placental sections

For a first characterization, a cell surface marker staining was performed with a specific antibody against c-Kit, the receptor of stem cell factor (SCF). This surface marker-receptor protein is present on human ES cells, primordial germ cells and many somatic stem cells, including, but not limited to, those of the neural crest.

Later in pregnancy, c-Kit is expressed in the cytotrophoblast cells, the intermediate trophoblast cells, Hofbauer cells, and decidual macrophages and is not expressed in the syncytiotrophoblast cells. In our samples, a considerable staining pattern was detected in fibroblast-like cells around the blood vessels. These fibroblast-like cells, as described by Castrechini (2010) are consistent with the proposed model of mesenchymal stem cells lying in, or attached to, the basement membrane opposed to the endothelial cells. Mesenchymal stem cells showed a perivascular cell phenotype both *in vitro* and *in vivo*.

A population of placenta c-Kit<sup>+</sup> cells detected in the chorionic villi is composed by pericyte-like cells: *in vivo* studies provided evidence that pericyte may act as a source of undifferentiated cells during adipose or osseous tissue repair (Diaz-Flores L *et al.*, 1992).

There may be multiple sub-populations of mesenchymal stem cell in the chorionic side of term placenta. In fact, the staining distribution showed another specific spatial location of c-Kit positive cells: a cell subpopulation, with a strong cytoplasmatic c-Kit<sup>+</sup> staining, was localized in the peri-chorionic connective tissue and was characterized by big cells surrounded by Langhans fibrinoid deposit, close to the connective tissue.

A further aim of the present study was to investigate whether cells with phenotypic and functional characteristics of pluripotent stem cells were present within human placenta (chorionic side). We detected the presence of Oct4, a transcription factor that plays a crucial role in maintaining pluripotency of embryonic stem cells. Down-regulation of Oct4 is associated with the differentiation of trophoblast cell lineage, from which the normal placenta derives. Oct4-positive cells were localized in the same region of a subpopulation of c-Kit<sup>+</sup> cells: positive-nuclei were detected in single-round cells, with an extended cytoplasm, similar to c-Kit<sup>+</sup> cells located in the connective tissue, in the peri-chorionic side.

Another Oct4<sup>+</sup> population, distinct from the Oct4<sup>+</sup> and c-Kit<sup>+</sup> population previously described, were additionally found close to the syncytiotrophoblast layer. Isolated Oct4<sup>+</sup> cells were present both in the connective-mesenchymal layer (Fig. 23D), as near the edge of the villus, close to the syncytiotrophoblast cells (Fig. 23C-E). Immunohistochemical analysis showed a strong nucleoplasmatic staining, in cells with a

simple cytoplasmatic organization, as a sign of multipotentiality, typical of cells metabolically quiescent.

Little is known about the functional role of these pluripotency markers in adult stem cells. It has been shown that knockdown of Oct4 in human bone marrow MSCs induces changes in cell morphology, decrease growth rates and shifts cells from a cycling to a non-cycling state. Therefore, similar regulatory mechanism has been suggested for Oct4 in both ESCs and MSCs (Riekstina U *et al.*, 2009). As well as for the AFS, the surface marker profile and the expression of the transcription factor Oct4 suggest that they may represent an intermediate stage between pluripotent ES cells and lineage-restricted adult stem cells.

To identify the stem potentiality of these cells, various combinations of intracellular markers or cells surface markers were studied in order to characterize the placental cells phenotype (Castrechini NM *et al.*, 2010).

Stella, a pluripotent marker expressed in primordial germ cells, in oocytes and in cells of the inner mass of blastocyst, shows nuclear staining in cells close to the syncytiotrophoblast layer, that, as reported by Ahamed *et al.* (2005), during normal gestation, derived by the fusion of the cytotrophoblastic cells. The nuclear staining of these cells is of high intensity, as well as Oct4 positive staining. Positive cells were solitary and scattered on the mesenchyma, close to the syncytiotrophoblast: surrounded by fibrinoid deposits, Stella positive cells are identically distributed as Oct4 positive cells, but they did not express c-Kit.

In summary we localized cells at different stages of differentiation in distinct part of the tissue analyzed: c-Kit positive cells in the peri-vascular tissue, big-round cells in the

connective tissue positive both for c-Kit as for Oct4 and cells that expressed positive staining for Oct4 and Stella, close to the syncytiotrophoblast. Cytotrophoblasts, a self-renewing population located in the proliferation zone, form the multinucleate syncytiotrophoblasts (Spitalieri P *et al.*, 2009): syncytiotrophoblast cells are terminally differentiated, but our results showed Oct4 and Stella nuclear positive staining in the same region.

#### -Characterization of placental-cells *in vitro*

The distribution of stem cells marker-positive cells in the chorionic tissue, is important to better understand their function and their potentiality; at the same time, it is necessary to isolate and then characterized chorion-derived stem cells from the term placenta at delivery, for further clinical applications. Our immunohistochemical/immunofluorescence results, as previously described, suggests that isolated cells from chorionic membrane may be stem cells.

