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Biochimica, Biologia Molecolare e Biotecnologie

CICLO XXIII

COORDINATORE Prof. Francesco Bernardi

Mesenchymal stem cells from Wharton's Jelly and periodontal ligament: reliable not controversial sources for osteogenic differentiation and regenerative medicine.

Settore Scientifico Disciplinare BIO/11

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Anni 2008/2010

Ma le cose, dentro di noi,
sono sempre maledettamente complicate;
e tanto più inganniamo noi stessi, o tentiamo,
quanto più evidente e immediato
si presenta il disinganno.

Leonardo Sciascia

Summary

Mesenchymal stem cells (MSCs) are uniquely capable of crossing germinative layers borders (these cell populations are able to differentiate towards ectoderm-, mesoderm- and endoderm- derived lineages) and are viewed as promising cells for regenerative medicine approaches in several diseases.

Some undoubtedly limiting factors for the clinical use of MSCs, i.e. for the repair of bone defects, are related to many different problems, that only partially today can be overcome through ex-vivo expansion and cells modification strategies.

Considering MSCs sources, the use of fetal annexes-derived MSCs, such as MSCs from Wharton' jelly of umbilical cord (WJMSCs), or the recruitment of MSCs from "stem cell niches" in adult discard tissues, like periodontal ligament (PDL) of extracted teeth (PDLMSCs), could be promising in compensating limits of MSCs traditional sources, i.e. Bone Marrow, like small cells number, high harvesting technique morbility, difficult cells commitment due to early senescence.

Uniforming and investigating best cells culture conditions with in vitro different experimental conditions is useful in our job to understand and control differentiation mechanisms and finally to influence the yield and proliferation rate of these MSCs populations, together with their osteogenic potential.

The aim of our study is briefly sumarized:

- To isolate and culture MSCs cells from human Umbilical Cord Wharton's Jelly and periodontal ligament;
- To compare characteristics between all samples recruited and to link them to clinical aspects of tissues donors;
- To characterise both MSCs population in regard to their proliferation and differentiation potential;
- To investigate their functional characteristics before and after alginate microbeads encapsulation;

- To investigate effects of three-dimensional and microgravity on MSCs before and after differentiation.

WJMSCs and PDLSCs were analyzed for the expression of MSC markers, and then committed to osteogenic differentiation. Before and after differentiation, alkaline phosphatase (ALP) activity, the expression level of a specific osteoblast transcription factor (Runx2), and mineralization status were evaluated. The performance of WJMSCs and PDLSCs was then compared in 3-D culture systems (alginate beads and microgravity system) in terms of viability, proliferation, secretive profile, expression of markers and effectiveness of phenotype modulation.

Characterization of MSCs population was succesfull for all samples investigated, and for largest samples cohort (WJMSCs) it was possible to correlate cells biochemical parameters to clinical donors features.These findings may help during selection of best donors to combine them with scaffolds and biomaterials to promote tissue regeneration.

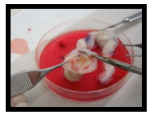
For both MSCs populations, we demonstrated cells can live and grow in 3-D systems. All MSCs samples analyzed showed a substantial osteogenic potential, before and after encapsulation in alginate microbeads.

Modulation of cells culture conditions, with the use of nanotechnologies strategies or the use of bioreactors, like rotating bioreactor (HARV bioreactor, Synthecon, Inc., Houston, TX, U.S.A.) has been shown to be a useful and promising approach to investigate characteristics and environmental effects of cells/biomaterials combinations, in order to predict their effect and potential for regenerative medicine strategies.

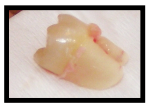
Key words:

Mesenchymal stem cells, Wharton's jelly, Periodontal ligament, Osteogenic differentiation markers, Regenerative medicine.

Thesis subjects overview



Wharton's Jelly



periodontal ligament

MSCs isolation

80-90% subconfluence

flow cytometric analysis

MSCs expansion

2 D

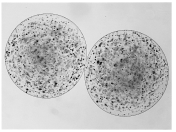
osteogenic differentiation:
-traditional
- Polimeric Micelles (PMs)

PI staining
Rna
AR colorimetric assay

proliferation assay
-wound assay
-MTT test

3 D

cryopreservation

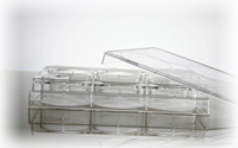


encapsulation

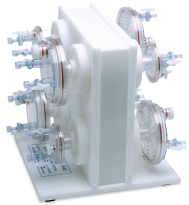
6 well plate

PI staining
Rna
secretive profile

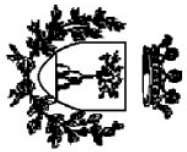
rotating vessels



6 well plate



Bioreactor
HARV
Sinthecon technology



osteogenic differentiation

PI staining
Rna
AR colorimetric assay

INTRODUCTION

Chapter 1

Mesenchymal stem cells (MSCs): isolation, characterization and differentiation potential.

The use of living cells as therapeutic agents for the maintenance, regeneration, or replacement of malfunctioning tissues has been proposed in last decades [Kirouac et al, 2008]. Stem cells are the basis for cell therapy. They are functionally undifferentiated cells that retain the ability to differentiate in one or more mature cell types under appropriate conditions, and to self-renew, representing a potentially inexhaustible cell source [Alberts et al, 2002].

Stem cells are defined simply as cells meeting three basic criteria:

- stem cells renew themselves throughout life, i.e., the cells divide to produce identical daughter cells and thereby maintain the stem cell population,
- stem cells have the capacity to undergo differentiation to become specialized progeny cells [Burns et al. 2010],
- when stem cells differentiate, they may divide asymmetrically to yield an identical cell and a daughter cell that acquires properties of a particular cell type,
- stem cells is that they may renew the tissues that they populate. All tissue compartments contain cells that satisfy the definition of “stem cells”, and the rate at which stem cells contribute to replacement cells varies throughout the body.

List of abbreviation

ALP	Alkaline Phosphatase
AML	Acute Myeloid Leukemia
BM-MSCs	Bone Marrow Mesenchymal Stem Cells
BMP-2	Bone Morphogenetic Protein-2
BSP	Bone Sialoprotein
CD 3	Cluster Of Differentiation 3
CD 14	Cluster Of Differentiation 14
CD 31	Cluster Of Differentiation 31
CD 34	Cluster Of Differentiation 34
CD 45	Cluster Of Differentiation 45
CD 73	Cluster Of Differentiation 73
CD 90	Cluster Of Differentiation 90
CD 105	Cluster Of Differentiation 105
CD 146	Cluster Of Differentiation 146
Col1a1	Collagen type I alpha 1
D-MEM HG	Dulbecco's Modified Essential Medium High Glucose
D-MEM LG	Dulbecco's Modified Essential Medium Low Glucose
ECM	Extracellular Matrix
EDTA	EthyleneDiamineTetraacetic Acid
ESCs	Embryonic Stem Cells
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
GSK3β	Glycogen Synthase Kinase 3 Beta
HA	Hydroxyapatite
HUC	Human Umbilical Cord

HERS	Hertwig's Epithelial Root Sheath
HSCs	Hematopoietic Stem Cells
HARV	High Aspect Ratio Vessel
Ibsp	Integrin-binding Sialoprotein
M-CSF	Macrophage Colony-Stimulating Factor
MNC	mononuclear cells
MSCs	Mesenchymal Stem Cells
mRNA	Messenger Ribonucleic Acid
NF-κB	Nuclear Factor kappa B
OC	Osteocalcin
OPN	Osteopontin
PDL	Periodontal Ligament
PDLABs	PDLMSCs alginate beads
PDLSCs	Periodontal Ligament Stem Cells
PDLMSCs	Periodontal Ligament Mesenchymal Stem Cells
PBS	Phosphate Buffer Solution
PTH	Parathyroid Hormone
RANK	Receptor Activator For Nuclear Factor K B
RANKL	Receptor Activator For Nuclear Factor K B Ligand
RT-PCR	Reverse Transcription Polymerase Chain Reaction
Runx2	Runt-related Transcription Factor 2
RWV	Rotating Wall Vessel
Spp1	Secreted Phosphoprotein-1
STRO-1	Stromal Factor-1
TCP	Tricalcium Phosphate
UC	Umbilical Cord
UCM	Umbilical Cord Matrix
UC-WJMSCs	Umbilical Cord Wharton's Jelly Mesenchymal Stem Cells

WJ	Wharton's Jelly
WJABs	WJMSCs alginate beads
WJMSCs	Wharton's Jelly Mesenchymal Stem Cells
Wnt signaling	Wingless signaling pathway

To describe different culture conditions for cells encapsulated in alginate microbeads, combinations of some different abbreviations were choosen:

- To indicate WJABs cultured in 6 well-plate (We):
    - "control" culture medium (CCM): WJABs/We/CCM
    - osteogenic differentiation medium (ODM): WJABs/We/ODM
- To indicate PDLABs cultured in 6 well-plate (We):
    - "control" culture medium (CCM): PDLABs/We/CCM
    - osteogenic differentiation medium (ODM): PDLABs/We/ODM
- To indicate WJABs cultured in rotating HARV bioreactor 10 ml vessels (HARV):
    - "control" culture medium (CCM): WJABs/HARV/CCM
    - osteogenic differentiation medium (ODM): WJABs/HARV/ODM
- To indicate PDLABs cultured in rotating HARV bioreactor 10 ml vessels (HARV):
    - "control" culture medium (CCM): PDLABs/HARV/CCM
    - osteogenic differentiation medium (ODM): PDLABs/HARV/ODM.

Cell-based therapies use either autologous or allogeneic stem cells, depending if the recipient is the same as the donor or not, respectively. While autologous cell therapy has the advantage of complete immunological compatibility, there are many situations where allogeneic cell transplantation is necessary for therapeutic efficacy [Cortesini, 2005]. Therapeutic strategies that make use of stem cells have been proposed for many clinical applications including Parkinson's and Alzheimer's diseases, spinal cord injury, stroke, burns, heart disease, diabetes, osteoarthritis, and rheumatoid arthritis (for an updated list visit **stemcells.nih.gov**).

Stem cells that may differentiate into tissues derived from all three germ layers, ectoderm, endoderm, and mesoderm, are called “pluripotent” [Weiss & Troyer 2006] (see **FIGURE 1**).

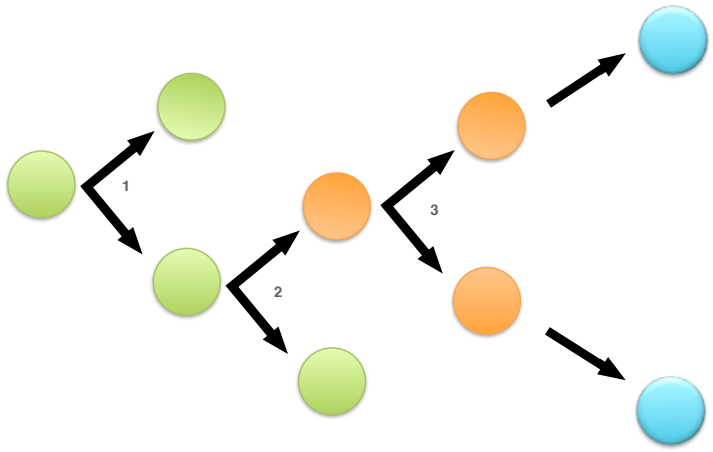


Figure 1: Stem cell division. Green dots: stem cells; Orange dots: progenitor cell; Light blue dots: differentiated cell; 1 - symmetric stem cell division; 2 - asymmetric stem cell division (Green dot is still stem cells, while Orange dot goes on through differentition pathway); 3 - progenitor division and terminal differentiation.

Embryonic Stem Cells (ESCs) are the “de facto” pluripotent cells for biomedical research. In contrast, biomedical research with postnatally collected tissues and stem cells has generated less controversy and enjoyed more therapeutic applications to date. This is likely owing to the fact that blood and bone marrow

stem cells were found to rescue patients with bone marrow deficiencies [Weiss & Troyer 2006 (**a**)].

Many recent evidences demonstrated that in all organisms, stem cells live in specialized “niches,” microenvironments including stem cell support cells and extracellular matrix. The niche micro-environment regulates the growth and differentiation of stem cells [Xie et al. 2000; Shinohara et al. 2001; Shinohara et al. 2002].

The definition of a mesenchymal stem cell would be “ the putative marrow cell that can self renew and give rise to a one or more mesenchymal tissues” [Friedenstein et al. 1968]. However, marrow stromal cells, the population of cells within which the mesenchymal stem cell is thought to exist, can also differentiate to tissues other than those that originate in the embryonic mesoderm, raising questions about the appropriate use of the term mesenchymal stem cell.

In addition to hematopoietic stem cells (HSCs), the bone marrow contains mesenchymal stem cells (MSCs). These cells were first recognised by Friedenstein and colleagues more than 30 years ago, who identified a non-phagocytic, adherent, fibroblast-like population that *in vitro* could regenerate bone, cartilage, adipose and stroma [Friedenstein et al. 1968].

The cells were thoroughly characterised in regard to their colony forming efficiency, strain generation and differentiation into bone, cartilage, adipose tissue and myelo-supporting stroma. After transplantation of these cells under the kidney capsule, they engrafted in and differentiated into different connective tissue lineages [Friedenstein et al. 1974], which indicates that a common progenitor cell exists that gives rise to these tissues.

These cells are multipotent stem cells that can differentiate into a variety of cell type, such as osteoblasts, chondrocytes, adipocytes, myocites [Wobus 2008;

Kadiyala et al. 1997; Dennis et al. 1999; Bruder et al. 1997; Jaiswal et al. 1997; Ferrari et al. 1998; Gang et al. 2004; Young et al. 1998] (see **FIGURE 2**).

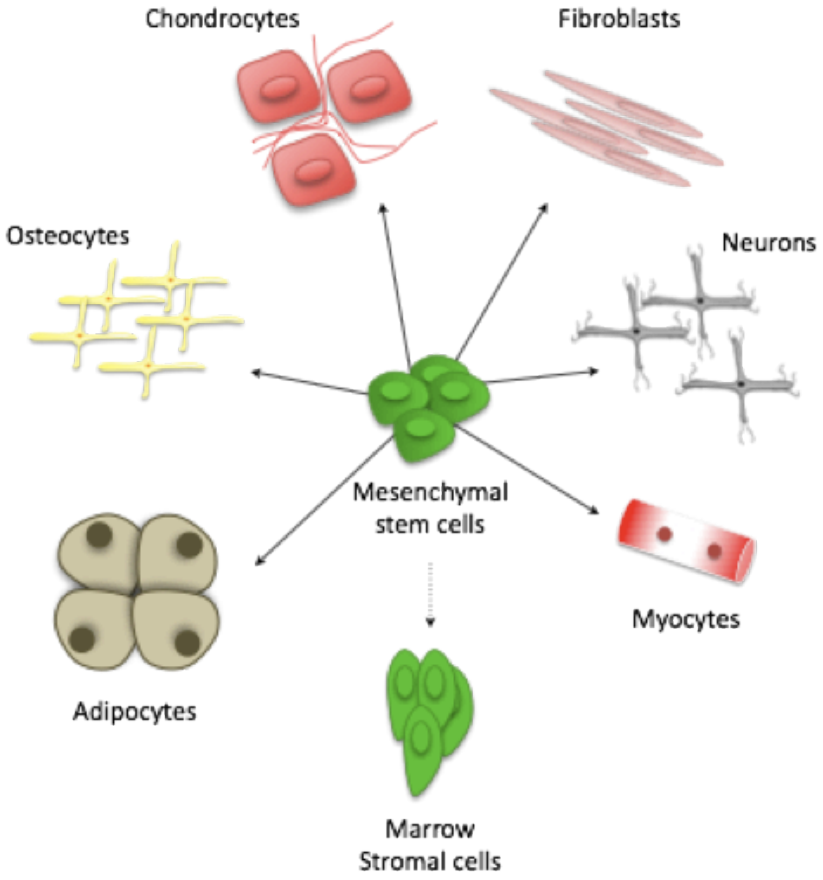


Figure 2: Mesenchymal stem cells can differentiate into several mesenchymal tissues such as bone, cartilage, muscle, bone marrow stroma, adipose tissue and tendon.

The term Mesenchymal Stem Cell is descriptive for:

- *Mesenchymal status:* from Mesenchyme, the embryonic connective tissue that is derived from the mesoderm and that differentiates into hematopoietic and connective tissue, whereas MSCs do not differentiate into hematopoietic cells.
- *Stemness*: stem cells are capable of dividing and renewing themselves for long periods, they are unspecialised and they can give rise to specialised cell types. Their self-renewal potential ensures that they are available for the demands over a normal adult lifespan.

- *Lack of specialisation*: a stem cell is that it does not have any tissue-specific structure, so they can give rise to specialised cells.
- *Potential*: three major classes of stem cells are now recognised: totipotent, pluripotent and multipotent stem cells. Multipotent cells, like hematopoietic and mesenchymal stem cells, are committed to give rise to cells that have a particular function.

MSCs can undergo over 25 passages in vitro (more than 50 cell doublings). This demonstrates a high capacity for self-replication; they can be generated frequently from a small amount of starting material and they don't seem to show signs of senescence and apoptosis until 50 population doublings [Campagnoli et al. 2001 Bruder et al. 1997; Digirolamo et al. 1999; Conget & Minguell 1999]. An overview of MSCs hallmark are sumarized in **Table 1**. MSCs are identified through a combination of physical, phenotypic, and functional properties [Giordano et al. 2007]. The classical assay utilized to identify MSCs is the colony forming unit (CFU). This assay identifies adherent spindle shaped cells that proliferate to form colonies and can be induced to differentiate into adipocytes, osteocytes, and chondrocytes [Prockop, 1997; Bianco & Gehron Robey 2000; Beyer Nardi & da Silva Meirelles 2006; Sethe et al. 2006; Pountos & Giannoudis 2005].

Isolation		Differentiation
Bone marrow		Osteoblasts
Trabecular bone		Chndrocytes
Periosteum		Adipocytes
Articular cartilage		Cardiac myocytes
Synovium		Fibroblasts
Synovial fluid		Myofibroblasts
Muscles		Skeletal myocytes
Adipose tissue	***Mesenchymal stem cells***	Pericytes
Tendons		Tenocytes
Blood		Retinal cells
Blood vessels		Neural cells
Umbilical cord vessels		Astrocytes
Fetal tissues		Hepatocytes
Spleen and Tymus		Pancreatic cells
Skin		Hematopoetic supporting stroma

Table 1: Sources and cell types derived from MSCs (all references in: Pountos & Giannoudis. Injury 2005;36 Suppl 3:S8-S12.)

Great effort has been applied to identify specific surface markers on MSCs for definition and identification of the cells in vivo and in vitro. The in vivo phenotype of MSCs has not been entirely established. However, MSCs expanded in vitro do not express the hematopoietic or endothelial surface markers CD11b, CD14, CD31, CD34 or CD45 but are positive for CD29, CD44, CD73, CD105 and CD166 [Pittenger et al. 1999; Conget & Minguell 1999; Haynesworth et al. 1992; Noort et al 2002]. Molecules suggested to be specific for primitive MSCs and their more differentiated progeny are STRO-1, CD63, CD49a and CD166 [Stewart et al. 2003].

1.1 MSCs from different sources

Human MSCs were first identified in postnatal bone marrow and later in a variety of other human adult tissues, including muscle, connective tissue, skin, adipose tissue, perichondrium, trabecular bone [Young et al. 2001; Zuk et al. 2002; De Ugarte et al. 2003; Arai et al. 2002; Sakaguchi et al. 2004].

Bone marrow-derived stem cells (BM-MSCs) are reported to be more difficult to extract from the marrow cavity in normal aging because the red marrow space changes to a yellow marrow (fat-filled) as a consequence of aging [Weiss & Troyer 2006 (**a**)]. Optimal stem cell aspirates from the marrow are found in young donors. Although BM-MSCs become senescent (cease to divide in vitro) by passage 6–10.

Moreover MSCs take part in matrix remodelling and possess paracrine activity particularly in the environment of a wound [Fathke et al. 2004; Neuss et al. 2009]. These characteristics make them an interesting tool for wound healing applications. However, any medical application is hampered by the low amount of MSC in bone marrow, in particular in older patients, as well as the high cell

senescence, limited proliferation and differentiation capacity of these cells [Bruder et al. 1997; Mendes et al. 2002].

It is generally thought that stem cells derived from “younger” tissues, for example, tissues derived from the early embryo or fetus, would have longer telomeres and have the capacity for extended expansion in culture prior to becoming senescent (see **FIGURE 3**).

In the last 10 years, umbilical cord blood has been shown to be therapeutically useful for rescuing patients with bone marrow- related deficits and inborn errors of metabolism. Umbilical cord blood offers advantages over bone marrow because cord blood does not require perfect human leukocyte antigen (HLA) tissue matching, has less incidence of graft vs host disease, and may be used allogenically [Weiss & Troyer 2006 (**b**)].

MSC-like cells can be isolated from so many different tissues discard at the moment of delivery, and they could be considered as young as the new born. The collection of MSC-like cells from tissues that are discarded at birth is easier and less expensive than collecting MSCs from a bone marrow aspirate. During the collection of these tissues, there is no health impact on either the mother or the newborn. At least these cells may be stored frozen and then thawed to provide stem cells for therapeutic use decades after cryogenic storage.

Fetal annexes, such as UC, UC blood, placenta, amniotic fluids, have been shown to be good MSCs sources [Romanov et al. 2003; Sarugaser et al. 2009; Soncini et al. 2007]. They can also be isolated from several fetal tissues, such as blood, bone marrow and liver, kidney, lung, spleen, pancreas, blood and brain [Yu et al. 2004; Campagnoli et al. 2001; in 't Anker et al. 2003; Yu et al. 2003; Airey et al. 2004 Kægler G & Wernet P 2006].

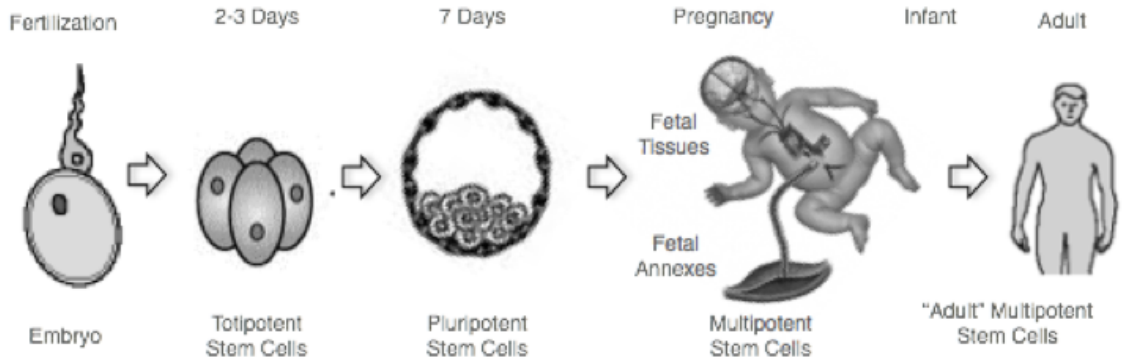


Figure 3: Adult and fetal stem cells can be considered "multipotent".

The differentiative capacity of fetal and adult MSCs may vary. D'Ippolito et al. examined the osteogenic differentiation of postnatal MSCs of different age and found that younger individuals exhibit increased osteogenic potential than older individuals [D'Ippolito et al 1999]. Also, MSCs from various fetal tissues exhibit diverse differential potential; osteogenic differentiation was reduced in MSCs derived from liver and adipogenic differentiation was less in spleen-derived MSCs, compared to bone marrow and lung- derived MSCs [in 't Anker et al. 2003].

It has been shown, that in contrast to its adult bone marrow (BM) counterpart, the stem cell compartment in CB is less mature [Kægler G & Wernet P 2006].

Considering adipose tissue and his procurement, it can be considered a discard tissue, but its procurement from living patients involves an invasive and painful surgical procedure [Seshareddy et al. 2008].

Contrarily MSCs can be isolated from UC without any risk for both patients, mother and newborn, avoiding morbility and senecence problems related to different MSCs sources.

Different works demostrated that the ontological and anatomical origins of MSCs, i.e. Bone Marrow Mesenchymal Stem Cells (BM-MSCs) compared to UC MSCs or mandibular-derived MSCs, have influence on the differentiation capacities and behaviour both in a 3-dimensional matrix or in monolayer [Schneider et al. 2010; Aghaloo et al. 2010].

MSCs are low-immunogenic both in vitro and in vivo [Horwitz 2001; Newman 2009], suggesting their utility for autologous as well as allogeneic transplantations.

Most of these data have been carried out in vitro and as of yet, MSCs have not been completely shown to be capable of regeneration or maintenance of a tissue in vivo.

Differences between fetal and adult MSCs

Several differences between fetal MSCs and adult MSCs were noted:

- fetal MSCs appear to have greater expansion capacity in vitro and faster doubling times than adult MSCs and this may be due to their having longer telomeres than adult MSCs [Guillot et al. 2007];
- fetal MSCs appear to lack some of the immune suppression properties observed in adult MSCs [Gotherstrom et al. 2003].
- fetal MSCs appear to lack HLA class II, in contrast to adult MSCs [Gotherstrom et al. 2003]
- fetal MSCs appear to synthesize HLA- G, which is absent in adult MSCs [Gotherstrom et al.2003].
- fetal MSCs express slightly different cytokine profile than adult MSCs. In summary, primitive MSCs have a greater ability to expand in culture perhaps due to their relative youth and have different physiology that is likely due to their naïve status.

These differences are similar those observed between umbilical cord blood and adult peripheral blood [Troyer & Weiss 2008].

Considering markers expression, WJ cells are CD45, CD34 and HLA class II negative; CD73, CD90, CD105 can HLA-class I positive. Similar to early passage both adult MSCs and WJC cells, they grow robustly, can be frozen/thawed, and

engineered to express exogenous proteins [Troyer & Weiss 2008]. Thus, the similarities of WJ cells and MSCs can be summarized as follows: WJ cells share the basic criteria used to define adult-derived MSCs. Additionally, stromal support, specific immune properties of low immunogenicity and immune suppression and the ability to migrate to pathology are taken to be properties of adult MSCs. These properties are observed in WJC cells too [Troyer & Weiss 2008].

1.1.1 Adult MSCs

Adult stem cells have been isolated from different tissues, such as bone marrow, brain, liver, skin, skeletal muscle, gastrointestinal tract, pancreas, eye, dental pulp, appendix, peripheral blood, blood vessels, teeth, heart, gut, liver, ovarian epithelium, and testis [Atala et al. 2007; De Coppi et al. 2006]. They are thought to reside in a specific area of each tissue (called a "stem cell niche"). Stem cells may remain quiescent (non-dividing) for long periods of time until they are activated by a normal need for more cells to maintain tissues, or by disease or tissue injury.

Scientific interest in adult stem cells has centered on their ability to divide or *self-renew* indefinitely, and generate all the cell types of the organ from which they originate, potentially regenerating the entire organ from a few cells (for an updated list visit **stemcells.nih.gov**).

The use of adult stem cells in research and therapy is considered to be non-controversial as they are derived from adult tissue samples. Their use avoid all the ethical problems associated with ESCs and so far they have been the only stem population to be employed in the clinical setting [Segers & Lee 2008].

Typically, there is a very small number of stem cells in each tissue, and once removed from the body, their capacity to divide is limited, making generation of large quantities of stem cells difficult. Scientists in many laboratories are trying to find better ways to grow large quantities of adult stem cells in cell culture and to

manipulate them to generate specific cell types so they can be used to treat injury or disease. Some examples of potential treatments include regenerating bone using cells derived from bone marrow stroma, developing insulin-producing cells for type 1 diabetes, and repairing damaged heart muscle following a heart attack with cardiac muscle cells.

Although there are several reports of pluripotent cells being isolated from adults, this work is in need of verification [Young et al. 1999; Young et al. 2001; Jian et al. 2002; Reyes et al. 2001; Kogler et al. 2004]. Such verification is important because an alternative source of pluripotent cells, cells derived from adults, offers the best of both worlds: pluripotent cells for therapeutics and cells that are collected with consent from adults (no controversy there).

1.1.2 Fetal MSCs

MSCs represent a minor fraction in bone marrow and other tissues. The exact amount is difficult to calculate since different methods used to collect the bone marrow affect the harvest, but it is estimated that MSCs comprise 0.001% to 0.01% of the total bone marrow, [Caplan 1994; Pittenger et al. 1999]. Furthermore, the prevalence of MSCs declines with advancing age. In the marrow of a newborn, one MSC is found among 10,000 nucleated marrow cells, as compared to one MSC per 250,000 nucleated marrow cells in the adult bone marrow and one per 2×106 in a 80-year old [Caplan 1994]. In 1st trimester fetal blood, one MSCs is found among 3000 nucleated cells and frequency declined with advancing gestation. [Campagnoli et al. 2001]. The amount of MSCs in different fetal tissues also varies. In 2nd trimester fetal tissues, one MSC is estimated to be present among 400 cells in the bone marrow, 1/700 in lung, 1/600 in spleen and 1/3500 in liver [in 't Anker et al. 2003]. The decrease of circulating MSCs in fetal blood during gestation and the higher frequency of MSCs in the 2nd trimester bone marrow

might be related to their migration from one hematopoietic site to another in the developing fetus. Different fetal tissues have been shown rich sources of MSCs and useful for therapies.

It's nevertheless true that cells residing in fetal organs (liver, lung, brain, etc.) are impossible to collect without causing serious health complications for the fetus/ baby/mother, so they have to be carefuly considered as interesting from the practical point of view.

Human ESCs and stem cells derived from human fetal tissues have raised moral/ ethical concerns that have yet to be adequately discussed and addressed by our society [Weiss & Troyer 2006]. So many different and useful sources which do not affect baby or mother can be considered, as fetal annexes, like cord blood, amniotic fluid, umbilical cord and placenta [Weiss & Troyer 2006].

1.2 Umbilical Cord Wharton's Jelly MSCs

Umbilical Cord tissues overview

The UC represents the link between mother and fetus during all pregnancy. It is composed of a special embryonic mucous connective tissue, called Wharton's jelly (WJ), lying between the covering amniotic epithelium and the umbilical vessels. The main role of this jelly-like material is to prevent the compression, torsion, and bending of the enclosed vessels, two arteries and one vein, which provide blood flow between fetal and maternal circulation [Can & Karahuseyinoglu 2007].

The human UC weighs approximately 40 g, its length reaches to approximately 60 cm, and it has a mean diameter of 1.5 cm at term [Raio et al. 1999; Di Naro et al. 2001]. It is covered by a single/multiple layer(s) of squamous-cubic epithelial cells

[Copland et al. 2002; Mizoguchi et al. 2004] called amniotic epithelium. Those epithelial cells display ultrastructural and functional characteristics to those seen in keratinocytes [Sanmano et al. 2005] and were shown to possess stem cell characteristics [Miki et al. 2005].

The inner tissue architecture includes a mucous connective tissue comprised of specialized fibroblast-like cells and occasional mast cells embedded in an amorphous ground substance rich in proteoglycans, mainly hyaluronic acid. Neither capillaries nor lymphatics are found in the UC (see **FIGURE 4**).

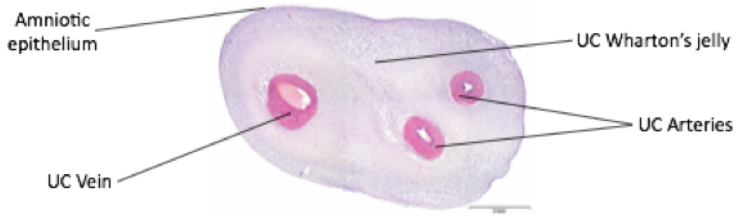


Figure 4: The umbilical cord (UC) in cross section.(modified from http://www.med.uio.no/dlo/mikro/Images/img03748.jpg)

MSCs isolation from Umbilical cord compartments: Wharton's Jelly MSCs

MSCs can be isolated from UC blood, placenta, perivascular areas, amniotic fluid, and from the tissue surrounding the UC vessels, the WJ [Weiss & Troyer 2006].

Different groups focused on different extraction protocols for MSCs from UC. As a result of short enzymatic digestion, Romanov et al. obtained cell populations positively differing from similar cell populations isolated from other sources alternative to bone marrow, like lipoaspirate [Romanov et al. 2003]. They reported only one contaminating cell type present (endothelial cells) that essentially does not affect the final outcome, while other cellular components of the venous wall or

Wharton's jelly were reported to be absent in the cell suspensions, due to the short time of incubation with collagenase [Romanov et al. 2003].

In 2006 several groups demonstrated that cells from UC's WJ share properties of MSCs, like similar surface phenotype and differentiation capability [Weiss & Troyer 2006; Conconi et al. 2006; Lu et al. 2006]. Moreover these cells are more similar to fetal MSCs in terms of their in vitro expansion potential.

While MSCs have been isolated from several UC compartements [Weiss et al. 2006 (**a**); Can & Karahuseyinoglu 2007; Secco et al. 2007], the choice of focusing on only one of them, the mesodermal one, could provide a purer derived population, avoiding any contamination and selecting a source embryologically nearer to MSCs tissue of origin [Baksh et al. 2007; Secco et al. 2007].

WJ is named for the English physician and anatomist Thomas Wharton (1614-1673) who first described it in his publication Adenographia, or "The Description of the Glands of the Entire Body", first published in 1656 (see **FIGURE 5**).

Figure 5: Thomas Wharton.

Due to many reports and careful examination of cellular and ECM components, human UC shows a tissue compartmentalization in which cellular characteristics and ECM elements differ from each other.

In past five years three different research labs have published on the isolation and characterization of cells from the Wharton's jelly: Dr. Weiss' lab at the Kansas State University, Dr. Davies' lab at the University of Toronto and Dr. Y. S. Fu at the National Yang-Ming University, Taipei [Weiss et al. 2006 (**a**); Sarugaser et al. 2005; Fu el al. 2006]. All three groups reported that Umbilical Cord Matrix (UCM) cells are MSC-like cells and are robust. These cells can be isolated easily, frozen/ thawed, clonally expanded, engineered to express exogenous proteins, and extensively expanded in culture. In addition, human UCM cells express a marker of neural precursors, nestin, without exposure to differentiation signals [Weiss et al. 2006 (**a**); Fu el al. 2006].

Whether UCM cells are MSC-like or fit into a unique niche is currently not clear.

It has been demonstrated that UCM cells express the pluripotency gene mar- kers Oct-4, nanog, and Sox-2 at low levels relative to ESCs [Carlin et al. 2006]. On the other hand, these findings may serve as evidence that the cord matrix cell population has a subset of primitive stem cells.

From these findings, it is suggested that UCM cells offer advantages over stem cells as a source of therapeutic cells. First, UCM cells are derived from a noncontroversial, inexhaustible source, and can be harvested noninvasively at low cost. Second, unlike human ESCs, UCM cells did not induce teratomas or death after 1×10^6 to 6×10^6 human UCM cells were transplanted either intravenously or subcutaneously into severe combined immunodeficient mice [Weiss et al. 2006 (**b**)].

A major potential application of these cells in medicine is for "tissue engineering," in which the ultimate goal is to provide "off-the-shelf tissues" and organs. UCM cells demonstrate excellent cell growth properties on bioabsorbable polymer constructs [Kadneret al. 2004].

In recent years, several investigators published protocols for isolating MSCs from the UC tissue [Karahuseyinoglu et al. 2007; Lund et al. 2007]. Basically, the isolation procedure starts with the removal of umbilical vessels. The cord is then cut down to smaller segments or chopped into small pieces which are subsequently enzymatically digested [Fu et al. 2006]. Alternative isolation methods without removal of vessels and without enzymatic digestions or explant cultures have also been described [Moretti et al. 2010]. To isolate cells from the perivascular tissue or the subendothelium of the umbilical vein, further methods have been established [Sarugaser et al. 2005].

Moretti et al. have used a protocol without enzymatic digestion and without removal of umbilical vessels to isolate MSC-like cells from whole UC tissue in an explant culture approach [Moretti et al. 2010].

Technical procedures to isolate MSCs from the WJ are still poorly investigated and vary dramatically depending on the authors [De Bruyn et al. 2010]. Trypsin or other enzymes such as hyaluronidase have been frequently but not systematically added to the collagenase, and the incubation time also varies from 4 to >24h. Moreover, some authors have removed the cord vessels by stripping them manually before enzymatic treatment. Several groups do not use enzymatic treatment, but most of them dissected the cord segment into very small pieces (1–3 mm^3), with or without discarding the cord vessels [De Bruyn et al. 2010].

Furthermore, UC-derived cells could be efficiently cryopreserved and revitalized. Using a cryo-medium containing 80% human serum, 10% culture medium, and 10 % DMSO Moretti et al. reported cell survival rate after rapid thawing at 37°C of 75 $\pm$12.8% [Moretti et al. 2010].

UC stromal cells characterization

In contrast to other progenitor cell populations, such as, for instance, hematopoietic stem cells, there is currently no specific marker available defining human MSCs. Several authors have contributed to the definition of markers useful for BM-MSC immunophenotyping. Several recent reports have extended the range of markers that help characterize and identify MSC from different sources. In particular, WJ-derived MSC have undergone an unprecedented characterization process further extending the similarities (and differences) with respect to other MSC populations [Anzalone et al. 2010].

The expression of a set of markers combined with the demonstration of in vitro multi-lineage differentiation potential is necessary to identify MSCs in UC- derived cell populations [Dominici et al. 2006].

The surface antigen CD105, CD73, and CD90 are widely used for the identification of UC-derived stromal cells, as these markers are proposed by the ISCT as positive markers for human MSCs [Dominici et al. 2006].

However, these epitopes are also expressed by hematopoietic and endothelial cells, which are two potential contaminants in UC-derived cell populations. Consequently, it is necessary to carefully exclude cells from hematopoietic or endothelial origin using surface markers, such as CD45, CD34, or CD31. UC-derived stroma cells were found positive for pluripotency markers usually expressed by ESCs such as Sox-2, which underlines their primitive nature [Moretti et al. 2010]. With growing evidence that MSC-like cell population isolated from UC tissues are rather heterogeneous, at least in regard to primitive marker expression, the identification of a universal marker defining primitive human MSCs remains challenging (see **Table 3**). The most convincing biological property for the identification of MSCs remains the capability to differentiate into mesodermal lineages.

Markers	BM-MSCs	WJ-MSCs
CD10	+	+
CD13	+	+
CD14	-	-
CD29	+	+
CD31	-	-
CD33	-	-
CD34	-	-
CD44	+	+
CD38	-	-
CD45	-	-
CD49e	+	+
CD51	+	+
CD54	+	NA
CD56	+	-
CD59	+	NA
CD68	NA	+
CD71	+	NA
CD73	+	+
CD79	-	NA
CD80	-	+
CD86	-	-
CD90	+	+
CD105	+	+
CD106	-	+/-
CD117	-	+
CD163	NA	-
CD166	+	+
CD235a	-	NA
HLA-A	+	+
HLA-B	+	+
HLA-C	+	+
HLA-DR	-	-
HLA-G	+	+
HNF-4α	NA	+
Vimentin	+	+
Sox2	+	+
Nestin	+	+
Gd2	+	+
STRO-1	-	+/-
NA: not applicable		

Table 3: Intra- and extra-cellular markers of BM- and WJ- derived MSCs are reported. (all references in: Anzalone et al. Stem Cells Dev 2010; 19:423-38; Moretti et al. Adv Biochem Eng Biotechnol 2010;123:29-54)

UC stromal cells differentiation

The differentiation repertoire of stroma cells derived from UC tissue reported in the literature till July 2009 is summarized in **Table 4**.