Cells were isolated from placental tissue after a mechanical peeling of amnion membrane from the underlying chorion; a small sample of chorion (a cube of 2cm) was digested in a enzymatic solution, centrifugated and the released cells were cultured (all protocols are described in M&M) on untreated plastic cultureware.

Using a similar protocol as described by De Coppi (2007), we sorted c-Kit<sup>+</sup> cells, with MACS® technology. C-Kit positive cells represented about 90 % of the selected cells, as confirmed by the FACS analysis: this percentage showed an homogeneous population with stem characteristics. In fact, CD117 (c-Kit), the surface marker used for immunoselection of AFS cells, plays an important role in gametogenesis, melanogenesis and hematopoiesis. This receptor protein is present on human ES cells,

primordial germ cells and many somatic stem cells, including, but not limited to, those of the neural crest. Cultured human c-Kit<sup>+</sup> placental-cells were still positive for c-Kit<sup>+</sup> also after 20 days of culture, in  $\alpha$ -MEM medium supplemented with 20% FCS. The immunophenotype of chorionic-derived stem cells was similar to that of adult bone marrow mesenchymal stem cells, as described by Habdulrazzak *et al* (2010). Double immunofluorescence labeling was carried out to confirm the membrane-localization of c-Kit in cultured cells.

When cultured *in vitro*, c-Kit<sup>+</sup> cells maintain undifferentiated characteristics and pluripotency: this was confirmed by the presence of a subpopulation of c-Kit<sup>+</sup> cells that expressed transcript for Oct4 (as reported in PCR results), which is crucial for maintaining stemness, both in mouse and human embryonic stem cells (Spitalieri P *et al.*, 2009).

WB analysis showed that Oct4 was expressed both in c-Kit<sup>+</sup> and in “not selected” protein extracts. In c-Kit<sup>-</sup> lysates Oct4 expression was not detectable. These data confirmed the immunohistochemical results: in the connective tissue some cells were c-Kit<sup>+</sup> and Oct4<sup>+</sup> (see Fig. 22-23 Results). The whole lysates of “not selected” populations, showed a slightly positivity for Oct4, due to the co-presence of the two populations, c-Kit<sup>+</sup> and c-Kit<sup>-</sup>.

The staining distribution and the expression of c-Kit<sup>+</sup> and Oct4<sup>+</sup> in isolated placenta cells suggests a spatial location or a stem cell niche within the chorionic villi. This subpopulation may act as a source of undifferentiated cells, with a gradient of stemness inside the chorionic tissue: pericyte-like cells are c-Kit<sup>+</sup>, mesenchymal stem cells in the

connective tissue are c-Kit<sup>+</sup> and Oct4<sup>+</sup>, while cells close to the edge of the chorionic villi, are Stella<sup>+</sup>, like germ cells.

### **-Conclusions**

Among the many cell types that may prove useful to regenerative medicine, mounting evidence suggests that human term placenta-derived cells will join strategies in clinical reality. Cells can be isolated during ongoing pregnancy using minimally invasive techniques such as chorionic villus sampling (CVS), or isolated from placental tissues readily available at delivery (Abdulrazzak H *et al.*, 2010). The availability of placenta-derived progenitor/stem cells are useful characteristics for cell therapy and tissue engineering.

The development of cell therapy approaches using these cells may also benefit from the fact that placental tissues harbor different cell types that may complement each other in a clinical setting. Furthermore, aside from being easily procured in a painless and noninvasive manner, placental cells also offer additional advantages over stem cells from other sources such as bone marrow, which carry a risk of viral infection and show decreasing differentiation capacity with increasing donor age. Finally, it is tempting to speculate that placenta-derived cells may also be preferable from an immunological point of view, given the unique role of this tissue in maintaining fetomaternal tolerance throughout pregnancy and supported by the finding that placental cells show a greater capacity to down-regulate T-cell proliferation *in vitro* compared to bone marrow-derived cells (Parolini O *et al.*, 2008).

## REFERENCES

1. Abdulrazzak H, Moschidou, D, *et al.* Biological characteristics of stem cells from foetal, cord blood and extraembryonic tissues. *J R Soc Interface.* 689-706,2010.
2. Adewumi O, *et al.* Characterization of human embryonic stem cell lines by the International Stem Cell Initiative. *Nat Biotechnol.* 25(7): 803-16, 2007.
3. Alison MR, Islam S. Attributes of adult stem cells. *J Pathol.* 217: 144–160, 2009.
4. Altman GH, Diaz F, *et al.* Silk-based biomaterials. *Biomaterials.* 24(3): 401–16, 2003.
5. Anderson DJ, Gage, FH, *et al.* Can stem cells cross lineage boundaries? *Nat Med.* 7: 393–395, 2001.
6. Arai F, Hirao A, *et al.* Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell.* 118: 149–161, 2004.
7. Aronow MA, Gerstenfeld LC, *et al.* Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvaria cells. *J Cell Physiol.* 143: 213-221, 1990.
8. Assady S, Maor G, *et al.* Insulin production by human embryonic stem cells. *Diabetes.* 50: 1691– 1697, 2001.
9. Atala A. Tissue engineering in the genitourinary system. In *Tissue Engineering.* Edited by Atala A, Mooney DJ. Birkhauser Press 149, 1997.
10. Atala A. Engineering Organs. *Current Opinion in Biotechnology.* 20: 575–592, 2009.