Cell type	
Mesodermal lineage	Adipocyte Chondromyocyte Osteocyte Cardiomyocyte Skeletal myocyte Endothelial myocyte
Ectodermal lineage	Neuronal cells
Endodermal lineage	Islet-like cells Immature hepatocyte

Table 4: Differentiation potential of stroma cells derived from human umbilical cord tissue reported in the literature till July 2009 (all references in: Moretti et al. Adv Biochem Eng Biotechnol. 2010;123:29-54).

As the UC stromal cells originated from extraembryonic mesoderm, adipogenic, chondrogenic, osteogenic, cardiomygenic, and skeletal myogenic inductions have been the most studied cell lineages with *in vitro* and *in vivo* studies [Beyer Nardi & da Silva Meirelles 2006; Can & Karahuseyinoglu 2007; Weiss & Troyer 2006; Weiss et al. 2006 (**a**); Troyer & Weiss 2008]. Some of these findings are summarized in **Table 5**.

Differentiation type	Cell type
In vitro	Adipocyte Chondrocyte Osteocyte Cardiomyocyte Skeletal myocyte Neuronal/glial precursor Dopaminergic neuron Endothelial cell
*In vivo**	Dopaminergic neuron Photoreceptor rescue Endothelial cell Skeletal myocyte
* in rodents	

Table 5: In vitro and in vivo differentiation competence of the human umbilical cord stromal cells (all references in: Can & Karahuseyinoglu Stem Cells 2007;25:2886-2895.)

Increasing evidence, indeed, indicates that MSC populations are heterogeneous with coexisting subsets having varying potency; this applies to bone marrow MSCs as well as to MSCs from other tissues. I.e. Karystinou et al. reported that human synovium-derived clonal MSCs were all capable of osteogenic and chondrogenic differentiation although with varying potency, but only 30% of the clonal populations tested were able to differentiate into adipocytes [Karystinou et al. 2009].

Considering UC stromal cells, most studies agree that WJ-MSC can be successfully induced toward connective tissue phenotypes (osteoblasts, adipocytes,

and chondrocytes), thus opening new paths in regenerative medicine applications , i.e., to the musculoskeletal system [Anzalone et al. 2010].

Cells isolated from perivascular tissues of the umbilical vein showed a high osteogenic potential with spontaneous formation of bone nodules [Sarugaser et al. 2005], which was even evaluated higher than the potential of bone-marrow MSCs in a comparative study [Baksh et al. 2007]. Recently, two sub-populations were evidenced in cultures of WJ-derived MSCs with regard to the expression of vimentin and pan-cytokeratin filaments [Karahuseyinoglu et al. 2007].

In 2003, Mitchell et al. reported that UCM cells can be induced in vitro to become cells with morphological and biochemical characteristics of “neural-like” cells expressing neuron-specific enolase, as well as other neural cell markers [Mitchell et al. 2003]. The multilineage potential of UCM stem cells was also verified by induction in vitro into chondrogenic, osteogenic, and adipogenic lineages [Wang et al. 2004; Fu et al. 2006]. Differentiation to cardiomyocytes was also reported but remains controversial. I.e. Wang et al. demonstrated for instance that WJCs could be induced to cells exhibiting cardiomyocyte morphology and expressing specific markers (N-cadherin and cardiac troponin) using 5-azacytidine or cardiomyocyte-conditioned medium [Wang et al. 2004]. I.e. Kadner et al. minced either UC vessels or whole cord to derive an autologous cell source of myofibroblasts for cardiovascular tissue engineering [Kadner et al. 2002], and WJ cells as well were described as “smooth muscle cells”, and named “myofibroblasts” after in situ labeling of vimentin, desmin, α-actin, and myosin [Kadner et al, 2004]. Sarugaser et al. investigated human umbilical cord perivascular cells, which were discarded, or not specifically isolated, in the previous studies, but they identified it as a subpopulation that, when isolated, would be capable of exhibiting a functional mesenchymal phenotype [Sarugaser et al. 2005].

In addition, it has been shown that UC-MSCs can successfully differentiate i.e. to endothelial cells after addition of VEGF and b-FGF [Kestendjieva et al. 2008] and

can form vessel-like structures in matrigel cultures [Kestendjieva et al. 2008; Chen et al. 2009]. For instance, WJ cells (WJCs) could be induced to skeletal myocytes when placed in myogenic medium [Conconi et al. 2006].

Different reports about trans differentiation of UCB cells into neural cells aroused great interest for investigators for their clinical implication and significance in central nervous system, and pushed them to focus on UC stromal cells as source for isolation, expantion and differentiation into neural cells [Jeong et al. 2004; Ma et al. 2005]. WJ MSCs after induction expressed nestin, beta-tubulinIII, neurofilament and glial fibrillary acidic protein [Ma et al. 2005].

Several attempts have been made to use stem cells from different origins, including bone marrow stem cells, neuroepithelical stem cells, fetal neural stem cells, and umbilical cord blood cells, for treatment of focal ischemia [Jomura et al. 2007]. Therapeutic effects of rat umbilical cord matrix cells transplantation in treating cerebral global ischemia were evaluated using a reproducible model in rats, with promising results (indirect reduction of the percentage of damaged hippocampal neurons after cardiac arrest and resuscitation) [Jomura et al. 2007].

Mitchell et al., using a similar approach, reported that the fibroblast-like cells of WJ could be induced to differentiate into “neural-like” cells expressing neuron-specific enolase, as well as other neural cell markers.

Transplanted human umbilical cord (HUC) MSCs migrated towards the ischemic boundary zone and differentiated into glial, neuronal, doublecortin^{+}, CXCR4^{+}, and vascular endothelial cells to enhance neuroplasticity in the ischemic brain. In addition, HUCMSCs transplantation promoted the formation of new vessels to increase local cortical blood flow in the ischemic hemisphere [Ding et al. 2007].

Recent findings suggest that UC-MSCs can differentiate into endodermal lineages. Campard et al. reported that UC-matrix cells constitutively expressed markers of hepatic lineage, such as albumin, alpha-fetoprotein, cytokeratin-19, connexin-32,

and dipeptidyl peptidase IV. After in vitro hepatic induction, cells exhibiting a hepatocyte-like morphology with hepatic features such as specific markers up-regulation and urea production were observed. However, the authors pointed out that their cells lack important characteristics of functional liver cells and thus conclude that UC-matrix cells can be differentiated at least to immature hepatocytes [Campard et al. 2008].

At present time, results of studies comparing the differentiation potential of UC-derived MSCs with other sources should be carefully interpreted. More work is needed to attest whether cells isolated from a defined compartment of the UC is more suitable for a specific differentiation lineage. This information would be of tremendous importance for clinical applications of UC-derived MSCs, WJ cells too.

1.3 Periodontal Ligament MSCs

Periodontal Ligament overview

Periodontium refers to the specialized tissues that both surround and support the teeth, maintaining them in the maxillary and mandibular bones (see **FIGURE 6**).

The periodontal ligament (PDL) is a connective tissue attached to tooth and alveolar bone. The alveolar process is the thickened ridge of bone that contains the tooth sockets on bones that bear teeth. It is also referred to as the alveolar bone. Bundle bone is a histologic term for the portion of the bone of the alveolar process that surrounds teeth and into which the collagen fibers of the periodontal ligament are embedded. It can also be referred to as alveolar bone proper [Mörmann & Ciancio 1997; Beertsen et al.1997].

Teeth are epithelio-mesenchymal organs developing and functioning in intimate coordination with periodontal tissues and bone [Fleischmannova et al 2010]. The

tooth-alveolar bone complex develops in the context of continuing epithelio-mesenchymal interactions between the oral ectoderm and the mesenchymal cells of neural crest origin.

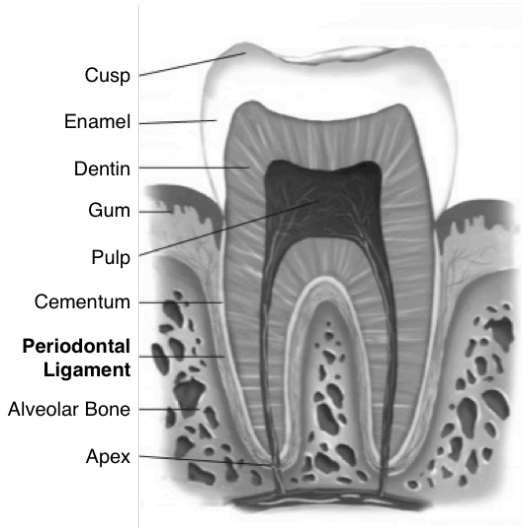


Figure 6: Periodontal ligament anatomy (modifed by: http://www.studiodentaire.com/en/glossary/tooth.php)

Tooth development starts with a locally circumscribed thickening of the oral ectoderm that induces condensation of the local neural-crest-derived mesenchyme. Subsequently, at the bud stage, the odontogenic potential shifts to the dental mesenchyme, and signals from the dental mesenchyme induce formation of the epithelial signaling center regulating the bud-to-cap transition, the primary enamel knot [Jernvall et al., 1998]. During the bell stage, mesenchymal cells in contact with the dental epithelium differentiate into dentin- producing odontoblasts, and the adjacent layer of inner enamel epithelium cells differentiates into enamel-producing ameloblasts. The most superficial mesenchymal part of the tooth germ, the dental follicle surrounding the tooth epithelium and mesenchymal papilla, gives rise to periodontal structures: cementum, PDL and alveolar bone.

During the eruption process, tooth roots are formed, and molecular interactions between tooth root epithelium and the dental follicle mesenchymal cells direct periodontium formation [Fleischmannova et al 2010] (see **FIGURE 7**).

Signaling interactions between dental follicle cells and the Hertwig's epithelial root sheath (HERS) result in the development of periodontium cementoblasts, osteoblasts, and fibroblasts from the mesenchymal cells of the dental follicle [Nakatomi et al. 2006]. Fibroblasts give rise to periodontal ligament fibers that are embedded in cementoblast-produced cementum at the side adjacent to the root dentin and in alveolar bone produced by dental follicle osteoblasts at the side adjacent to the jawbone.

Figure 7: Tooth development is traditionally considered a series of stages that reflect key processes.

Cementoblasts are believed to differentiate from dental follicle cells migrating through the increasing space between the HERS cells during HERS fenestration [Diekwisch 2002]. Between cementoblasts and osteoblasts of the alveolar bone, the PDL is maintained. The PDL is highly vascularized and innervated region with heterogeneous populations, as well as a source of signaling factors and immature cells for periodontal homeostasis and regeneration.

Different periodontal structures develop from initially homogenous mesenchymal cells of the dental follicle. The tightly balanced interaction in the development and maintenance of the tooth-periodontium-bone complex can be shown by how the failure of one component affects the others [Blair et al., 2008]. Disturbances in bone homeostasis causing decreased systemic or periodontal bone mineralization (periodontitis, osteoporosis, osteolysis) may result in premature tooth loss.

Periodontal ligament stem cells

Human periodontal ligament stem cells (PDLSCs) are a unique population of MSCs which demonstrate the capacity to generate cementum- and periodontal ligament-like structures in vivo. As such, PDLSCs represent a promising cell-based therapy in reconstructive dentistry for the treatment of periodontal disease. [Mrozik et al. 2010]

The presence of multiple cell types (fibroblasts, cementoblasts, and osteoblasts) within the postnatal periodontal ligament suggests that these cells may share common ancestors. The possibility that progenitor cells might exist in the postnatal periodontal ligament has been recognized but not even demonstrated in the past [Melcher et al. 1985]. Anyway these cells were believed to provide a renewable cell source for normal tissue homeostasis and periodontal wound healing [Pitaru el al. 1994; Gould et al. 1980].

Recently, multipotent stem cell populations, named periodontal ligament stem cells (PDLSCs), have been isolated from the periodontal ligament of extracted human third molar teeth [Seo et al. 2004]. These PDLSCs have been shown able to get rise to adherent clonogenic clusters that resemble fibroblasts; to be capable of developing into adipocytes, osteoblast- and cementoblast-like cells in vitro; to be capable of producing cementum- and periodontal ligament-like tissues in vivo [Seo et al. 2004; Gronthos et al. 2006; Shi et al. 2005]. PDLSCs express an array of cementoblast and osteoblast markers as well as several bone marrow-derived mesenchymal stem/stromal cell associated markers [Gronthos et al. 2000; Trubiani et al. 2005]. The similarity between PDLSCs and bone marrow MSCs suggested that PDLSCs represent another MSC-like population. On the other hand also research efforts to identify markers uniquely expressed by PDLSCs has been useful to discriminate these cells from other types of MSC-like cells in dental tissues [Chen et al. 2006].

The first reported isolation and identification of MSCs in human periodontal ligament was in 2004 [Seo et al. 2004]. Since then, there has been considerable activity trying to understand the function of these cell populations and their interactions with each other with a view to laying the fundamental groundwork for clinical applications in regenerative periodontics. A number of studies have now been carried out to confirm the presence of MSC-like cells in the periodontal ligament. These have not been limited to human but also include mouse, rat, and sheep [Gronthos et al. 2006; Trubiani et al. 2005; Nagatomo et al. 2006; Jo et al. 2007; Ivanovski et al. 2001; Luan et al. 2006; Techawattanawisal et al. 2007]. All of these studies have confirmed the multipotent nature of PDLSCs, and while the initial studies indicated this to include an ability to differentiate into osteoblast, cementoblast, or adipogenic phenotypes, at least one recent study has indicated an ability of these cells to also differentiate into neuronal precursors [Techawattanawisal et al. 2007]. Importantly, cryopreservation does not seem to alter the functional properties of PDLSCs [Seo et al. 2005]. This will have significant relevance should “banking” of these cells become a clinical necessity.

Identification of stem cells in postnatal dental tissues has presented exciting possibilities for the application of tissue engineering as well as gene- and cell-based therapies in reconstructive dentistry. The use of stem cells with these technologies may constitute novel strategies for regenerative periodontal therapy.

Periodontal diseases are infectious diseases characterised by irreversible destruction of supporting tissue for tooth, including periodontium, cementum, alveolar bone, and gingiva [Seo et al. 2004; Philstrom et al. 2005]. These changes often lead to an aesthetically and functionally compromised dentition. Thus, regeneration of these tissue lost through periodontitis is the ultimate goal of periodontal therapy [Yang et al. 2010]. Periodontal regeneration can be defined as the complete restoration of the lost tissues to their original architecture and function by recapitulating the crucial wound healing events associated with their

development [Polimeni et al 200; Lin et al. 2008]. The isolation of adult stem cells from human periodontal ligament has presented new opportunities for tissue engineering [Gronthos et al. 2006; Nagatomo et al. 2006] (see **FIGURE 8**).

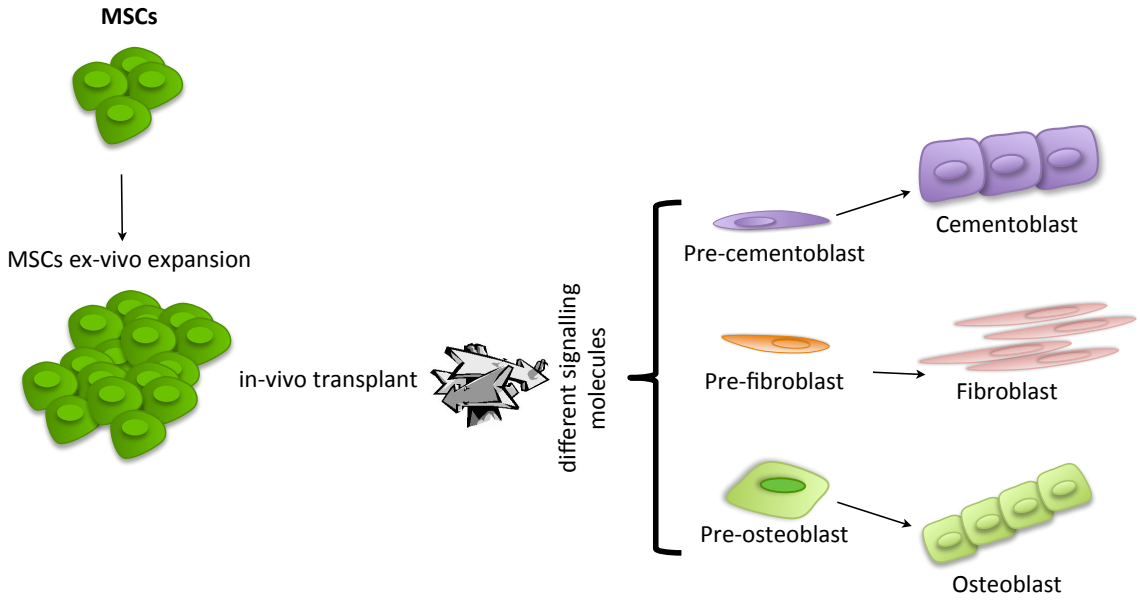


Figure 8: Strategies to promote periodontal regeneration and possible differentiating directions of adult bone.

Periodontal ligament stem cells characterization and differentiation

The interest in dental stem cells has risen during last years and several reports on characterisation and differentiation of dental stem cells from different sources have been published [Lindroos et al. 2008]. To date, the surface marker expression data published on dental stem cells do not allow extensive comparison between other sources of stem cells in the body.

The FACS analysis demonstrated that PDLSCs showed positive expression for the putative stem cell marker STRO-1; adhesion molecules CD9, CD29, CD49d, CD105, CD106, and CD166; receptor molecule CD44; surface enzymes CD10

and CD13, extracellular matrix protein CD90; complement regulatory protein CD59; and hFSP. PDLSCs lacked expression of CD31 and CD45, suggesting lack of cells of haematopoietic and angiogenic lineages [Lindroos et al. 2008].

PDLSCs have the capacity to commit to the cementoblastic, osteoblastic, and adipogenic lineages [Seo et al. 2004; Jo et al. 2007].

Ex-vivo expanded PDLSCs formed mineralized nodules with the presence of calcium in the extracellular matrix and expressed an array of cementoblastic/ osteoblastic markers, including alkaline phosphatase, MEPE (matrix extracellular phosphaglycoprotein), bone sialoprotein, osteocalcin, and TGFβ receptor type I. Moreover, PDLSCs cultured with an adipogenic inductive cocktail could differentiate into Oil red O-positive lipid-laden fat cells, and this was correlated with an upregulation in the expression of two adipocyte specific transcripts—PPRAγ2 and lipoprotein lipase, as detected by Reverse trancriptase-polymerase chain reaction (RT-PCR) [Silverio et al. 2008; Jo et al. 2007].

To explore the role for enamel matrix proteins in periodontal regeneration, the effect of enamel matrix derivative (EMD) on PDL cells in vitro was examined largely [Gestrelius et al. 1997; Lyngstadaas et al 2001]. EMD in vitro formed protein aggregates, providing a unique environment for cell-matrix interaction. Under these conditions, BMD enhanced proliferation of PDL cells, and promoted mineral nodule formation [Gestrelius et al. 1997; Lyngstadaas et al 2001].

Through *in vivo* experiments, PDLSCs transplanted into immunocompromised mice and rats were shown off to be able to promote tissue regeneration and periodontal repair [Morsczeck et al. 2008].

Different studies have shown also that human periodontal ligament cells produce NGF and express trkA mRNA [Kurihara et al. 2003]. These findings suggest that NGF modulates the differentiation and proliferation of periodontal ligament cells by paracrine and autocrine functions in vitro and in vivo. RT-PCR showed that NGF

increased mRNA levels ALP, bone morphogenetic protein (BMP-2), and osteopontin (OPN), which are involved in periodontal tissue regeneration [Kurihara et al. 2003].

Chapter 2

MSCs and osteogenic differentiation

Bone is a hallmark of all vertebrates and absolutely essential in terms of organ protection and support, brain and lung function, locomotion, support of haematopoiesis in the bone marrow, storage of minerals (i.e. calcium, phosphate) and providing attachment to muscles [Proff & Römer 2010]. Bone tissue is largely composed of type I collagen amounting to about 90% of total bone protein. The remaining organic component consists of non-structural proteins like growth factors, blood protein, osteonectin and osteocalcin (see **FIGURE 1**).

The inorganic phase of bone is mainly composed of mineral hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$). Due to its material consistency, bone has a relatively flexible character as well as compressive strength. The mature bone is composed of two different types of tissue:

- *cortical bone*: responsible for the stability of the skeleton, it appears smooth, white and solid. The cortical bone contains osteons (Haversian systems), which are composed of a central canal (Haversian canal) surrounded by lamellae of bone matrix. Within the lamellae, there are osteocytes embedded in tiny spaces (lacunae). The Haversian canal encompasses blood vessels and nerve cells throughout the bone and communicates with osteocytes in lacunae through canaliculi. Moreover, the corticalis has an outer membrane, the periosteum. The periosteum consists of an outer fibrous layer and an inner one that has

osteogenic potential and enables the bone to enlarge [Katagiri & Takahashi 2002].

- *cancellous bone*: the inside of bone is assembled by a trabecular network (spongiosa) and harbours bone marrow or embryonic connective tissue. The spongiosa ensures elasticity and stability of the skeleton and accounts for the main part (about 70%) of bone metabolism [Neumann & Schett 2007].

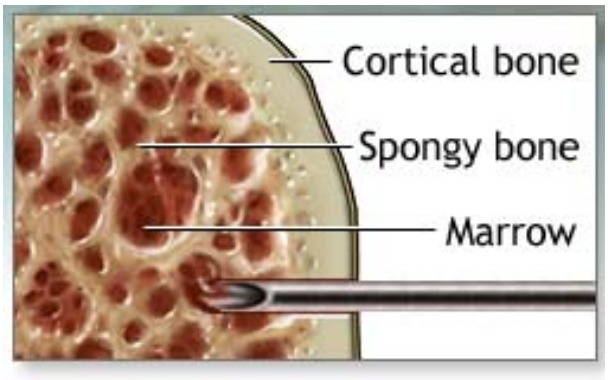


Figure 1: Bone shematic rapresentation (modified by http://www.nlm.nih.gov/medlineplus/ency/images/ency/fullsize/1129.jpg)

Under control of specific growth and transcriptional factors, mesenchymal stem cells differentiate toward osteogenic lineage during a number of developmental stages, starting from commitment to osteoprogenitors, through preosteoblasts and osteoblasts and finally osteocytes or lining cells [Long 2001].

It is thought that during the early stages, commitment osteoprogenitors maintain certain degree of plasticity allowing de- and trans- differentiation to other mesenchymal lineages, whereas osteoblasts and osteocytes represent a terminal differentiation stage as they become specialized functional cells [Rottmar et al. 2010]. However, it has also been suggested that even mature osteoblasts are being able to transdifferentiate to other phenotypes [Aubin 1998].

2.1 Bone modeling and remodeling

In order to maintain stability and integrity, bone is constantly undergoing remodelling, with about 10% of bone material being renewed each year [Lerner 2006].

Bone remodelling is a complex process that involves bone resorption performed by osteoclasts, followed by bone formation carried out by osteoblasts. In this process, these cells closely collaborate in basic multicellular units (BMU) [Hadjidakis & Androulakis 2006; Hill 1998]. The bone remodelling cycle involves different sequential steps, namely resorption, reversal and formation [Hadjidakis & Androulakis 2006; Hill 1998; Neumann & Schett 2007]. Different cells populations are involved (see **FIGURE 2**):

- *Osteoblasts*: Active osteoblasts are cuboidal, polarized bone matrix producing cells. Similar to fibroblasts, myoblasts, chondrocytes and adipocytes, osteoblasts originate from MSCs located in the bone marrow, endosteum and periosteum. During differentiation of multipotent mesenchymal cells into several lineages, the progenitors of these lineages acquire specific phenotypes under the control of regulatory factors of the restricted lineages [Karsenty & Wagner 2002, Rosen & Spiegelman 2000, Arnold & Winter 1998]. Osteoblasts deposit osteoid, the unmineralized extra cellular matrix (ECM), which subsequently becomes calcified.
- *Osteocytes*: During bone formation process, a proportion of cells becomes trapped within the lacunae of the matrix and are termed osteocytes. Osteocytes are connected by a system of canaliculi, and their proposed function is to regulate the response of bone to mechanical stimuli [Ehrlich & Lanyon 2002].
- *Lining cells*: The other proportion of osteoblasts becomes bone-lining cells, which are flat cells lining the surface of bone [Aubin 1998].

- *Osteoclasts*: bone resorbing cells belong to the family of monocyte/macrophage lineage. Osteoblasts express in vivo the receptor for activation of nuclear factor kappa B (NF-κB) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) , which in turn activate a number of signaling pathways in osteoclasts, such as NF-κB pathway [Teitelbaum & Ross 2004].

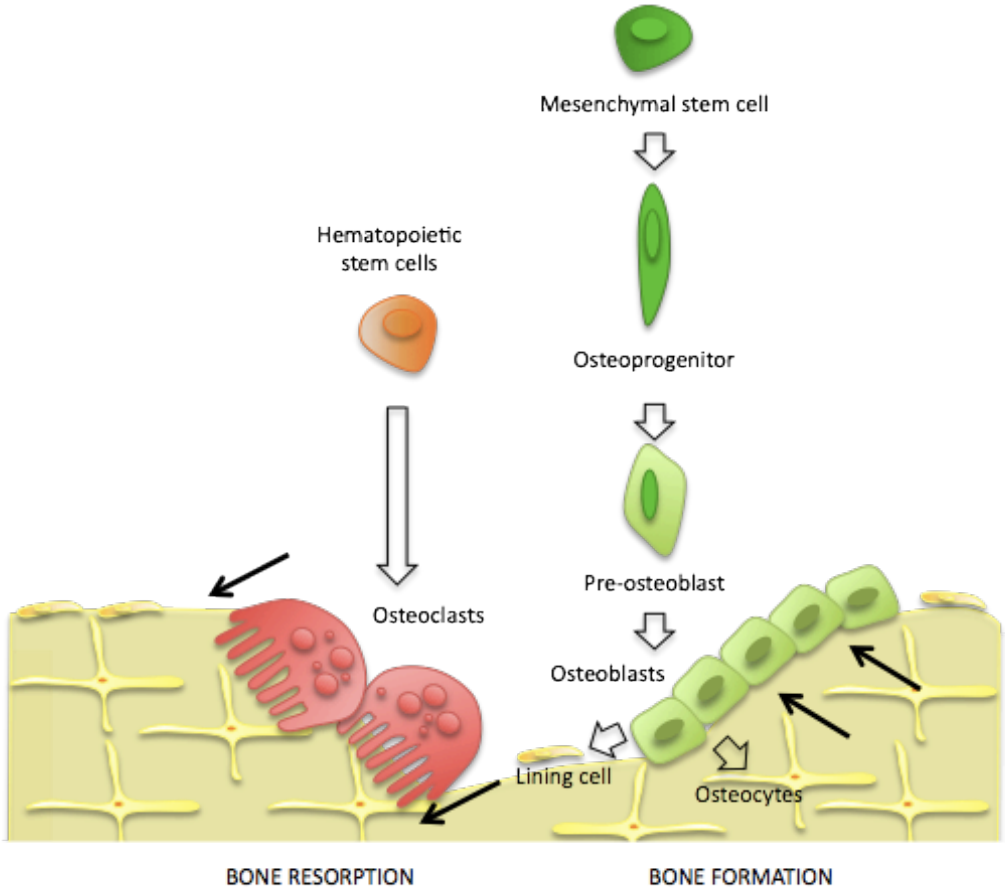


Figure 2: Overview of bone remodelling. During resorption phase osteoclasts degrade old bone matrix; after bone removal, osteoclasts undergo apoptosis and osteoblasts become activated and lay down new bone material in the trench. With the replacement of old bone by new one, the osteoblasts form resting flattened lining cells on the surface of bone, while the embedded in bone matrix one evolve to osteocytes.

2.2 MSCs osteoblast differentiation: different signalling pathways.

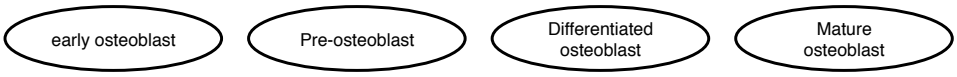
The population of cells that is committed to the osteoblastic phenotype are called osteoprogenitors. Such cells divide and differentiate into osteoblasts forming bone. Analysis of fetal rat calvaria-derived osteoblast cultures (RC cells) has indicated that less than 1% of cells are actually destined to form bone [Aubin et al. 1995; Malaval et al 1999].

Continuous recruitment, proliferation and differentiation of cells within bone tissue is regulated by the expression of genes providing the characteristics to the bone phenotype. A precise pattern for the expression of marker genes encoding the osteoblast phenotype has been shown [Calvi et al. 2003; Heino & Hentunen 2008; Lo Celso et al. 2009], which can be subdivided in three chronologically related distinct stages:

- *proliferation phase*: high mitotic activity that is accompanied by the expression of cell-cycle genes, such as those encoding for histones, and cell growth genes, such as C-myc, C-fos, and C-jun. During this period, genes associated with the formation of ECM, such as collagen type I, osteopontin (OPN), and fibronectin are actively expressed, then gradually down regulated; following the down-regulation of the proliferation genes, an increase in ALP activity is evident.
- *matrix development phase*: composition and organization of the ECM is greatly modified, providing an environment favourable for mineralization; an increase in ALP activity is still evident.
- *mineralization phase*: coordinated by the osteoblasts and involves the deposition of a calcium phosphate apatite within an organic framework.

Commitment of bone marrow originated MSCs to tissue-specific cells is orchestrated by transcriptional and biochemical regulators [Marie 2008; Katagiri & Takahashi 2002]. Osteoblast differentiation, recruitment, function and maturation is tightly promoted and regulated by the secretion of lipid-modified glycoproteins of

the wingless (Wnt) family, BMP-2 and several transcription factors [Proff & Römer 2010]. An overview of these modification is represented in **Figure 3**.



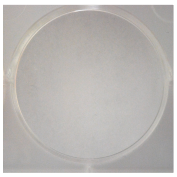
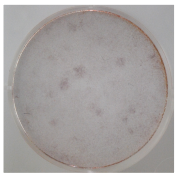
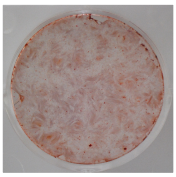
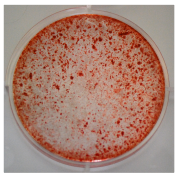
Markers	Runx2	Runx2 Col II Col IX	Osteopontin Col II Col IX	Osteopontin BspII
Alp activity	-	+	++	++
Mineralization				

Figure 3: Markers modification during MSCs differentiation through osteoblast lineage.

A central regulator of bone formation is Runx2, also known as Core-binding factor α1 (Cbf α1), a member of the Runx (Runt-related factors) family of transcription factors. The family members, Runx1 (Acute myeloid leukemia gene (AML) -1), Runx2 (AML3), and Runx3 (AML2), are encoded by distinct genes but share a common DNA recognition motif. Runx2 activates the OCN and collagen type I- α 1 genes [Komori 2008], and serves as an initial marker of the osteogenic cell lineage [Franceschi 2007]. Runx2 is abundantly expressed in calcified cartilage and bone tissues and is transcribed from two separate promoters. The upstream promoter drives the expression of osteoblast-specific isoforms, whereas the second promoter drives the expression of isoforms that are mainly expressed in T-cells, but they can be found also in osteoblasts and other mesenchymal cells [Xiao et al. 1998; Ogawa et al. 2000].

During osteoblast differentiation, RUNX2, and canonical Wnt signaling play essential roles in the commitment of pluripotent mesenchymal cells to the osteoblastic lineage [Komori et al. 2006]. After commitment into the osteoblastic lineage, the osteoblasts express bone matrix protein genes at various expression

levels depending on the maturation level of the cells. Immature mesenchymal cells and preosteoblasts weakly express Col1a1, and its expression is upregulated in immature osteoblasts [Aubin & Triffitt 2002; Inada et al. 1999]. Immature osteoblasts express Spp1 and then Ibsp, and maturated osteoblasts strongly express Bglap (bone gamma-carboxyglutamate [gla] protein/ osteocalcin) [Aubin & Triffitt 2002; Maruyama et al. 2007]. Mature osteoblasts are embedded into the bone matrix and finally become osteocytes, which express Dmp1 (dentin matrix protein 1) [Toyosawa et al. 2001] (see **FIGURE 4**).

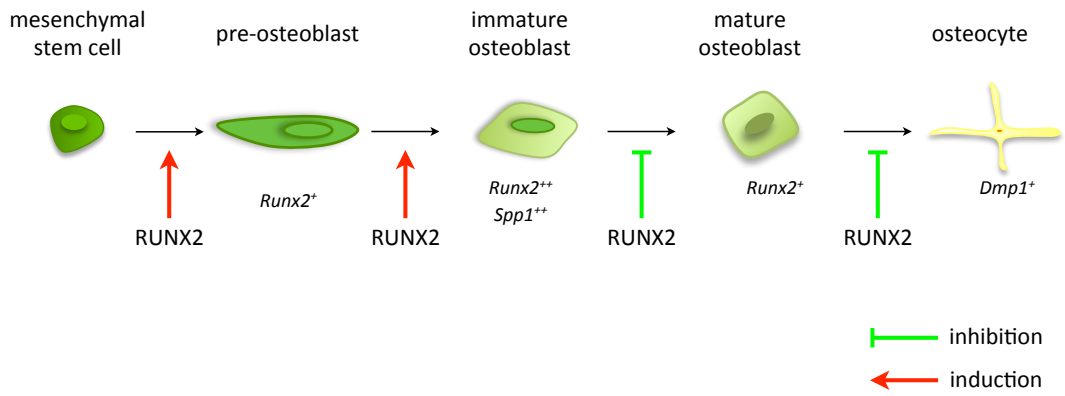


Figure 4: RUNX2 directs pluripotent mesenchymal cells to the osteoblast lineage, increases the number of immature osteoblasts, but inhibits osteoblast maturation. Preosteoblasts express Runx2. Immature osteoblasts express Runx2 and Spp1. Mature osteoblasts Runx2 expression is down-regulated. Osteocytes express Dmp1+. The transition of immature osteoblasts to osteocytes occurs at an early stage of bone development.

Targeted disruption of Runx2 results in the complete lack of bone formation by osteoblasts, revealing that Runx2 is essential for both endochondral and intramembranous bone formation [Komori 2008].

Runx2 has been designated as the most pleiotropic regulator of skeletogenesis [Karsenty & Wagner 2002], it functions as an inhibitor of proliferation of progenitors [Pratap et al. 2003], and is also required for osteoblast function beyond differentiation [Ducy et al. 1999; Liu et al. 2001].

Even if several findings support this biochemical function of Runx2, the number of Runx2-modulated genes that have been identified thus far is limited [Lambertini et

al. 2007]. Bone is also a specific estrogen target tissue. Both isoforms of estrogen receptors alpha and beta (ERα and ERβ), through which estrogen is involved in the regulation of factors associated with bone formation were detected in functional osteoblasts [Sutherland et al. 1996; Sasaki-Iwaoka et al. 1999; Nilsson et al. 2009].

2.3 MSCs and tooth tissue development.

Facial bone and dental development

Teeth are generated through highly orchestrated mutual inductive interactions between stomodeal ectoderm and cranial neural crest-derived ectomesenchyme cells [Koussoulakou et al. 2009]. Genes encode transcription factors that regulate the synthesis of various signaling factors [Thesleff et al. 1995].

Proper development of all tooth-periodontium-alveolar complex structures is achieved by reiterative temporo-spatially well-coordinated signaling interactions [Thesleff & Sharpe, 2006; Hu & Simmer, 2007].

These signaling factors mediate inductive interactions between the odontogenic tissue layers and affect cell multiplication, cell death and cytodifferentiation [Matalova et al. 2004].

Interestingly, genes and signaling factors playing leading roles in teeth morphogenesis are also involved in the development of many other organs [Koussoulakou et al. 2009; Matalova et al. 2004].

The plethora of molecules involved [e.g., fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), sonic hedgehog (SHH), wingless integrated (Wnts)] and the complexity of interactions (e.g., activation, inhibition, regulatory loops) inevitably lead with some frequency to homeostatic disorganization, which

results in congenital abnormalities, such as tooth agenesis, which is the most commonly inherited disorder [Line 2003; Kapadia et al. 2007]. Most human congenital teeth malformations are caused by mutations in develop- mentally regulated genes [Smith & Coates 1998]. The fact that, an embryonic tooth bud can develop in vitro indicates that the expression of teeth-related genes is not restricted only in vivo [Thesleff et al. 1995]. Mutations that alter teeth act at many levels of control, i.e., the development of the embryonic bud, the morphogenesis of the bell stage, the production of enamel and dentin and the forma- tion of the roots [Townsend et al. 2008].

Tooth morphogenesis starts with an epithelial thickening at the areas that have been pre-patterned as odontogenic. Paracrine growth factors—mainly from the hedgehog, Wnt (wingless-type MMTV integration site gene), BMP and FGF families—homeobox transcription factors, and extracellular matrix molecules are activated to control tooth bud formation, and the development of the tooth cap and bell [Fleischmannova et al 2010].

Differentiation of dental hard tissue producing odontoblasts and ameloblasts is directed by reciprocal interactions between inner enamel epithelium (future ameloblasts) and the mesenchymal cells directly facing inner enamel epithelium (future odontoblasts), mediated mainly by molecules from Wnt, Runx2, and Tgf (transforming growth factor) protein families [Bègue-Kirn et al., 1994].

The RANK/RANKL/OPG (receptor activator of NFκB/ RANK ligand/ osteoprotegerin) signaling pathway seems to pro- vide temporo-spatial coordination of tooth-bone development. Expression of Rankl in the early alveolar bone ossification cen- ters is a critical contributor to tooth development [Fleischmannova et al 2010].

The presence of RUNX2 in fully differentiated cells supports the concept that RUNX2 is also required in maintaining fully functional cells, at least in bone [Ducy et al. 1999; Quack et al. 1999].

RUNX2 has also been identified as essential for tooth formation [Ducy et al. 1999; D'Souza et al. 1999] (see **FIGURE 5**).

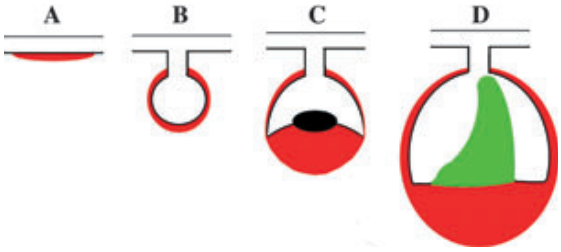


Figure 5: Patterns of Runx2 expression (red areas). (A) Initiation stage. Expression is induced in the mesenchyme by the odontogenic epithelium. (B) Bud stage. Expression is maintained around the ingrowing dental epithelium. (C) Cap stage. Expression is sus- tained in the mesenchyme and the dental follicle. RUNX2 is necessary for Sonic Hedgehog (Shh) expression and for formation of the enamel knot. (D) Bell stage. In this stage, expression is down-regulated in the dental papilla but is maintained in the dental follicle and surrounding mesenchyme.

Type II Runx2 is also expressed in periodontal ligament (PDL) fibroblasts, although BSP, a marker of osteoblast differentiation and biomineralization, is not [Jiang et al. 1999]. The action of RUNX2 seems to be suppressed by a mechanism designed to maintain PDL width [Saito et al. 2001]. However, these cells maintain the potential to differentiate to osteoblasts under certain conditions, such as mechanical stress [Yang et al. 2006].