11. Atala A, Freeman MR, *et al.* Implantation in vivo and retrieval of artificial structures consisting of rabbit and human urothelium and human bladder muscle. *J Urol.* 150: 608-612, 1993.
12. Bailo M, *et al.* Engraftment potential of human amnion and chorion cells derived from term placenta. *Transplantation.* 78: 1439–1448, 2004.
13. Banerjee C, Javed A, *et al.* Differential regulation of the two principal Runx2/Cbfa1 N-terminal isoforms in response to bone morphogenetic protein-2 during development of the osteoblast phenotype. *Endocrinology.* 142: 4026-4039, 2001.
14. Battula VL, *et al.* Human placenta and bone marrow derived MSC cultured in serum-free, b-FGF-containing medium express cell surface frizzled-9 and SSEA-4 and give rise to multilineage differentiation. *Differentiation.* 75: 279–291, 2007.
15. Bellows CG, Aubin JE, *et al.* Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells in vitro. *Endocrinology.* 121: 1985–1992, 1987.
16. Benirschke K, Kaufmann P. *Pathology of the human placenta.* New York; Springer-Verlag, 42– 46; 116: 281–297, 2000.
17. Bianco P, Riminucci M, *et al.* Bone marrow stromal stem cells: nature, biology, and potential applications. *Stem Cells.* 19: 180–192, 2001.
18. Bieback K, Kern S, *et al.* Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. *Stem Cells.* 22: 625–634, 2004.

19. Blau HM, Brazelton TR, *et al.* The evolving concept of a stem cell: Entity or function? *Cell*. 105: 829–841, 2001.
20. Bondar B, Fuchs S, *et al.* Functionality of endothelial cells on silk fibroin nets: comparative study of micro- and nanometric fibre size. *Biomaterials*. 29(5): 561–72, 2008.
21. Boukhechba F, Balaguer T, *et al.* Human Primary Osteocyte Differentiation in a Three-Dimensional Culture System. *J Bone Miner Res*. 24(11): 1927-35, 2009.
22. Brooke G, Rossetti T, *et al.* Manufacturing of human placenta-derived mesenchymal stem cells for clinical trials. *Br J Haematol*. 144: 571 – 579, 2009.
23. Cananzi M, Atala A *et al.* Stem cells derived from amniotic fluid: new potentials in regenerative medicine. *Reprod Biomed Online*. 18(1): 17–27, 2009.
24. Caplan AI. Mesenchymal stem cells. *J Orthop Res*. 9: 641–650, 1991.
25. Caplan AI. Mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. *Tissue Eng*. 11: 1198–211, 2005.
26. Castrechini NM, Murthi, P, *et al.* Mesenchymal stem cell in human placental chorionic villi reside in a vascular Niche. *Placenta*. 31: 203-212, 2010.
27. Choumerianou DM, Dimitriou H, *et al.* Stem Cells: promises versus limitations tissue engineering. Part B Volume 14, 2008.
28. Cilento BG, Freeman MR, *et al.* Phenotypic and cytogenetic characterization of human bladder urothelia expanded in vitro. *J Urol*. 152: 665-670, 1994.
29. Civitelli R. Cell-cell communication in the osteoblast/osteocyte lineage. *Arch Biochem Biophys*. 473(2): 188-192, 2008.

30. Csaki C, Matis U, *et al.* Co-culture of canine mesenchymal stem cells with primary bone-derived osteoblasts promotes osteogenic differentiation. *Histochem Cell Biol.* 131: 251–266, 2009.
31. Cunningham FG, MacDonald PC, *et al.* The placenta and fetal membranes. *Williams Obstetrics.* 20th ed. Stamford, CT: Appleton and Lange, 95–125, 1997.
32. Czyz J, Wiese C. Potential of Embryonic and Adult Stem Cells *in vitro*. *Biol Chem.* 384: 1391–1409, 2003.
33. D’Aquino R, Papaccio G, *et al.* Dental Pulp Stem Cells: a promising tool for bone regeneration. *Stem Cell Rev.* 4: 21–26, 2008.
34. Daley GQ, Scadden DT, *et al.* Prospects for stem cell-based therapy. *Cell.* 132(4): 544–548, 2008.
35. De Coppi P, Bartsch G, *et al.* Isolation of amniotic stem cell lines with potential for therapy. *Nature Biotechnology.* 25(1): 100–106, 2007.
36. Deliloglu-Gurhana SI, Vatanseverb HS, *et al.* Characterization of osteoblasts derived from bone marrow stromal cells in a modified cell culture system . *Acta Histochemica.* 108: 49–57, 2006.
37. Dezawa M, Ishikawa H, *et al.* Bone marrow stromal cells generate muscle cells and repair muscle degeneration. *Science.* 309(5732): 314–317, 2005.
38. Donzelli E, Salvadè A, *et al.* Mesenchymal stem cells cultured on a collagen scaffold: In vitro osteogenic differentiation. *Arch Oral Biol.* 52(1): 64-73, 2007.
39. Fang D, Nguyen TK, *et al.* A tumorigenic subpopulation with stem cell properties in melanomas. *Cancer Res.* 65: 9328 –9337, 2005.