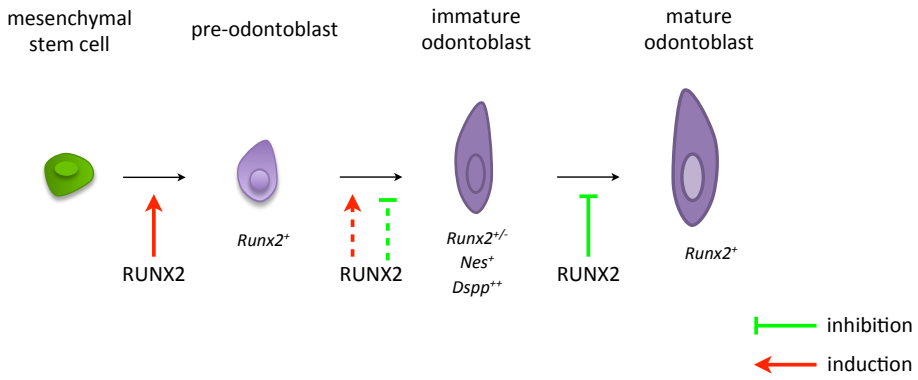


Figure 6: Preodontoblasts differentiate from neural-crest-derived pluripotent mesenchymal cells. RUNX2 is essential for differentiation of pluripotent mesenchymal cells into preodontoblasts. RUNX2 also probably induces the differentiation of preodontoblasts into immature odontoblasts at an early stage but is inhibitory at a late stage. Preodontoblasts express Runx2, immature odontoblasts express Dspp and Nes but Runx2 weakly, and mature odontoblasts express Dspp and Nes but not Runx2. Runx2 expression is downregulated during odontoblast differentiation, and RUNX2 inhibits terminal differentiation of odontoblasts. (Dspp dentin sialophosphoprotein, Nes nestin)

Wnt signaling plays a fundamental role in facial and tooth development [Galli et al 2010]. Several Wnt genes and Lef1 are expressed in the dental epithelium and in the immediately adjacent mesenchyme [Kratochwil et al. 1996; Dassule & McMahon, 1998; Chen et al. 2009], and the control of their expression yields dramatic effects on tooth formation: inhibition of Wnt signaling in explants from mandibular arch primordia by deletion of β-catenin in tooth mesenchyme [Chen et al., 2009] or in dental epithelium [Liu et al. 2008] blocked tooth development.

Besides playing a fundamental role in tooth morphogenesis, however, Wnt canonical signaling has also been demonstrated to be active in adult periodontal fibroblasts and con- tributes to periodontal tissue growth and remodeling during tooth eruption [Rooker et al. 2009]. Moreover, this signaling cascade can orchestrate the activity of several cell types in tooth and periodontal tissues, because it can up-regulate the proliferation and decrease the differentiation of cementoblasts [Nemoto et al. 2009], while it negatively controls the odontoblastic differentiation of dental pulp stem cells [Scheller et al. 2008]. Amelogenin, a critical pro- tein for the formation and mineralization of tooth enamel, is also capable of stimulating β-catenin/TCF signaling in human periodontal ligament cells and in mouse calvaria osteoblastic cells [Matsuzawa et al. 2009].

Chapter 3

MSCs for bone tissue regenerative medicine

Due to their abilities (steamness and self-renewal properties) and because MSCs can be isolated and expanded ex vivo without modification in the phenotype or loss of function, MSCs are considered to be very important for the development of cell-based therapies and tissue repair in regenerative medicine.

3.1 Bone repair

After completion of the bone remodeling cycle, most osteoblasts undergo apoptosis [Clines 2010]. Some cells will remain at the bone surface to form lining cells that may serve as osteoprogenitors or even prepare bone for osteoclast attachment [Everts et al. 2002]. A few osteoblasts become encased in their own matrix to become osteocytes, the most prevalent cell in bone.

After a bone fracture, both endochondral and intramembranous ossification re-emerge. This process of fracture repair begins with the immediate formation of a hematoma at the fracture site. Through release of cytokines, cells are recruited to the fracture site that marks the site for repair. Hypoxia and vascular disruption at the fracture site are stimuli for chondrocyte recruitment and initiation of endochondral ossification. Adjacent to the fracture, fibroblastoid periosteal bone lining cells differentiate into osteoblasts and begin the process callus formation.

The fracture gap is eventually filled with immature woven bone that is remodeled over time to more structurally sound lamellar bone [Clines 2010].

Fracture repair in most situations is a predictable process. However, the risk of fracture repair failure increases with aging, diabetes and smoking [Clines 2010].

Osteoporosis, from estrogen deficiency, glucocorticoids or inflammatory states, can also be viewed as a condition whereby bone fails to optimally ossify. Anti-resorptive agents such as bisphosphonates are effective in reducing fracture risk but potential side effects have recently gained more attention [Khosla et al. 2007].

Conventional treatment for bone fracture is to join or fixate the fractured ends and allow physiologic ossification and remodeling to occur. The application of BMP-2 or biodegradable scaffolds enhances repair times. However, failure of fracture repair is still common, especially when associated with chronic disease [Clines 2010].

Several types of approach are of a particular interest in improving bone repair (see **FIGURE 1**).

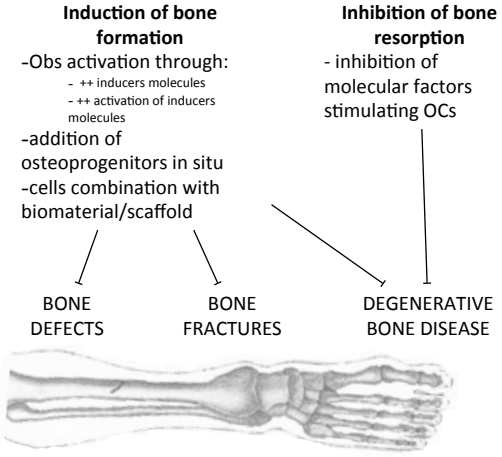


Figure1: Different strategies can be used and combined to treat diseases that weaken bones. Bone formation can be activated (i) by enhancing osteoblasts (Obs) activation (ii) by suppressing the activity of osteoclasts (Ocs) (iii) by adding growth factors and hormones (so called "inducers molecules"). Osteoprogenitors cells (like MSCs) within bone defects can be added with or without the use of biomaterial (synthetic biomaterials, demineralized bone). (modified by: Deschaseaux et al. 2009 Trends Mol Med. 15:417-29)

One approach involves strategies to target molecular pathways to increase the number of osteoblasts or their maturation. The US Food and Drug Administration approved the use of parathyroid hormone (PTH) as a highly efficient anabolic agent for osteoporosis. Although the exact mechanisms underlying these effects of PTH are unknown, experiments have shown that intermittent injection of PTH increased the number of osteoblasts and their maturation, probably through different molecular pathways [Datta et al. 2009]. However, recent data suggest that PTH administration in rats can generate osteosarcoma [Tashjian et al. 2008]. Whether such an adverse effect could occur in humans remains undetermined.

An other clinical condition that results in altered bone metabolism is rheumatoid arthritis (RA). RA is a chronic debilitating autoimmune disease that results in inflammation and structural destruction of the joints. A hallmark of RA pathogenesis is an imbalance of the osteoblast–osteoclast axis driven by inflammatory processes, resulting in elevated bone resorption by osteoclasts. Current therapies used to treat this disease have focused on inhibition of synovitis, but such treatments do not adequately repair damaged bone. A key pathway of osteoclast formation involves the receptor activator of NF-κB ligand pathway acting on myeloid progenitor cells [Choi et al. 2009]. The Wnt pathway has been shown to be important for the differentiation of osteoblasts from mesenchymal lineage precursors, and endogenous Wnt inhibitor, such as sclerostin might have important roles in osteoclast dysregulation in RA.

Targeted deletion of sclerostin in mice results in increased bone volume, bone mineral density and bone strength compared with wild type controls [Choi et al. 2009]. Sclerostin-deficient animals had increased osteoblast activity as well as increased serum levels of osteocalcin, an osteoblast marker.

The use of molecules to intervene in the Wnt pathways as anabolic agents seems to be more complex because their activities depend on differentiation stage of cells and their concentration. Furthermore, this pathway is extensively described as

being involved in tumorigenic processes. Nevertheless, targeting the Wnt pathway offers several options to treat bone diseases [Deschaseaux et al. 2009].

Initially used in patients with bipolar disease, lithium was found to be a Glycogen synthase kinase 3 beta (GSK3β) inhibitor. Surprisingly, in such bipolar patients, oral lithium treatment was shown to be associated with a lower risk of fracture, although 25% of patients treated with lithium developed parathyroidism and hypercalcemia [Chen & Alman 2009]. Other Wnt antagonists are being studied such as sclerostin proteins. Sclerostin was found to be a Wnt pathway inhibitor; bone mass, bone formation and bone strength are increased in rats treated with anti-sclerostin antibody. Clinical trials in post-menopausal women are under investigation [Li et al. 2009]. However, in addition to cancer, long-term activation of the Wnt/β-catenin path- way can have other drawbacks because osteoarthritis symptoms and osteophytes were observed in animal models [Deschaseaux et al. 2009].

The osteogenic potential of MSCs has been well defined, as evidenced by bone formation following transplantation of MSC in vivo. Moreover, in vitro experiments demonstrate the formation of mineralized nodules, along with elevated production of alkaline phosphatase, osteopontin, bone sialoprotein, osteocalcin, types I and III collagen, and generation of PTH and estrogen receptors, during the differentiation of MSC into osteoblasts [Tsuchida et al. 2003]. Although there is enthusiasm for attempting to utilize MSC stherapeutically, methods, either in vitro or in vivo, which provide an adequate commitment to direct MSC differentiation into osteoblastic cells need to be defined. Different groups has shown the chance of transfering the gene for BMP-2 into bone marrow MSC and mesenchymal progenitor cells via recombinant adenovirus. These gene-engineered cells continuously expressed BMP-2 protein both in vitro and in vivo, and facilitate cell differentiation into cells of the osteoblast lineage [Cheng et al. 2001].

Considering others molecular pathways, and the chance to “inhibit or enhance them”, several studies have shown that Wnt proteins inhibit the ability of human MSCs to differentiate to osteoblasts [Boland et al. 2004; de Boer et al. 2004], while others show the opposite [Luo et al. 2004, Bain et al. 2003]. In the absence of ß-catenin, osteoprogenitors differentiate into chondrocytes [Hill et al. 2005], thus ß-catenin seems to be required for osteoblast differentiation at a very early stage.

The bone repair process in adults closely resembles the normal development of the skeleton during embryogenesis, even though some aspects differ. After injury, hypoxia and inflammation induce vascularization, and in vessels growing within injured tissue, perivascular MSCs populate the wound site under hypoxic conditions [Deschaseaux et al. 2009]. MSCs proliferate and then differentiate along a cartilaginous or an osteogenic lineage in response to growth factors and cytokines released by platelets, inflammatory cells, and neighboring cells and tissues. Nowadays crucial data regarding the native properties of MSCs and osteoblasts and osteoclasts progenitors too are still needed to elucidate the mechanisms of bone formation and bone remodeling.

3.2 Dental tissue disease and regeneration

Disturbances in bone homeostasis causing decreased systemic or periodontal bone mineralization (periodontitis, osteoporosis, osteolysis) may result in premature tooth loss [Fleischmannova et al 2010].

Periodontal diseases are infectious diseases characterised by destruction of supporting tissue for tooth, including periodontium, cementum, alveolar bone, and gingiva [Seo et al. 2004; Philstrom et al. 2005]. Thus, regeneration of these tissue lost through periodontitis is the ultimate goal of periodontal therapy [Yang et al. 2010] (see **FIGURE 2**).

When PDL tissues loss occurs, because of inflammatory disease or traumatic reasons, dental root ankylosis and root resorption occur both [Nyman et al. 1980; Andreasen & Kristerson, 1981].

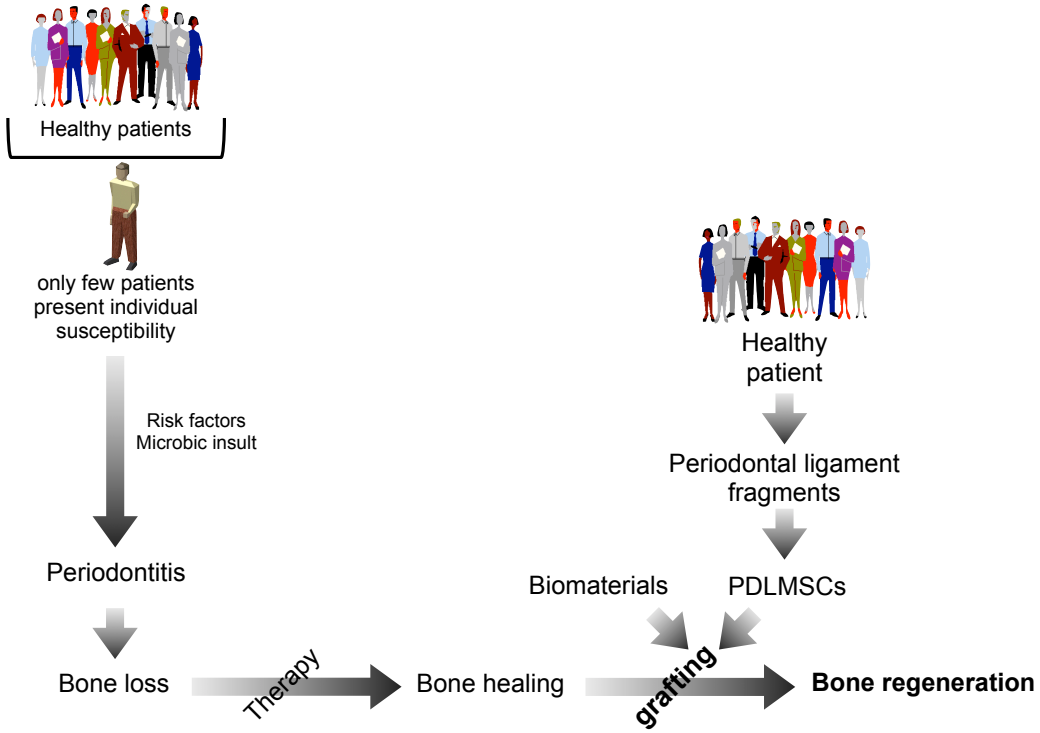


Figure 2: Periodontal tissue damage and healing strategies. Within population some patients develop periodontitis. Regenerative approach has the aim to rebuilt all tissues lost (hard and soft too), instead of achieving only reparation of tissues.

Periodontitis is an inflammatory disease caused by bacterial infection, mainly Aggregatibacter actinomycetemcomitans or Porphyromonas gingivalis. Characteristic of periodontitis are periodontal pocket formation and excessive alveolar bone resorption, followed by tooth loss. Chronic inflammation is, in many cases, coupled with excessive bone resorption, most likely resulting from up-regulation of RANK/RANKL signaling. Other studies showed that not only bone resorption but also new bone production are affected by P. gingivalis infection. Bacterial lipopolysaccharide was reported to increase the proliferation of progenitor cell populations, however, delaying their subsequent dif- ferentiation, which may potentially be reflected in alveolar bone extracellular matrix formation [Roberts et al. 2008]. Additionally, premature tooth loss may be associated with

generalized bone density disorders resulting from both congenital genetic defect and disease. Several studies have reported that post-menopausal women without estrogen substitution therapy have more teeth missing than do women with estrogen substitution therapy. Moreover, syndromes such as familial expansile osteolysis or Paget's disease, caused by RANKL pathway defects resulting in high activity of osteoclasts that predominantly affect long bones, often involve loss of dentition [Fleischmannova et al 2010].

At the present time, periodontal regeneration, considered as the the formation of new bone and new cementum with supportive periodontal ligament [Iwata et al. 2010], is a process achieved only if some conditions, local and sistemic, are present at the moment of healing.

Considering tissue engineering field, it has been defined as "understanding the principles of tissue growth, and applying this to produce functional replacement tissue for clinical use" [MacArthur & Oreffo 2005]. After tooth loss, this therapeutical approach offers a new hope to both patients who suffered it and who is involved in treating the disease as well [Peng et al. 2009].

All these findings support the hypothesis that it would be conceivable to use PDLSCs for in vivo regeneration of the periodontium in patients with periodontal destruction [Arnold et al. 2010].

Between healthy donors, it could be also useful to develop selective strategies based on predictable elements, such as clinical and biological paramenters, to discriminate cells with most promising regenerative potential, as done before with different cells donors.

The development of tooth tissue engineering strategies and cell therapy mainly focuses on three aspects: seeding cells, scaffolds and biomolecules (see **FIGURE 3**).

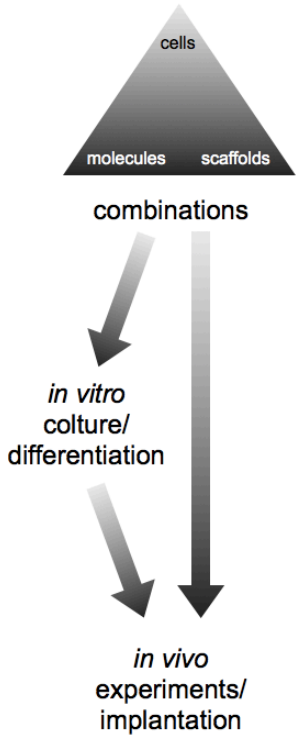


Figure 3: cells/biocomponents combinative options and their implication in experimental strategies.

Traditional 2-D culture well were chosen first to test PDLSCs osteogenic differentiation potential.

Analysis of bone markers ALP and Runx-2, OC, and OP showed PDL as reliable source of osteogenic progenitors [Lekic et al. 2001; Lindroos et al. 2008], and well staining revealed presence of sparse calcified nodules after 21 days osteogenic differentiation [Seo et al, 2004].

Both mineralized tissues, bone and cementum, reconstitution is necessary to achieve complete periodontal regeneration, and it is known that some progenitor cells niches, scattered in the whole tissue, possess the ability to differentiate, when properly stimulated, in osteoblasts, fibroblasts and cementoblasts [Bartold et al. 2006; Seo et al. 2004; Pitaru et al. 1994].

To overcome some problems connected to the small amount of MSCs in the adult tissue, and the difficult recruitment of them during healing processes, due to

inflammation and microenvironement, all knowledge and mechanisms comprehension, only cells ex vivo expansion protocols and biomaterials combination have been shown useful for both tesearch and devepoling therapies.

Combining traditional culture conditions and different new technologies or biomolecular approach, cells phenotype enhancement can be achieved through in vitro and in vivo experiments, as seen for BM MSCs [Park et al. 2005].

Some molecules family, such as amelogenin-like molecules and bone morphogenetic proteins (BMPs) have been demonstrated having a critical role in controlling periodontal tissue development first and after tissue damage, in periodontal regeneration [Foster & Somerman 2005].

Although their role in this mechanism is not well understood, there are evidences of how their presence could provides an environment that enables PDL cells to speed up their metabolism, growth rate and growth factors release [Lyngstadaas et al. 2001; Duan el al. 2011].

Instead of molecular environment modification, physical and mechanical modification have been proposed to modify cells proliferation, differentiation and molecular pattern expression.

Osteoinductive medium combined with HA-containing composite microsphere material affected encapsulated cells and allowed their differentiation into osteoblastic lineage in vitro, both under 2D and 3D culture conditions [Inanç et al. 2007].

When ex-vivo expanded and transplanted in immunocompromised mice with hydroxyapatite/tricalcium phosphate carrier particles, PDLSCs generated a typical cementum/PDL-like structure [Seo et al. 2004].

Some promising results come also from clinical outcome of patients previously treated with autologous PDLPs combined with bone grafting material [Feng et al. 2010].

Chapter 4

MSCs culture systems: 2-D vs. 3-D

The main drawback in MSCs transplantation is sometimes the low quantity of MSCs. Since the biology of MSCs and their microenvironment are not totally understood it has not been easy to overcome this issue. Ex vivo expansion of MSCs from UC or other sources became an alternative to increase the cell-dose available for transplants and to further research on MSCs. There is evidence that even if short-term expansion may modify MSCs properties, it is strongly probable that the engraftment characteristics remain unaltered [Zhai et al. 2004].

The differentiation of MSCs has been extensively studied, using mainly well-established *in vitro* assays with culture-expanded MSCs. Findings, therefore, have the caveat that they may not always be reliable and fully reproducible because of the vast heterogeneity of *in vitro* culture conditions and MSC types [Augello & De Bari 2010]. The *in vitro*-obtained data are overly dependent on culture conditions for derivation and expansion of MSC populations and, therefore, are unlikely to be extrapolated to the native cells.