40. Fauza D. Amniotic fluid and placental stem cells. *Best Pract Res Clin Obstet Gynaecol.* 18: 877–891, 2004.
41. Ferrera D, Biassoni C, *et al.* Three-dimensional cultures of normal human osteoblasts: proliferation and differentiation potential *in vitro* and upon ectopic implantation in nude mice. *Bone.* 5: 718-725, 2002.
42. Franceschi RT. The role of ascorbic acid in mesenchymal differentiation. *Nutrition Rev.* 50: 65–70, 1992.
43. Franceschi RT, Iyer BS, *et al.* Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. *J Bone Miner Res.* 9: 843–854, 1994.
44. Friedenstein AJ, Deriglasova UF, *et al.* Precursors for fibroblasts in different populations of hematopoietic cells as detected by the *in vitro* colony assay method. *Exp Hematol.* 2: 83–92, 1974.
45. Friedenstein AJ, Gorskaja JF, *et al.* Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol.* 4: 267–274, 1976.
46. Friedenstein AJ, Pettakova KV, *et al.* Heterotopic transplants of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation.* 6: 230-247, 1968.
47. Fukuchi Y, Nakajima H, *et al.* Human placenta-derived cells have mesenchymal stem/progenitor cell potential. *Stem Cells.* 22: 649–658, 2004.
48. Ganss B, Kim RH, *et al.* Bone sialoprotein. *Crit Rev Oral Biol Med.* 10(1): 79–98, 1999.

49. García-Pacheco JM, Oliver C, *et al.* Human decidual stromal cells express CD34 and STRO-1 and are related to bone marrow stromal precursors. *Mol Hum Reprod.* 7(12): 1151-7, 2001.
50. George J, Kuboki Y, *et al.* Differentiation of Mesenchymal Stem Cells Into Osteoblasts on Honeycomb Collagen Scaffolds. *Biotechnology and Bioengineering.* 95: 3, 2006.
51. Gericke A, Qin,C, *et al.* Importance of phosphorylation for osteopontin regulation of biomineralization. *Calcif Tissue Int.* 77(1): 45–54, 2005.
52. Gigante A, Manzotti S, *et al.* Adult mesenchymal stem cells for bone and cartilage engineering: effect of scaffold materials. *Eur J Histochem.* 52(3): 169-74, 2008.
53. Graziano A, D'Aquino R, *et al.* Dental pulp stem cells: a promising tool for bone regeneration. *Stem Cell Rev.* 4(1): 21-26, 2008.
54. Griffiths MJ, Bonnet D, *et al.* Stem cells of the alveolar epithelium. *Lancet.* 366: 249–260, 2005.
55. Gronthos S, Brahim J, *et al.* Stem cell properties of human dental pulp stem cells. *J Dent Res.* 81(8): 531-5, 2002.
56. Gronthos S, Graves SE, *et al.* The STRO-1<sup>+</sup> fraction of adult human bone marrow contains the osteogenic precursors. *Blood.* 84(12): 4164-4173, 1994.
57. Gronthos S, Mankani M, *et al.* Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *PNAS.* 97: 13625, 2000.