MSCs also interact physically with the surrounding environment. The first line of interaction for MSCs is with the extracellular environment, be it plastic, a resorbable bioscaffold, or a more rigid structure [Augello & De Bari 2010]. Evidence of how the extracellular matrix can control stem cell fate, inducing seeded MSCs toward osteogenesis or chondrogenesis depending on its physical

properties was reported [Engler et al. 2006]. These findings open up unprecedented avenues for the regulation of MSC differentiation in regenerative medicine using physical factors, avoiding or combining these conditions with the use of exogenous growth factors too (see **FIGURE 1**).

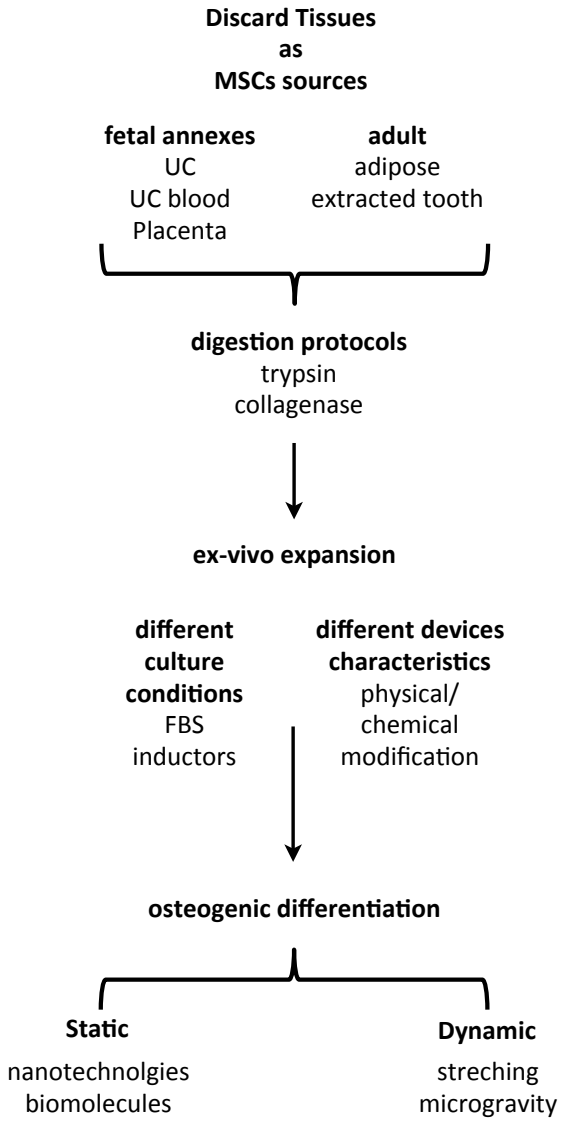


Figure 1: Modulation of cells phenotype could be reached at different levels through many different approaches, involving cell physical and biochemical environment modifications.

This knowledge leads to a new approach by researchers, of investigating all possible conditions *in vitro* that can simulate or mimic the *in vivo* ones.

4.1 Static conditions

Static culture systems, such as polystyrene Petri dishes, multiwells, or flasks, have been the most widely used culture devices. MSCs are conventionally cultured in these containers, and put inside a biological incubator, where two physical parameters are regulated: temperature and pH. Temperature is usually set at 37°C, whereas pH control is performed by maintaining an atmosphere at 5% CO_2 concentration, which corresponds to the physiological pH of 7.2. The main sources of variability among different culture protocols reside in medium composition, cell seeding density, and time intervals between cell dilutions, called cell passages, and medium changes. Conventional cultivation methods are in need of both optimized protocols and ad hoc technology [Cabrita et al, 2003].

Classically, osteogenic differentiation of human MSCs requires incubation in fetal bovine serum containing medium supplemented with ascorbic acid, β-glycerophosphate, and dexamethasone, resulting in an increase in alkaline phosphatase activity and calcium deposition [Augello & De Bari 2010]. The chondrogenic differentiation is performed with a high cell-density pellet or micromass culture treated with transforming growth factor (TGF)-β in serum-free medium; this results in production of cartilage-specific, highly sulfated proteoglycans and type II collagen. For adipogenic differentiation, MSCs are treated with dexamethasone, insulin, isobutyl-methyl-xanthine, and indomethacin (added to medium containing fetal bovine serum), and the differentiation is revealed by the detection of lipid vacuoles with oil red O staining [Augello & De Bari 2010].

Most investigators have used modified Dulbecco's Medium plus animal or human serum and combinations of cytokines. Alternatively, serum-free and animal

product-free media have been developed to avoid immunological issues affecting transplantation [Andrade-Zaldívar et al. 2008]. Different cytokine cocktails aim the proliferation of undifferentiated MSCs and the maintenance of their engraftment capacity. The optimal combination and concentration of growth factors to preserve the stem state has not been yet established.

When cells are cultured under static conditions, medium in the well is stagnant. Soluble factors are present in the medium, and are up taken and released by the cells, depending if they are exogenous or endogenous. The competitive processes in these culture conditions are transport by diffusion and cell uptake/release of soluble factors.

It is well known that bone biology basically depends on some molecular pivots that could represent disease targets as well as tools for new treatments [Deschaseaux et al. 2009] (see **Table 1**).

Recognized molecular factors	Observations
PTHrP/PTH	–Used for osteoporosis; efficient for increasing bone mass when intermittently administered
SDF1	–Allows MSCs homing both in vitro and in vivo
BMP 2	–Osteochondrogenic factor; might initiate bone formation and bone healing and can induce expression of other BMPs –Used for spine fusion, bone nonunion and bone defects; clinically efficient for bone repair and regeneration; some adverse effects observed (osteolysis and ectopic bone formation)
BMP 7	–Osteogenic factor in vivo and in vitro; active on more mature osteoblasts –Used for spine fusion and bone nonunion; clinically efficient for bone repair
Wnt–β-catenin	–crucial for osteoprogenitor proliferation; –LiCl used as a specific inhibitor of GSK3b to increase bone mass post-fracture and to diminish fracture risk: –Bortezomib, proteasome inhibitor used in treatment of multiple myeloma (MM); also increases bone mass –Anti-DKK1 monoclonal antibody (BHQ880) used to inhibit osteolysis in MM or to increase BMD –Anti-sclerostin antibody used to increase bone mass –GSK3b inhibitor (603281-31-8)
RANKL/OPG	–Targeting RANKL to treat osteoporosis
Biphosphonates	–Widely used for osteoporosis, bone necrosis, osteogenesis imperfecta and some osteolytic tumors (zoledronate, alendronate, risedronate); some adverse effects noted (osteonecrosis, inhibition of osteogenesis)
TGF β	–Can induce osteoblast differentiation at the early stage of immature cells but can also inhibit osteogenesis in committed cells –Used as a bone nonunion marker
Platelet-rich plasma (growth factor substitute)	–Used for bone defects treatment with or without biomaterials with or without osteoregenerative cells (randomized controlled trials still required)

Table 1: Key molecules involved in bone repair (complete references in: Deschaseaux et al. 2009 Trends Mol Med. 15:417-29).

In normal conditions, after damage or “de-novo”, bone formation begins when mesenchymal cells form condensations and in most condensations the endochondral process precedes bone generation.

Like other tissues, bone responds to bone-specific soluble growth factors. It is therefore important to understand that the appropriate growth factors are required to discover the osteogenic potential of isolated progenitor cells populations.

TGF-β have been shown able to affect cell growth and differentiation during developmental processes such as embryogenesis and tissue repair [Long 2001].

Also a large number of BMP have been cloned and involved in osteogenic differentiation. Additional growth factors also regulate bone development, and their role in MSCs differentiation is being investigated.

Considering PDL cells, different molecular factors have a significant role in controlling the behavior of cells within the periodontium [Foster & Somerman 2005]:

- BMPs

- Amelogenine-like molecules;

- Phosphate-regulating factors.

Several groups developed in vitro models for defining the role of PDL cells and cementoblasts [Zhao et al. 2002]. They combined and examined cells biomolecular interactions, focusing on molecules family above mentioned.

Results from numerous studies, in vitro and in vivo have shown that certain BMPs promote osteoblast maturation and induce mineral formation. In fact, recombinant human BMP2/absorbable collagen sponge was approved by the FDA this year for treatment of bone fractures in the US [Foster & Somerman 2005].

Different studies focused on monolayer culture conditions modification, for example through well coating strategies. The increased attachment rate of PDL

cells growing on EMD demonstrates that this enamel protein based matrix mimics an extracellular matrix that facilitates rapid attachment of these cells [Gestrelius et al. 1997; Lyngstadaas et al 2001].

Data are accumulating to suggest that amelogenins can regulate the behavior of mesenchymal cells and thus act as signaling molecules. Regardless of the role such molecules play during development of periodontal tissues it is clear that cells within the periodontium respond to these factors [Foster & Somerman 2005].

Different groups have noted considerable alterations in periodontal tissues in mice having mutations in or knockout of genes associated with phosphate regulation , providing novel insights into mechanisms controlling mineralization [Foster & Somerman 2005; Zhao et al. 2002].

Due to the side effects of uncontrolled release of these factors in some culture conditions, like in static conditions, many studies have focused on the creation of appropriate delivery systems in the form of nanoparticles, microspheres, and scaffolds. Although their design and compositions are unique to each other, they share the same goal: how to deliver factors to target sites to meet their temporal and spatial need [Bae et al. 2010].

Among these candidates biodegradable polymeric microspheres (PMS) have been widely utilized as a favorable vehicle in delivering various cytokines and proteins. The encapsulation of various drugs, bioactive proteins, or other molecules within degradable polymers has long been recognized an effective way to control the release profile of the retained substances [Bae et al. 2010].

Many efforts have been done to identify new drug delivery systems able to enhance drug permeation and to control drug delivery release rate. In this context stricking advantages can be given by nanotechnology [Civiale et al. 2009].

Pharmaceutical nanotechnologies including nanosuspensions, solid lipid nanoparticles, liposomes and polymeric micelles can allow to overcome some of

the inconveniences of conventional drug delivery and sometimes improve water solubility of poorly soluble drugs and their chemical stability [Kayser et al., 2005].

Natural polymers, such as pectin and gelatin, can be used to produce biocompatible and biodegradable microparticles, obviating the toxicity or biodegradability problems (i.e., formation of localized granulomatous inflammation) possibly related to the use of synthetic materials. Pectin in particular, due to its interesting features, such as hydrophilicity, biocompatibility, and biodegradability, has been proposed for the production of pharmaceutical formulations intended for controlled drug delivery [Esposito et al 2001].

Polymeric micelles are self-assembling colloidal systems obtained by assembling of block or graft amphiphilic copolymers. These systems seems to be very promising in drug delivery for many peculiarities, including their high kinetic and thermodynamic stability and able to give a slow drug release [Civiale et al. 2009].

Polyhydroxyethylaspartamide is a synthetic polymer having protein-like structure, obtained by the reaction of ethanolamine with polysuccinimide. It was largely investigated for ocular drug delivery also for its good biopharmaceutical properties as drug carrier material such as high water solubility, multifunctionality, biocompatibility, low cost of production [Civiale et al. 2009].

Alginate microspheres have been used for the encapsulation of a wide variety of biologically active agents, including proteins, antibodies, DNA and eventually cells [Capretto et al 2010].

Recently, the production of alginate microbeads was also accomplished by microfluidic procedures. Chang-Hyung and collaborators reported the preparation of alginate beads using a polydimethylsiloxane-based chip, including two injection lines for alginate solution and $CaCl_2$, and an injection line for the cell suspension [Chang-Hyung et al. 2007; Capretto et al. 2010] (see **FIGURE 2**).

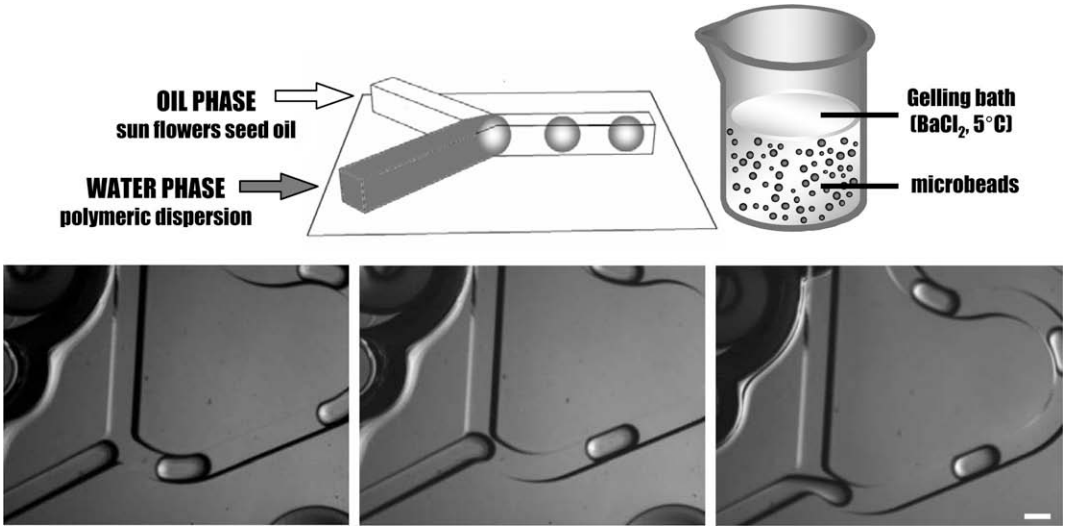


Figure 2: Schematic representation of the microfluidic system set-up for the preparation of polysaccharidic microbeads, including drawing of the multiphase flow generator by the "Y" junction squeezing mechanism (upper panel). Microphotographs of the multiphase flow formation, bar = 300 µn (lower panel) (from Capretto et al. 2010).

All these strategies try to obtain controlled and predictable factors delivery, targeting sites or cells in a temporal and spatial optimized condition. Considering static experimental conditions, the problem of the limited interactions of cells with the extra cellular matrix or environment is not overcame.

4.1.1 Scaffolds overview and alginate bead dissertation

Mammalian tissues rank among the most difficult tissues to grow *in vitro* conditions because of their important nutrient needs, their sensitivity to nitrogenated wastes and their high fragility to shear stresses. The exact requirements in nutrients, waste elimination, and shear stress also vary widely for the different types of tissues [Martin & Vermette 2005].

The first cultures of MSCs were performed in classic static culture dishes. However, static cultures do not provide the proper three-dimensional (3D) environment. Therefore, new strategies have been designed.

The microenvironment of MSCs has been mimicked with 3D-scaffolds.

Cells are often combined with artificial structures capable of supporting three-dimensional tissue formation structures, typically called *scaffolds*.

Scaffolds usually serve at least one of the following purposes:

- Allow cell attachment and migration
- Deliver and retain cells and biochemical factors
- Enable diffusion of vital cell nutrients and expressed products
- Exert certain mechanical and biological influences to modify the behaviour of the cell phase.

A scaffold should provide chemical stability and physical properties matching the surrounding tissue to provide cytocompatibility, adhesion support, proliferation and mechanical strength [Hollweck et al. 2010]. Scaffolds can be of biological origin, made of synthetic material or be some type of hybrid [Ahsan et al. 2005; Dawson et al. 2008]. Naturally occurring biomaterials may most closely stimulate the native cellular milieu, but large batch-to-batch variations upon isolation from biological tissues and poor mechanical strength are the main limitations for i.e. cardiac applications. In addition, biopolymers are often denatured at rates not matching those for tissue formation and often require chemical modifications, which can lead to toxicity [Vats et al 2003; Yang et al 2001]. Synthetic materials, like expanded polytetrafluoroethylene (ePTFE), confer non-biodegradability due to their highly crystalline nature. Additionally, these polymers are nonimmunogenic, antithrombotic, and easily available.

As both cell attachment and incorporation *in vitro*, as well as subsequent tissue maturation during in situ regeneration, are crucial features of tissue engineering, the amount of porosity and the pore size of the supporting 3D structure are important features that need to be taken into consideration when designing tissue-engineering scaffolds [Boyan et al. 1996].

With respect to the scaffolds for bone engineering, calcium phosphate bioceramics, such as hydroxyapatite (HA) and tricalcium phosphate (TCP), have been intensively investigated due to their similarity in composition to bone matrix, bioactivity and osteo-conductivity [Legeros 2002].

Numerous *in vivo* and *in vitro* assessments have reported that calcium phosphate materials, no matter of which form (bulk, coating, powder or porous) or phase (crystalline or amorphous), always support the attachment, differentiation and proliferation of relevant cells (such as osteoblasts and mesenchymal cells) [Brown 2001]. Also developed ceramic/polymer composite materials are attractive as bone substitutes because the novel biomimetic strategy used to generate them provides properties similar to natural bone [Liao et al. 2004].

Schneider et al. study compared the cell-mediated remodelling of 3-D collagen I/III gels during osteogenic differentiation of BM-MSCs and UC-MSCs. Results indicate that both cells populations display all features needed for effective bone fracture healing [Schneider et al. 2010]. The expression of ECM differs in both cell types considerably, suggesting different mechanisms for bone formation and significant impact for bone tissue engineering [Schneider et al. 2010].

Experimental injection of MSCs into injured regions ensured delivery to the damaged area but was hampered by significant cell loss [Hollweck et al. 2010]. An alternative approach to injection of isolated cells into the defect was to use artificially engineered tissues.

Recently, tissue engineering based on PDLSCs to enhance periodontal regeneration has been the focus of periodontal research. In situ engineered constructs containing in vitro expanded autologous cells have been used to regenerate periodontal defects [Li et al. 2009].

As an important biotechnology in tissue engineering, cell encapsulation has increasingly attracted interests in recent years [Abbah et al. 2008]. It promotes

tissue regeneration by facilitating the localized retention of entrapped cells, as well as controlling the release of therapeutic agents to the host. Microcapsules are important components for prolonging the viability and therefore sustaining the functions of confined cells [Abbah et al. 2008].

Also the protection of implanted cells from the host's immune response is of primary importance. Encapsulation protocols have also been proposed to maintain the cell phenotype longer than in monolayer, to prolong the cell viability and therefore to sustain cell function [Penolazzi et al. 2010].

Living cells embedded in microbeads, acting as scaffolds, have found application in many different fields, including cell culture, cell-based therapy and tissue engineering [Wang et al. 2003].

The main advantage of these devices is represented by the presence of an immunoisolating membrane, which enables the transplantation of non-self cells and tissues without the need for immunosuppressive regimens [[Abbah et al. 2008 ; Capretto et al 2010].

Successful clinical use of encapsulated cells strongly depends on a number of crucial characteristics:

- the morphological and dimensional properties;

- mechanical stability;

- biocompatibility;

- molecular exchangeability of microbeads.

As scaffold materials, polysaccharides have largely been used. Polysaccharides (such as alginate, agarose or chitosan) indeed possess adequate mechanical properties, permit the exchange of molecules and can be transformed into spherical gelled solid particles by mild procedures, preserving the cell viability [Capretto et al 2010].

Several groups showed that human MSCs encapsulated showed a higher proliferation rate than those seeded on scaffolds, and alginate beads were also tested to study the chondrogenesis of human MSCs [Yowa et al. 2009; Ma et al. 2000].

The use of alginate microbeads showed excellent biocompatibility and immunoprotection of embedded cells [Luca et al. 2005; Luca et al. 2007]; Abbah et al. showed that calcium cross-linked alginate microbeads can act as a scaffold for MSCs proliferation and osteogenic differentiation and has potential for use as 3D degradable scaffold [Abbah et al. 2008].

Despite static cultures have shown expansion of MSCs, the scaling-up represents a major problem because more volume means less oxygen flow and less nutrient availability in the system. Several dynamic models that incorporate gas flow have been used to overcome this problem [Andrade-Zaldívar et al. 2008]. Diverse bioreactors with specific characteristics have been designed for MSCs expansion since the 1990s. In addition, the latest designs have served as well to study the MSCs biology.