58. Gronthos S, Zannettino AC, *et al.* Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J Cell Sci.* 116: 1827–1835, 2003.
59. Guillot PV, Gotherstrom C, *et al.* Human first trimester fetal mesenchymal stem cells (MSC) express pluripotency markers, grow faster, and have longer telomeres compared to adult MSC. *Stem Cells.* 25: 646–654, 2007.
60. Haeckel E. (1877) *Anthropogenie*. Leipzig: Wilhelm Engelmann.
61. Haseltine W. A brave new medicine. A conversation with William Haseltine Interview by Joe Flower. *Health Forum J.* 42: 28-30, 1999.
62. Heng BC, Haider HK, *et al.* Strategies for directing the differentiation of stem cells into the cardiomyogenic lineage in vitro. *Cardiovasc Res.* 62: 34-42, 2004.
63. Hipp J, Atala A. Sources of Stem Cells for Regenerative Medicine. *Stem Cell Rev.* 4: 3–11, 2008.
64. Hochedlinger K, Jaenisch R, *et al.* Nuclear reprogramming and pluripotency. *Nature.* 29; 441(7097): 1061-7, 2006.
65. Hochedlinger K, Yamada Y, *et al.* Ectopic expression of Oct-4 blocks progenitor-cell differentiation and causes dysplasia in epithelial tissues. *Cell.* 6; 121(3): 465-77, 2005.
66. Hoffman LM, Carpenter MK. Characterization and culture of human embryonic stem cells. *Nat Biotechnol.* 23: 699–708, 2005.
67. Huang GT-J, Gronthos S, *et al.* Mesenchymal Stem Cells Derived from Dental Tissues *vs.* Those from Other Sources: Their Biology and Role in Regenerative Medicine. *J Dent Res.* 88(9): 792–806, 2009.

68. Hunter GK, Goldberg HA. Proc Natl Acad Sci USA. 90: 8562–8565, 1993.
69. Hunter GK, Hauschka PV, *et al.* Nucleation and inhibition of hydroxyapatite formation by mineralized tissue proteins. Biochem J. 317(1): 59-64, 1996.
70. Hwang WS, Roh, SI, *et al.* Science. 308: 1777–1783, 2005.
71. Ishida Y, Tertinegg I, *et al.* Progesterone and dexamethasone stimulate proliferation and differentiation of osteoprogenitors and progenitors for adipocytes and macrophages in cell populations derived from adult rat vertebrae. J Bone Miner Res. 11: 921–930, 1996.
72. Itskovitz-Eldor J, Schuldiner M, *et al.* Differentiation of human embryonic stem cells into embryoid bodies comprising the three embryonic germ layers. Mol Med. 6: 88–95, 2000.
73. Jäger M, Feser T, *et al.* Proliferation and osteogenic differentiation of mesenchymal stem cells cultured onto three different polymers in vitro. Ann Biomed Eng. 33(10): 1319-1332, 2005.
74. Kakishita K, Nakao N, *et al.* Implantation of human amniotic epithelial cells prevents the degeneration of nigral dopamine neurons in rats with 6-hydroxydopamine lesions. Brain Res. 980: 48–56, 2003.
75. Kärner E, Bäckesjö, CM. *et al.* Dynamics of gene expression during bone matrix formation in osteogenic cultures derived from human embryonic stem cells in vitro. Biochim Biophys Acta. 1790(2): 110-118, 2008.
76. Karsenty G, Wagner EF. Dev Cell 2(4): 389–406, 2002.
77. Kaufman DS, Thomson JA. Human ES cells –haematopoiesis and transplantation strategies. J Anat. 200: 243–248, 2002.

- 78.** Kleinman HK, Martin GR. Matrigel: Basement membrane matrix with biological activity. *Semin Cancer Biol.* 15: 378–386, 2005.
- 79.** Koga T, Matsui M, *et al.* NFAT and Osterix cooperatively regulate bone formation. *Nat Med.* 11(8): 880–885, 2005.
- 80.** Koike M, Sakaki S, *et al.* Characterization of embryoid bodies of mouse embryonic stem cells formed under various culture conditions and estimation of differentiation status of such bodies. *J Biosci Bioeng.* 104: 294–299, 2007.
- 81.** Kolf C, Cho E, *et al.* Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. *Arthritis Res Ther.* 9: 204, 2007.
- 82.** Komori T. Regulation of osteoblast differentiation by transcription factors. *J Cell Biochem.* 99(5): 1233–1239, 2006.
- 83.** Kubo M, Sonoda Y, *et al.* Immunogenicity of human amniotic membrane in experimental xenotransplantation. *Invest Ophthalmol Vis Sci.* 42: 1539–1546, 2001.
- 84.** Kuhn NZ, Tuan RS. Regulation in stemness and Stem cell niche of mesenchymal stem cells: implication in tumorigenesis and metastasis. *J Cell Physiol.* 222: 268-277, 2009.
- 85.** Kuo MY, Lan WH, *et al.* Collagen gene expression in human dental pulp cell cultures. *Arch Oral Biol.* 37: 945-952, 1992.
- 86.** Laino G, D'Aquino R, *et al.* A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB). *J Bone Miner Res.* 20(8): 1394-1402, 2005.

87. Laino G, Graziano A, *et al.* An approachable human adult stem cell source for hard-tissue engineering. *J Cell Physiol.* 206: 693–701, 2006.
88. Langer R, Vacanti JP. Tissue engineering. *Science.* 260(5110): 920–6, 1993.
89. Laschke MW, Rucker M. Incorporation of growth factor containing Matrigel promotes vascularization of porous PLGA scaffolds. *J Biomed Mater Res.* 85A: 397–407, 2008.
90. Lavker RM, Tseng SC, *et al.* Corneal epithelial stem cells at the limbus: Looking at some old problems from a new angle. *Exp Eye Res.* 78: 433– 446, 2004.
91. Lee SY, Miwa M, *et al.* *In vitro* multipotentiality and characterization of human unfractured traumatic hemarthrosis-derived progenitor cells: a potential cell source for tissue repair. *J Cell Physiol.* 210: 561–566, 2007.
92. Li L, Xie T. Stem cell niche: Structure and function. *Annu Rev Cell Dev Biol.* 21: 605– 631, 2005.
93. Lian JB, Shalhoub V, *et al.* Species-specific glucocorticoid and 1,25-dihydroxyvitamin D responsiveness in mouse MC3T3-E1 osteoblasts: Dexamethasone inhibits osteoblast differentiation and vitamin D down-regulates osteocalcin gene expression. *Endocrinology.* 138: 2117–2127, 1997.
94. Liebert M, Hubbel A, *et al.* Expression of mal is associated with urothelial differentiation in vitro: identification by differential display reverse-transcriptase polymerase chain reaction. *Differentiation.* 61: 177-185, 1997.
95. Liu L, Sun Z, *et al.* *Ex vivo* expansion and *in vivo* infusion of bone marrow-derived Flk-1+CD31–CD34– mesenchymal stem cells: feasibility and safety from monkey to human. *Stem Cells Dev.* 15: 349–357, 2006.

- 96.** Liu W, Toyosawa S, *et al.* J Cell Biol. 155(1): 157–166, 2001.
- 97.** Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T) Method. Methods. 25: 402-408, 2001.
- 98.** Lutolf MP, Hubbell JA. Synthetic biomaterials as instructive extracellular microenvironments for morphogenesis in tissue engineering. Nature Biotechnology. 23(1): 47–55, 2005.
- 99.** Magatti M, De Munari S, *et al.* Human amnion mesenchyme harbors cells with allogeneic T cell suppression and stimulation capabilities. Stem Cells. 182-192, 2007.
- 100.** Maltepe E, Bakardjiev AI, *et al.* The placenta: transcriptional, epigenetic, and physiological integration during development. J Clin Invest. 120(4): 1016–1025, 2010.
- 101.** Marco F, Lopez-Oliva F, *et al.* Osteochondral allografts for osteochondritis dissecans and osteonecrosis of the femoral condyles. Int Orthop. 17: 104–108, 1993.
- 102.** Marcus A, Woodbury D. Fetal stem cells from extraembryonic tissues: do not discard. J Cell Mol Med. 12: 730–742, 2008.
- 103.** Marie PJ. Transcription factors controlling osteoblastogenesis. Arch Biochem Biophys. 473(2): 98-105, 2008.
- 104.** Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci USA, 78: 7634-7638, 1981.

- 105.** McCulloch CAG, Fair C, *et al.* The regulatory effect of phosphates on bone metabolism in vitro. *Cell Tissue Res.* 257: 555-563, 1989.
- 106.** McKee MD, Nanci A. Osteopontin and the bone remodeling sequence. Colloidal-gold immunocytochemistry of an interfacial extracellular matrix protein. *Ann. NY Acad Sci.* 760: 177–189, 1995a.
- 107.** Mimeault M, Batra SK. Concise Review: Recent Advances on the Significance of Stem Cells in Tissue Regeneration and Cancer Therapies. *Stem Cells.* 24: 2319–2345, 2006.
- 108.** Mitsui K, Tokuzawa Y, *et al.* The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. *Cell.* 30; 113(5): 631-42, 2003.
- 109.** Miura M, Gronthos S, *et al.* SHED: stem cells from human exfoliated deciduous teeth. *Proc Natl Acad Sci USA.* 100: 5807, 2003.
- 110.** Mizuno H, Zuk PA, *et al.* Myogenic differentiation by human processed lipoaspirate cells. *Plast Reconstr Surg.* 109: 199–209, 2002.
- 111.** Modlin IM, Kidd M, *et al.* Gastric stem cells: An update. *Keio J Med.* 52: 134 – 137, 2003.
- 112.** Morsczech C, Gotz W, *et al.* Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth. *Matrix Biol.* 24: 155–165, 2005.
- 113.** Nagata T, Bellows CG, *et al.* Biosynthesis of bone proteins ISPP-1 (secreted phosphoprotein-1, osteopontin), BSP (bone sialoprotein) and SPARC (osteonectin) in association with mineralized-tissue formation by fetal-rat calvarial cells in culture. *Biochem J.* 274: 513–20, 1991.