Differences between tissues suggest that reactor design considerations and operating conditions can be different when dealing with a specific tissue. However, every type of tissue beneficiates from controlled shear stresses and optimal nutrient availability and wastes elimination [Martin & Vermette 2005].

The term “bioreactor” has been frequently used, but often to mean different things. Bioreactors can be defined ‘‘as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions (e.g., pH, temperature, pressure, nutrient supply, and waste removal)’’. Different bioreactor systems matching this definition are currently being used in a wide range of biotechnological applications including industrial

fermentation processing, wastewater treatment, food processing, and manufacturing of biopharmaceuticals. However, many systems encountered in the tissue-engineering literature are composed of vessels of few milliliters, which, under many aspects, do not fit in the presented definition of a bioreactor [Martin & Vermette 2005].

Dynamic bioreactor culture systems are essential for the *in vitro* cultivation and maturation of bone tissue engineering grafts, especially for larger grafts where the core of the scaffold is more than 200 mm from the surface [Chen et al. 2006]. Bioreactors improve the mass transport of nutrients and allow the diffusion limitation of traditional static culture, which is generally taken to be around 200 mm, to be overcome [Ishaug et al. 1997; Martin et al. 1999]. In addition, the dynamic media flow applies a mechanical stimulus to the cells, enhancing cellular osteogenesis and mineralization through triggering of mechano-transduction signaling pathways [Gomes et al. 2003; Chen et al. 2006].

Currently, several types of bioreactors have been developed for bone tissue engineering applications, providing truly *microgravity* environement (see **FIGURE 3**).

This includes the Spinner Flask bioreactor, Perfusion bioreactor, Biaxial and Rotating Wall Vessel (RWV) bioreactor, all of which have been shown to be useful to expand MSCs as well as to study their *in vitro* biology, and to promote cellular osteogenic differentiation [Gomes et al. 2003; Martin et al. 2004; Stiehler et al. 2008].

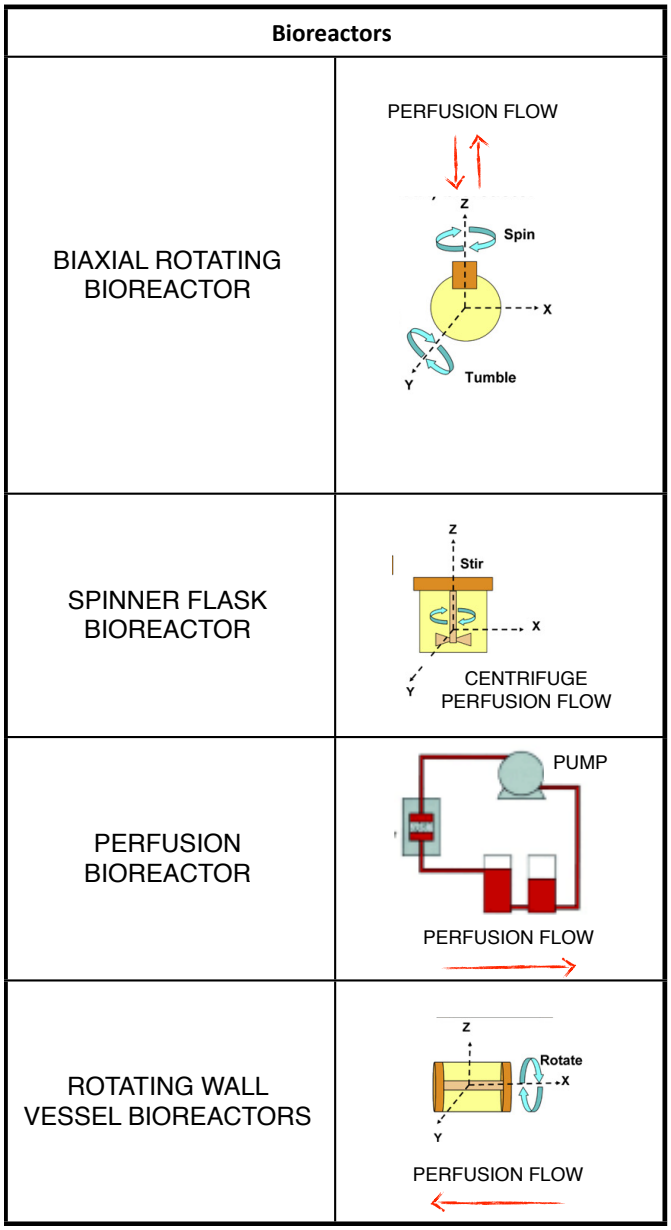


Figure 3: designs of bioreactor systems.

Microgravity is any condition equivalent to experiencing little or no acceleration or gravitational attraction, e.g., far from a planet, star, or other massive body. The term microgravity is used to describe a condition where gravity is not small, but appears to be small. This occurs on an orbiting spacecraft, such as the International Space Station (ISS), and all objects in free-fall. NASA used a variety of facilities to create microgravity conditions. The most famous way is by aircraft flying in parabolic arcs to create microgravity for tests and simulations that last

20-25 seconds. The facilities most-likely to be misconstrued as “anti-gravity chambers,” are NASA’s drop towers.

It has long been established that cells and tissue growing in microgravity - the weightless condition obtained in space - can grow and mutate in ways different than on Earth. A perpetual challenge for the experimental study of these phenomena has been simulating the conditions of space so that complete laboratory studies can be done by numerous investigators on Earth. The simulated growth of mammalian cells in tissue culture needed to duplicate the quiet conditions of orbital free-fall in a way that allowed for maintaining fresh media and oxygenation (see **FIGURE 4**). To solve the problem, NASA in the 1980s developed the bioreactor, a can-like vessel equipped with a membrane for gas exchange and ports for media exchange and sampling.

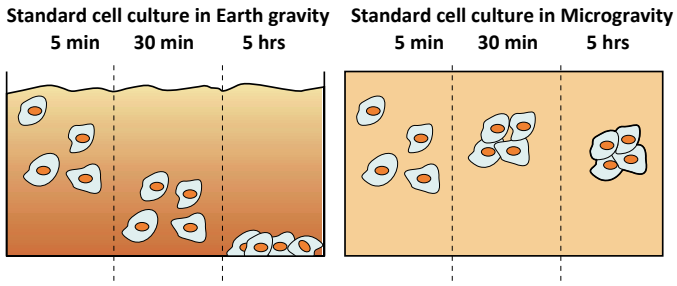


Figure 4: Cell constructs grown in a rotating bioreactor on Earth (left) eventually become too large to stay suspended in the nutrient media. In the microgravity of orbit, the cells stay suspended. Rotation then is needed for gentle stirring to replenish the media around the cells.

As the bioreactor turns, the cells continually fall through the medium yet never hit bottom. Under these quiet conditions, the cells "self- assemble" to form clusters that sometimes grow and differentiate much as they would in the body [Unsworth et al. 1998].

It has been well established that a number of cell types grow in the bioreactor on Earth for extended periods in ways that resemble tissue-like behavior. For this reason, the bioreactor also provides cell culture studies with a new tool for the study of three-dimensional (3-D) cell growth and differentiation. Bioreactors have

been used aboard the Mir Space Station to grow larger cultures than even terrestrial-bioreactors can support. Several cancer types, including breast and colon cancer cells, have been studied in this manner [Ingram et al. 1997].

A cell suspension bioreactor is a closed stirred vessel where it is possible to inline monitor and control some culture parameters, and to take samples from the vessel without disrupting culture conditions. It can be run in different configurations, such as batch, fed-batch, and in perfusion mode, with a system to prevent cell wash-out [Cabrita et al, 2003]. The main advantage of a suspension bioreactor is related to the reduction of spatial gradients. The homogeneity in the vessel ensures more defined and repeatable culture conditions, making easier the scale-up of the process with a consequent cost reduction. Other advantages are the possibility to control the process inline, and to obtain higher cellular densities, because, as cells are suspended, the culture becomes three-dimensional [Blanch et al, 1997].

One approach to maintain homogeneous environment with low stress, is the use of rotating wall vessels (RWV).

The RWV has proven beneficial not only to microgravity sciences but also to the field of tissue engineeing. The reconstituted tissue formed in the RWV is characterized by more extensive 3D growth, tissue differentiation, and cell-cell and cell-matrix interactions than can be achieved in conventional bioreactors [Muhitch et al. 2000]. Multicellular aggregates of human prostate tumor cells were more differentiated in terms of mor- phology and cytokeratin expression when grown in a RWV than control cultures in a spinner flask or static Transwell [Clejan et al., 1996]. Likewise, reconstituted cartilage prepared from RWV cultures of chondrocytes had a higher content of matrix glycosaminoglycans relative to spinner-flask controls [Freed & Vunjak-Novakovic, 1995].

Liu et al. designed a RWV bioreactor to culture total mononuclear from UCB [Liu et al. 2006]. Cells were cultured at an increasing rotating speed from 0 to 6 rpm. The

RWV achieved large expansion of mononuclear from UCB. The authors suggested that the multi-step RWV bioreactor could expand a single cord blood to reach 1.2 x 10^9 mononuclear from UCB [Liu et al. 2006].

The National Aeronautics and Space Administration (NASA) developed two RWV bioreactors for tissue mass culture [Martin & Vermette 2005]. The slow turn lateral vessel (STLV) bioreactor has been used to culture several kinds of cells both on Earth and in space. The STLV was operated at 15–30 rpm on Earth and slower in space allowing a free-fall state, reducing the shear stress.

Gas exchange is provided by a 31.4 cm^2 gas exchange membrane at the inner concentric cylinder. On Earth, this reactor is often rotated at a speed of approximately 15–30 rpm as the tissues grow, so the constructs in the reactor are maintained ‘‘stationary’’ in a state of continuous free-fall. Solid body rotation of the constructs at higher rotating speeds is also reported in space and Earth experiments. Both the inner and outer cylinders can be independently rotated [Martin & Vermette 2005].

The high aspect ratio vessel (HARV) is similar to the STLV, but its design lowers the speed necessary to maintain the constructs stationary (typical speeds of 12–15 rpm) and enhances gas exchange. It is enhanced by the presence of a 78.5 cm^2 gas exchange membrane at one end of the vessel, the area for exchange being more than twice as large than the one in the STLV [Prewett et al.1993]. The principle of operation of this reactor is identical to that of the STLV. Both the STLV and HARV are commercialized by Synthecon Inc. (http:// www.synthecon.com/, Houston, TX) [Schwarz & Wolf 1991; Schwarz et al. 1991] (see **FIGURE 5**).

Both systems were used in the space and on earth to culture human embryonic stem cells (hESC), showing that STLV reduced the aggregation of hESC and they attained a 4-fold increase in productivity respect to the Petri dish cultures [Gerecht-Nir et al. 2004]. The NASA- RWV systems have been used to study the

effects of microgravity on murine HSC and evaluating the hematopoietic homeostasis during long space expeditions [Ohi et al. 2004].

Earth's gravity provides additional shear to cell aggregates in the vessel as cells, which are about 4% denser than the fluid media, fall through the fluid at their free-fall terminal velocity (i.e., drag and gravitational forces on the constructs are equal but opposite).

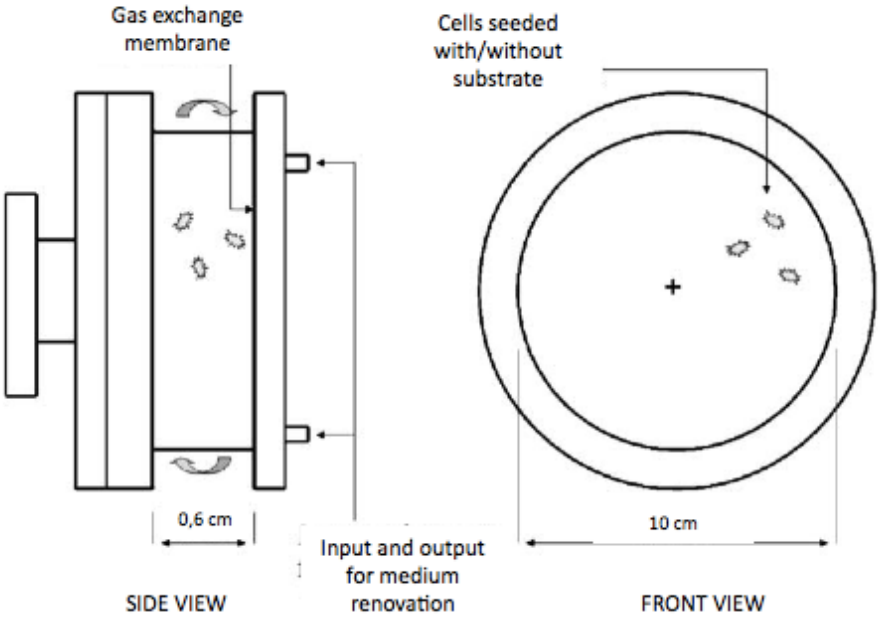


Figure 5: NASA-RWV bioreactors. High aspect ratio vessel: (a) side view (b) front view (adapted from Gerecht-Nir et al. 2004).

Rotating-wall reactors have also been shown to attenuate heart tissue development by disturbing the cytoskeleton–cell surface–ECM interface of embryonic cells [Lwigale et al. 2000]. The HARV reactor, while not altering the morphology of the cells, significantly decreased the number of contracting heart cells and the quantity of fibronectin relatively to a control culture. Fibronectin is a glycoprotein responsible in part for linking cells cytoskeleton to the ECM. Bone tissue cultured in the rotating-wall reactor also presented major differences

compared to a control culture, noticeably in the expression of many proteins [Teti et al. 2002]. The growth rate was found to be non-uniform across the faces of the cell carriers [Lappa et al. 2003].

Li et al. investigated the biological effect of three-dimensional dynamic simulated microgravity induced by rotary cell culture system on human periodontal ligament stem cells in vitro. PDLMSCs were carried on Cytodex 3 micro-carriers and cultured in HARV vessels. Dynamic simulated microgravity affected the biology of cells as indicated by promotion of proliferation and viability, alterations of morphology and disorganization of microfilament system. Besides, simulated microgravity treated cells presented increased matrix mineralization and up-regulated expression of mineralization associated genes after incubation in osteogenic medium [Li et al. 2009].

AIM

Aim of work

- To isolate and culture MSCs cells from human Umbilical Cord Wharton's Jelly (WJMSCs) and periodontal ligament (PDLMSCs);
- To compare characteristics between all samples recruited and to link them to clinical aspects of tissues donors;
- To characterise both MSCs population in regard to their proliferation and differentiation potential;
- To point out culture strategies in order to modify osteogenic cells differentiation pattern;
- To investigate their functional characteristics before and after alginate microbeads encapsulation
- To investigate effects of alginate microbeads encapsulation on MSCs osteogenic differentiation potential, in different culture conditions (out and within rotating vessel bioreactor).

Experimental strategies

Cells Characterization

All experiments were performed after ex-vivo expansion and mesenchymal characterization of WJMSCs and PDLMSCs.

- WJMSCs and PDLMSCs were analyzed for mesenchymal hallmarks;
- WJMSCs: in order to find predictable criteria in selection of WJMSCs were cultured in 6,12 or 24 well-plate in control and osteogenic conditions (2-D stiren adhesion culture condition) and analyzed for clinical obstetric parameters of donors. Viability, markers expression and Calcium deposition ability were tested;
- PDLMSCs: in order to find culture conditions able to optimize osteogenic differentiation, PDLMSCs were cultured in 6,12 or 24 well-plate in control and different osteogenic conditions (2-D stiren adhesion culture condition, in presence of Polymeric Micelles (PMs) osteogenic inductors complexes or in presence of traditional osteogenic inductors). Viability, citotoxicity and Calcium deposition ability were tested.

Alginate beads encapsulation and different culture systems

WJMSCs and PDLMSCs were encapsulated in alginate beads after ex-vivo expansion.

- WJMSCs alginate beads (WJABs): viability, citotoxicity, markers expression and beads secretive profile were tested before and after cells encapsulation;
- WJMSCs and alginate beads (WJABs) and PDLMSCs alginate beads (PDLABs): Beads were split and cultured in 6 well-plate (We) or poured in rotary cells culture system (RCCS-4™ Bioreactor, Synthecon, Inc., Houston, TX, U.S.A.), in High Aspect Ratio Vessel (HARV™). Viability, markers expression and Calcium deposition ability were tested after cells encapsulation, in two different culture

conditions: “control” culture medium (CCM) and osteogenic differentiation medium (ODM).

MATERIALS AND METHODS

Cells characterization

Methods for the isolation, ex vivo expansion and characterization of WJMSCs from umbilical cord and PDLMSCs from human periodontal ligament are described.

Isolation of MSCs populations from Wharton's Jelly

Samples

In the obstetric department, UC were collected only after having obtained informed consent from the mother. Following delivery of the baby, the UC were collected from the into special bags containing sterile phosphate-buffered saline (PBS). WJMSCs cultures were initiated directly from UC samples, with a total generation frequency of 90%.

Human umbilical cords were collected after surgical and natural full-term deliveries with informed consent of the mothers. Long segments from 10 to 20 cm were sectioned and conserved at 4° C into sterile PBS until they were used in the laboratory (within 4h). Ethics approval was obtained from the institutional ethics committee.

Isolation and culture of WJMSCs

MSCs culture medium

- Dulbecco's Modified Essential Medium Low Glucose
- 10% (v/v) fetal bovine serum
- penicillin 100 mg/mL
- streptomycin 10 mg/mL

Protocol choosen followed several steps:

1. The umbilical cord segments were sectioned longitudinally to expose the WJ. Some incisions were made on the matrix with a sterile scalpel to expose a wider area of tissue. Forceps provided the isolations of vessels, both vein and arteries.
2. The WJ was minced into very fine pieces from 1 to 2 mm^3 avoiding any endothelial and epithelial contamination, mantaining all vessels integrity.
3. Small pieces were directly plated in T-25 cm^2 culture flasks, in Dulbecco's modified Eagle's medium low-glucose (D-MEM LG), supplemented with 10% fetal calf serum (Euroclone S.p.A., Milan, Italy), 1 mM L- Glutamine and antibiotics (penicillin 100 mg/mL and streptomycin 10 mg/mL).
4. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. At subconfluence (80–90%) cells were scraped off by 0.05% trypsin/ethylene diamine tetraacetic acid (EDTA) (Gibco, Grand Island, NE) (2min), washed, counted by hemocytometric analysis, assayed for viability, and thereafter used for in vitro experiments or encapsulation procedures.

Isolation of MSCs populations from periodontal ligament (PDL)

Samples

Following informed consent, teeth with healthy periodontal ligament were obtained as a result of tooth extraction for orthodontic purposes or removal of totally bone impacted lower or upper third molars.

All patients selected were healthy patients with clinically healthy periodontium. The extent of experiments was explained to all patients and informed consent was obtained according to the Helsinki Declaration.

PDL tissue were all processed within 4 h from extraction.

Before each surgical procedure, each patient underwent to 60" clorexidine 0,12% mouthrinse to avoid any bacterial field contamination.

After surgical incision, flap elevation and surgical osteotomy, teeth were collected with proper forceps and put into 15 ml tube with sterile PBS avoiding any contact with oral cavity.

Isolation and culture of PDLMSCs

MSCs culture medium

- Dulbecco's Modified Essential Medium Low Glucose
  10% (v/v) fetal bovine serum
  penicillin 100 mg/mL
  streptomycin 10 mg/mL

Protocol choosen followed several steps:

1. In sterile laboratory conditions, teeth were washed several times and PDL samples were obtained from the mid-third of roots of extracted third molars.
2. Gently PDL was separated from the middle third of the tooth root surface using forceps and a size 15 surgical blade in a 10-cm tissue culture dish containing PBS.
3. Then PDL tissue was washed for 30", 5 times in culture medium, Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum, penicillin (100 mg/mL) and streptomycin (10 mg/mL)
4. PDL tissue was cut into small pieces (approximately 2 mm^2) and placed in 6-well culture dishes with 1mL culture medium. After 5 days of incubation, culture medium was gently changed and cell culture was continued with medium changes every 3 days until PDL cells were subconfluent. After reached subconfluency, cells were passaged using 0.05% trypsin– 0.1% EDTA solution (Invitrogen) and expanded in T-25 cm^2 flasks.
5. At subconfluence (80–90%) cells were scraped off by 0.05% trypsin/ethylene diamine tetraacetic acid (EDTA) (Gibco, Grand Island, NE) (2min), washed, counted by hemocytometric analysis, assayed for viability, and thereafter used for in vitro experiments or encapsulation procedures.