- 114.** Nakamura T, Arai Y, *et al.* PGC7/Stella protects against DNA demethylation in early embryogenesis. *Nat Cell Biol.* 9: 64–71, 2007.
- 115.** Nakashima K, Zhou J, *et al.* MANCA TITOLO *Cell.* 108(1): 17–29, 2002.
- 116.** Nelson TJ, Behfar A. Stem Cell Platforms for Regenerative Medicine. *Clin Transl Sci.* 1; 2(3): 222–227, 2009.
- 117.** Niwa H, Miyazaki J, *et al.* Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nat Gene.* 24(4): 372–6, 2000.
- 118.** O’Donoghue K, Fisk NM. Fetal stem cells. *Best Pract Res Clin Obstet Gynaecol.* 18: 853–875, 2004.
- 119.** Oyama S. The Ontogeny of Information. *Developmental Systems and Evolution.* Raleigh, NC: Duke University Press. 2000.
- 120.** Palumbo C, Ferretti M, *et al.* Osteocyte dendrogenesis in static and dynamic bone formation: an ultrastructural study. *Anat Rec A Discov Mol Cell Evol Biol,* 278(1): 474–480, 2004.
- 121.** Pandipati S, Driscoll JE, *et al.* Glucocorticoid Stimulation Of Na<sup>-</sup> Dependent Ascorbic Acid Transport In Osteoblast-Like Cells. *J Cell Physiol.* 176: 85–91, 1998.
- 122.** Papaccio G, Graziano A, *et al.* Long-term cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. *J Cell Physiol.* 208: 319–325, 2006.

- 123.**Parolini O, Alviano F, *et al.* Toward Cell Therapy Using Placenta-Derived Cells: Disease Mechanisms, Cell Biology, Preclinical Studies, and Regulatory Aspects at the Round Table Stem Cells And Development. Volume 19, Number 2, 2010.
- 124.**Parolini O, Alviano F, *et al.* Concise Review: Isolation and Characterization of Cells from Human Term Placenta: Outcome of the First International Workshop on Placenta Derived Stem Cells. *Stem Cells.* 26: 300–311, 2008.
- 125.**Patrick JG, Barry JJA, *et al.* Controlling protein release from scaffolds using polymer blends and composites. *European Journal of Pharmaceutics and Biopharmaceutics.* 68: 82–89, 2008.
- 126.** Payer B, Saitou M, *et al.* Stella is a maternal effect gene required for normal early development in mice. *Curr Biol.* 13: 2110–2117, 2003.
- 127.**Phinney DG. Building a consensus regarding the nature and origin of mesenchymal stem cells *J Cell Biochem.* 38: 7–12, 2002.
- 128.**Pierdomenico L, Bonsi L, *et al.* Multipotent Mesenchymal Stem Cells with Immunosuppressive Activity Can Be Easily Isolated from Dental Pulp. *Transplantation.* 80: 836–842, 2005.
- 129.**Pittenger MF, Mackay AM, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science.* 284: 143–147, 1999.
- 130.**Potten CS, Loeffler M. Stem cells: Attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development.* 110: 1001–1020, 1990.
- 131.**Pratap J, Galindo M, *et al.* Cell growth regulatory role of Runx2 during proliferative expansion of preosteoblasts. *Cancer Res.* 63(17): 5357-5362, 2003.

- 132.**Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science*. 276: 71–74, 1997.
- 133.**Ramalho-Santos M, Willenbring H. On the origin of the term “stem cell”. *Stem Cell*. 1: 335–338, 2007.
- 134.**Reubinoff BE, Itsykson P, *et al.* Neural progenitors from human embryonic stem cells. *Nature Biotechnol*. 19: 1134–1140, 2001.
- 135.**Reubinoff BE, Pera MF, *et al.* Embryonic stem cell lines from human blastocysts: somatic differentiation *in vitro*. *Nature Biotechnol*. 18: 399–404, 2000.
- 136.** Riccio M, Resca E, *et al.* Osteogenic differentiation of human dental pulp stem cells in 3D scaffolds. *EJH*. 115, volume 45, number 4, 2010.
- 137.**Riekstina U, Cakstina I, *et al.* Embryonic Stem Cell Marker Expression Pattern in Human Mesenchymal Stem Cells Derived from Bone Marrow, Adipose Tissue, Heart and Dermis. *Stem Cell Rev and Rep*. 5: 378–386, 2009.
- 138.**Seong JM, Kim BC. Stem cells in bone tissue engineering. *Biomed. Mater*. 5:062010, 2010.
- 139.**Shamblott MJ, Axelman J, *et al.* Derivation of pluripotent stem cells from cultured human primordial germ cells. *Proc Natl Acad Sci USA*. 95: 13726–13731, 1998.
- 140.**Sila-Asna M, Bunyaratvej A, *et al.* Osteoblast differentiation and bone formation gene expression in strontium-inducing bone marrow mesenchymal stem cell. *J Med Sci*. 53(1-2): 25-35, 2007.

- 141.**Simmons PJ, Torok-Storb B. CD34 expression by stromal precursors in normal human adult bone marrow. *Blood*. 78(11): 2848-2853, 1991.
- 142.**Sinanani AC, Hunt NP, *et al.* Human adult craniofacial muscle-derived cells: neural-cell adhesion molecule (NCAM; CD56)-expressing cells appear to contain multipotential stem cells. *Biotechnology and Applied Biochemistry*. 40: 25–34, 2004.
- 143.**Soncini M, Vertua E, *et al.* Isolation and characterization of mesenchymal cells from human fetal membranes. *J Tissue Eng Regen Med*. 1: 296–305, 2007.
- 144.**Sonoyama W, Liu Y, *et al.* Mesenchymal stem cell-mediated functional tooth regeneration in Swine. *PLoS*. 1:79, 2006.
- 145.**Sonoyama W, Liu Y, *et al.* Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study. *J Endod*. 34: 166–171, 2008.
- 146.**Spitalieri P, Cortese G, *et al.* Identification of Multipotent Cytotrophoblast Cells from Human First Trimester Chorionic Villi. *Cloning and Stem Cells*. 11(4): 535-556, 2009.
- 147.**Stewart K, Walsh S, *et al.* Further characterization of cells expressing STRO-1 in cultures of adult human bone marrow stromal cells. *J Bone Miner Res*. 14: 1345–1356, 1999.
- 148.**Stojkovic M, Lako M, *et al.* Derivation, growth and applications of human embryonic stem cells. *Reproduction*. 128: 259–267, 2004.

- 149.**Sudo K, Kanno M, *et al.* Mesenchymal progenitors able to differentiate into osteogenic, chondrogenic, and/or adipogenic cells in vitro are present in most primary fibroblast-like cell populations. *Stem Cells.* 25: 1610–1617, 2007.
- 150.**Sun H, Feng K, *et al.* Osteogenic differentiation of human amniotic fluid-derived stem cells induced by bone morphogenetic protein-7 and enhanced by nanofibrous scaffolds. *Biomaterials.* 31: 1133–1139, 2010.
- 151.**Thomas ML. The Leukocyte Common Antigen Family Annual Review of Immunology. 7: 339-369, 1989.
- 152.**Thomson JA, Itskovitz-Eldor J, *et al.* Embryonic stem cell lines derived from human blastocysts. *Science.* 282, 1145–1147, 1998.
- 153.**Till JE, McCulloch EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res.* 14: 213–222, 1961.
- 154.**Tsai M, Lee J, *et al.* Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. *Hum Reprod.* 19: 1450–1456, 2004.
- 155.**Valenti MT, Dalle Carbonare L, *et al.* Gene expression analysis in osteoblastic differentiation from peripheral blood mesenchymal stem cells. *Bone.* 43(6): 1084-1092, 2008.
- 156.**Walsh S, Jordan GR, *et al.* High concentrations of dexamethasone suppress the proliferation but not the differentiation or further maturation of human osteoblast precursors in vitro: relevance to glucocorticoid-induced osteoporosis. *Rheumatology.* 40(1): 74–83. 2001.
- 157.**Wang X, Kua HY, *et al.* *J Cell Biol.* 172 (1): 115–125, 2006.

- 158.**Wanga Y, Kimj HJ, *et al.* Stem cell-based tissue engineering with silk biomaterials. *Biomaterials*. 27: 6064–6082, 2006.
- 159.**Weiss ML, Troyer DL. Stem cells in the umbilical cord. *Stem Cell Rev*. 2: 155–162, 2006.
- 160.**Wenisch S, Trinkaus K, *et al.* Immunochemical, ultrastructural and electrophysiological investigations of bone-derived stem cells in the course of neuronal differentiation. *Bone*. 38(6): 911-21, 2006.
- 161.**Wilson JX, Dixon SJ. High-affinity sodium-dependent uptake of ascorbic acid by rat osteoblasts. *J Membrane Biol*. 111: 83–91, 1989.
- 162.**Yoshimura K, Shigeura, T, *et al.* Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction spirates. *J Cell Physiol*. 208: 64–76, 2006.
- 163.**Yu J, He H, *et al.* Differentiation potential of STRO-1<sup>+</sup> dental pulp stem cells changes during cell passaging. *BMC Cell Biol*. 8:11-32, 2010.
- 164.**Zavan B, Giorgi C, *et al.* Osteogenic and chondrogenic differentiation: comparison of human and rat bone marrow mesenchymal stem cells cultured into polymeric scaffolds. *Eur J Histochem*. 51(1): 1-8, 2007.
- 165.**Zhang W, Walboomers XF, *et al.* Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation. *Tissue Eng*. 12(10): 2813-2823, 2006.
- 166.**Zhao HX, Li Y, *et al.* Rapid and efficient reprogramming of human amnion-derived cells into pluripotency by three factors OCT4/SOX2/NANOG. *Differentiation*. 80(2-3): 123-9, 2010.

**167.**Zhou J, Zhang Y, *et al.* Embryoid bodies formation and differentiation from mouse embryonic stem cells in collagen/Matrigel scaffolds. *J Genet Genomics.* 37: 451–460, 2010.