Cryopreservation of Ex Vivo-Expanded MSCs

For all samples the same protocol for cryopreservation was chosen:

1. suspensions of culture-expanded MSCs were prepared by 0.05% trypsin/0.02% EDTA digestion and cells were enumerated. Viability was assessed using 0.4% trypan blue/PBS as described above. Cells were centrifuged and resuspended

in 0,9 mL of FBS and kept on ice. Cells were then resuspended in a 90% FBS, 10% dimethyl sulfoxide (DMSO) solution (Sigma Aldrich, St Luis, MO, USA).

2. Cryovials were placed at –20◦C for 1 hr, –80◦C overnight before transferring the cryovials into liquid nitrogen for long-term storage.
3. Recovery of the cryopreserved stock was achieved by rapidly thawing the cells in a 37◦C water bath. The cells were resuspended in 45 mL cold MEDIUM and and spin at 1500 rpm for 5 min.
4. Viability of cells was assessed using 0.4% trypan blue/PBS.

Flow cytometric analysis

Homogeneous populations of PDLMSCs and WJMSCs were analyzed for markers expressed on the cell surface.

WJMSCs were fixed in 4% paraformaldehyde and incubated at 4°C for 30' with 5µg/ml of the following monoclonal antibodies: anti-human-CD3, -CD34, -CD45, (DAKO Cytomation, Glostrup, Denmark), CD31 (Chemicon International, temecula, CA), -CD73, -CD90, -CD146 (Becton Dickinson, Mountaine View, CA), -CD105 (produced from the hybridoma cell line, clone SH2, ATCC, Rochville, MD). The cells were washed twice and incubated with 2.4 µg/ml of a polyclonal rabbit anti-mouse immunoglobulins/FITC conjugate or with 8 µg/ml of a polyclonal rabbit anti-rat immunoglobulins/FITC conjugate or with 1µg/ml of a polyclonal swine anti-rabbit immunoglobulins/FITC conjugate (DAKO Cytomation) at 4°C for 30 min. After two final washes, the cells were analysed using a FACStar plus Cytometer (Becton Dickinson). For isotype control, FITC-coupled non-specific mouse IgG was substituted for the primary antibody.

The PDLMSCs were analyzed for expression of MSC surface marker molecules, by direct immunofluorescent staining. Briefly, cell pellets were resuspended in PBS and incubated with fluorescein iso-thiocyanate (FITC)– or phycoerythrin (PE)–conjugated mouse anti-human antibodies CD45-PE, CD34-FITC, CD90-FITC, CD105-PE and Stro-1-FITC (DakoCytomation; Dako, Glostrup, Denmark) for 15min at 4°C. Monoclonal antibodies with no specificity were used as negative control.

Antibody-treated cells were then washed with PBS and spinned down. Cell pellets were resuspended in 400mL of PBS and analyzed by FACS Scan (Becton Dickinson, Franklin Lakes, NJ). For each sample, 20,000 events were acquired and analyzed using the CellQuest software (Becton Dickinson European HQ, Erembodegem Aalst, Belgium).

Viability analysis of MSCs populations

Before and after all experiments, the viability of the cells was analyzed by double staining with propidium iodide (PI) and Calcein-AM according to the manufacturer's instructions. For the PI and Calcein-AM analysis, cells were visualized under a fluorescence microscope (Nikon, Optiphot-2; Nikon Corporation, Tokyo, Japan) using the filter block for fluorescein. Dead cells stained red, while viable ones appeared green.

Adipogenic differentiation

To induce adipogenic differentiation, cells were treated with adipogenic medium for three weeks with medium changes twice a week. The adipogenic medium consisted of DMEM supplemented with 1 µM dexamethasone (Sigma Aldrich, St. Louis, MO, USA), 5 µg/mL bovine insulin (Sigma Aldrich, St. Louis, MO, USA), 0.5

mM 3-isobutyl-1- methyl-xanthine (IBMX) (Sigma Aldrich, St. Louis, MO, USA), 60 µM indomethacin (Sigma Aldrich, St Louis, MO, USA) and 10% FCS. On the 21st day the cells were fixed in 10% formalin neutral solution (Merck, Darmstadt, Germany) for 30 min and stained with fresh 0.6% oil red O solution to show lipid droplets in induced cells.

Cytotoxicity studies

The cytoxicity analysis was done on in vitro cultured MSCs before and after experiments. Determination of viable cells was done with [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) colorimetric assay (thiazolyl blue). MTT assay is based on the conversion of the yellow tetrazolium salt MTT to purple formazan crystals in the mitochondria of living cells [Denizot et al. 1986]. MTT provides a quantitative determination of viable cells.
After 72 hrs of treatments in triplicate, 200 µL of a solution of MTT (5 mg/ml) was added to each well of cells, and the plate was incubated for 2 hr at 37°. The medium was removed, and the MTT crystals were solubilized with 50% dimethylformammide (DMF). Spectrophotometric absorbance of each sample was measured at 570 nm.

Alkaline phosphatase analysis

For alkaline phosphatase staining, the Alkaline phosphatase (ALP) Leukocyte kit (Sigma) was used. To perform the test, prefixed mono-layered cells were incubated at room temperature in a solution containing naphthol AS-BI phosphate and freshly prepared fast blue BB salt buffered at pH 9.5 with 2-amino-2-methyl-1,3-propanediol (AMPD). The presence of sites of ALP activity appeared as blue cytoplasmatic staining.

ALP activity, before and after osteogenic induction, was evaluated inMSCs population by the hydrolysis of p-nitrophenylphosphate (PNPP), according to literature [Ibbotson et al. 1986]. Cells enzime activity (expressed as U(nmol/min)/ µg of protein) was evaluated before and after osteogenic differentiation. One unit was defined as the amount of enzyme which hydrolyzed 1 nmol/PNPP per minute. Cell protein was determined according to the Lowry method [Waterborg et al. 1994].

Alizarin Red staining

The extent of mineralized matrix in the plates was determined by Alizarin Red S staining (AR-S, Sigma) at different time, and before and after osteogenic induction. Cells were fixed in 70% ethanol for 1 h at room temperature, washed with PBS, and stained with 40 mM AR-S (pH 4.2) for 10 min at room temperature. Next, cell preparations were washed five times with deionized water and incubated in PBS for 15 min to eliminate non-specific staining. The stained matrix was observed at different magnification using a Leitz microscope.

Oil Red O Staining

Lipid vacuole formation in the adipogenic cultures was determined by Oil Red O (Sigma- Aldrich) staining on day 14. The cells were gently rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 1 hour, rinsed with H_2O, and incubated with 60% isopropanol for 5 minutes, and stained with the Oil Red O working solution (30 ml of 3 mg/ml Oil Red O/isopropanol stock solution plus 20 ml H_2O) for 5 minutes at room temperature. After rinsing with water, the cultures were counterstained with haematoxylin for 1 minute at room temperature, rinsed again, and observed with phase contrast microscopy (Nikon TS100).

Immunocytochemistry

Cells grown in chamber slides were fixed in 4% PFA for 20 minutes at room temperature, washed twice in PBS, treated with 3% H2O2 (in PBS) and incubated in 2% normal horse serum (S-2000, Vector labs, CA, USA) for 15 min at room temperature. After the incubation in blocking serum, the slides were incubated with monoclonal antibody anti-human-STRO1 FITC conjugated (Santa Cruz biotec) for 1 hour at room temperature. The slides were washed three times with PBS 1X and then were visualized under a fluorescence microscope (Nikon, Optiphot-2, Nikon Corporation, Japan) using the filter block for fluorescein; cells were also counterstained with DAPI.

Real-time quantitative RT-PCR

Cells from wells were harvested and total RNA was extracted using an RNeasy Mini Kit (Qiagen GMBH, Hilden, GM) according to the manufacturer's instruction. 2 micrograms of total RNA were reverse transcribed with the improm II RT system (Promega, WI, USA).

Real-time PCR was carried out using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems Inc, Foster City, CA, USA), TaqMan technology, and the Assays-On-Demand kit for human Slug and Runx2. The mRNA levels of target genes were corrected for GAPDH mRNA levels (endogenous control). All PCR reactions were performed in triplicate for each sample and were repeated three times. All experimental data were expressed as the mean ± S.E.M.

Scratch Wound Migration Assay

24 h before siRNA transfection, PLDMSCs and WJMSCs were seeded in 12 well plates in duplicate at density of $15x10^3/cm^2$ in DMEM supplemented with 10% FCS. Cells were transfected with 30 nM siRNA using Lipofectamine RNAiMAX (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturers' instructions. After 24 hours a scratch was introduced to the cell monolayer using a sterile 200 ml pipette tip. Transfected cells were incubated for 6 days at 37°C. As a negative control for the siRNA treatment, PDLMSCs and WJMSCs were seeded in DMEM with 10% FCS. Scratch wound modifications were observed at 0, 24 and 48 h. Knockdown of Slug expression was verified by Real-Time RT-PCR.

Encapsulation of MSCs in alginate microbeads and bioreactor culture

Encapsulation of MSCs

Monodisperse alginate beads containing cells were prepared using an encapsulation device that is based on a vibrating nozzle (Encapsulator Research Inotech, Dottikon, Switzerland). The encapsulator is composed by a 2-L glass reaction vessel with stainless steel top and bottom plates. The top plate contains a feed-line connected to a syringe and a vibrating nozzle. A nozzle with an internal diameter of 300mm was used. The flow of alginate to the nozzle is achieved by a precision syringe pump. The production of WJMSC-filled alginate microcapsules was optimized by changing the following experimental parameters: vibrational frequency, vibrational amplitude, alginate pumping rate (pump), and distance between the nozzle and the surface of the gelling bath (height) (see Table 1).

Parameter	Abbreviation	Meaning	Range
frequency	freq	frequency of the vibration of the nozzle	100.0-140.0 hz
amplitude	amp	amplitude of vibration of the nozzle	1.0-6.0 mm
pump	pump	polymer pumping rate	7.5-9.5 mL/min
height	height	distance from nozzle to surface of gelling bath	100.0-140.0 mm

Table 1: Production of alginate microcapsules by vibrational encapsulation procedure: the investigated experimental parameters and their range of variation.

Before encapsulation, WJMSCs were suspended in a 1.5% (w=v) aqueous solution of highly purified sodium alginate (Stern Italia, Milano, Italy) at a concentration of 8–12x10^6 cells=mL. The generated microdroplets were dropped into an isotonic barium chloride solution (1.2%; w=v); after gelation (3 min), the microbeads were washed twice with saline and cultured in 10% fetal calf serum (Euroclone S.p.A.) Dulbecco's modified Eagle's medium supplemented with antibiotics (penicillin 100 mg/mL and streptomycin 10 mg/mL) at 37°C in an humidified atmosphere of 5% CO_2.

Dimensional and morphological characterization of microbeads

The morphology of Ba–alginate microbeads was evaluated by optical stereomicroscopy (Nikon SMZ 1500 stereo micro- scope, Tokyo, Japan). Microbead size and size distribution were determined by photomicrograph analyses (Eclipsenet version 1.16.5; Laboratory Imaging s.r.o. for Nikon Italia, Firenze, Italy). Microbead samples, immediately after preparation and at intervals after storage under different conditions, were deposited onto a microscope slide and examined microscopically. A sample of 100–300 beads was examined, and the mean size was determined.

Determination of MSCs encapsulated and free secretory pattern by Bio-Plex® analysis

After 72h of in vitro cell culture, the medium from MSCs growing as monolayer and embedded in Ba–alginate microbeads was collected and analyzed for a set of selected proteins. To compare and normalize the data of adherent and encapsulated MSCs, the microbeads were washed twice with PBS before the analyses and incubated at 37°C, for 15min, with 500µL of 50mM EDTA (Sigma, St. Louis, MO) in PBS buffer. EDTA was then neutralized with additional 10mL PBS, and cells were collected by centrifuging at 400 g for 15 min. Pellets obtained from MSCs growing as monolayer and embedded in Ba–alginate microbeads were lysed with 50 µL buffer (NaCl 150mM; Tris-HCl pH 7.4 20mM; EDTA 1mM; ethylene glycol tetraacetic acid (EGTA) 1mM; Triton X-100 1%; Na3VO4 0.1mM; phenylmethanesulphonyl fluoride (PMSF) 1 mM; proteinase inhibitory cocktail (PIC) 0.1% v=v). About 10 µL of each sample was tested with the Bradford method to determine proteins content. Concentrations of interferon- alpha2 (IFN-a2), interleukin-1alpha (IL-1a), IL-2 receptor a, IL-3, IL-12 (p40), IL-16, IL-18, cutaneous T-cell-attracting chemokine (CTACK), growth regulated oncogene-a (GRO-a), hepatocyte growth factor (HGF), intercellular adhesion molecule-1 (ICAM-1), leukemia inhibitory factor (LIF), monocyte chemotactic protein-3 (MCP3), macrophage colony stimulating factor (M-CSF), macrophage migration inhibitory factor (MIF), monokine induced by IFN-Gamma (MIG), β-nerve growth factor, stem cell factor, stem cell growth factor-β (SCGF-β), stromal cell-derived factor 1a (SDF-1a), tumor necrosis factor-β, tumor-necrosis-factor related apoptosis inducing ligand (TRAIL), and vascular cell adhesion molecule-1 (VCAM-1) were simultaneously evaluated using a commercially available multiplex bead–based sandwich immunoassay kit (Human 23-plex; Bio-Rad Laboratories, Milano, Italy). Bio-Plex® analysis was performed following the manufacturer's instructions. About 23 distinct sets of fluorescently dyed beads loaded with capture

monoclonal antibodies, specific for each cytokine, were used. Secretion and standard samples (50 µL/well) were incubated with 50 µL of premixed bead sets in a prewet 96-well microtiter plate. After incubation and washing, 25 µL of fluorescent detection anti-body mixture was added and left to react for 30 min under gentle shaking; samples were then washed and resuspended in the assay buffer. Standard calibration curves for each protein were used, ranging from 2 to 32000pg/µL; the minimum detectable dose was 2pg/µL. The formation of the different immunocomplexes was measured by the Bio-Plex® Protein Array System (Bio-Rad Laboratories). A 50 µL volume sample was withdrawn from each well, and the fluorescent signal of a minimum of 100 beads per region (chemokine/ cytokine) was measured. To compare directly secretive content of adherent and encapsulated MSCs, all values were normalized with respect to the total protein amount.

Bioreactor setup

Three-dimensional dynamic culture experiments were performed in Rotary Cell Culture System™ (RCCS-4™ bioreactor, Synthecon™, Inc., Houston, TX, U.S.A.), with High Aspect Ratio Vessel (HARV™).

For all experiments 10 ml HARV™ vessels were used.

RCCS™ is a technology for growing either a chorage dependent or suspension cells in laboratory. Two basic type of RCCS vessels are manifactured by Synthecon™, Inc.:

- Slow Turning Lateral Vessel (STLV™) is tubular shaped and has central gas transfer core;
- High Aspect Ratio Vessel (HARV™) has a disc shaped culture chamber with the oxigenator membrane forming the iside wall of the vessel.

HARV™ vessel consists of a horizontally rotated culture chamber, where the alginate beads were suspended, and a perfusion system with media continuously flowing through the culture chamber. The culture chamber can rotate in the X-axis at certain speed to suspend the cellular scaffolds in a free floating culture condition. Rotating-wall reactor need control systems to vary the rotation speed of the vessel in function of the cells culture system size (i.e. scaffold, tissue mass). When the rotation velocity is maintained constant, the cells/beads will see its terminal free-fall velocity to increase.

Alginate microbeads were inserted in each HARV™ 10 mL vessel filled with basal medium. 50 to 60 beads were used in each vessel.

After alginate microbeads insertion, HARV™ vessels were filled with basal medium, and all air bubbles were removed from the culture chamber. Then HARV™ vessels were located into incubator at 37°C and 5% CO_2 with the whole RCCS-4™. Cells were rotated at 25 rpm for all the experiment duration. Medium was replaced in vessel every three days.

Osteogenic differentiation

The ability of WJMSCs and PDLMSCs to differentiate into osteblastic cell lineages in vitro can be investigated by culturing under inductive conditions.

Osteogenic differentiation of WJMSCs and PDLMSCs

WJMSCs Differentiation medium:

- Dulbecco's Modified Essential Medium High Glucose
- 10% (v/v) fetal calf serum
- 1× L-glutamine
- 1× penicillin-streptomycin

- 0.1 μM dexamethasone
- 10 mM β-glycerophosphate
- 50 μg L-ascorbic acid-2-phosphate

PDLMSCs Differentiation medium:

- Dulbecco's Modified Essential Medium High Glucose
- 10% (v/v) fetal calf serum
- 1× L-glutamine
- 1× penicillin-streptomycin
- 0.1 μM dexamethasone
- 10 mM β-glycerophosphate
- 50 μg L-ascorbic acid-2-phosphate

Culture protocol

1. In vitro-expanded MSCs were seeded 2 × 10^4 cells/cm^2 D-MEM growth medium and incubate at 37°C in 5% CO_2 and >90% humidity.
2. Cells adhered well overnight in tissue culture incubator.
3. After 24 h, the D-MEM growth medium was aspirated and add an equivalent volume of osteogenic inductive medium.
4. the medium was changed twice a week.
5. Cells differentiate up to 3 weeks.

Osteogenic and control culture conditions

Three different culture conditions were established:

- two-dimensional culture as monolayer, in different osteogenic conditions. For the two-dimensional monolayer culture, confluent MSCs from the 2nd-4th passages were trypsinized and placed inside 6, 12 and 24-well plates. Cells were

maintained in the basal medium for 1–2 days until they reached confluence, and then the culture was continued with the osteogenic induction supplement-containing medium. Medium changes were performed twice a week.

- three-dimensional static culture in alginate microbeads. Alginate microbeads for three-dimensional static culture were placed in 6-well plate with basal medium for 48 h. Then, when encapsulated cells were induced to osteogenic differentiation, basal medium was removed and changed with osteogenic one. Osteogenic medium was used for the 21-days experimental period. Medium was changed twice a week, removing 2mL and adding 2 mL.
- three-dimensional dynamic culture in alginate microbeads using the RCCS-4™ (Synthecon™, Inc., Houston, TX, U.S.A.), with High Aspect Ratio Vessel (HARV™). For all experiments 10 mL HARV™ vessels were used. When osteogenic differentiation was performed, 48 hours after incubation the basal medium was changed with the osteogenic one in the vessel (an other vessel contained basal medium and beads as control). Medium was changed twice a week for the 21-days experimental period.

Batch Polymeric micelles (B-PMs) and Microfluidic Polymeric micelles (M-PMs) for osteogenic inducers entrapment

Polymeric Micelles (PMs) were designed and prepared by Prof. Claudio Nastruzzi at the Department of Pharmaceutical Science, University of Ferrara, Italy.

PMs were prepared in batch systems (B-PMs) using Pluronic F127® solutions in DMSO at a concentration of either $1.5 \cdot 10^{-2}$ M or $7.5 \cdot 10^{-3}$ M. PMs were formed by pipetting the polymeric solutions into deionized water (with a polymer solution to water volume ratio, R, ranging from 0.03 to 0.13) and stirred using the pipette.

Microfluidic synthesis of PMs (M-PMs) was carried out in microreactors consisting of three inlets and one main reaction channel with an outlet. A hydrodynamic flow

focusing configuration was created within the microreactor where the focused central stream of polymer-DMSO solution flowed along the main channel and met two lateral sheath streams of water side by side. Volumetric flow rate ratios, R, of polymer solution to water were varied by altering each stream's flow rate. The total flow rates for three different microreactors were controlled at 2.00, 0.65 and 0.30 mL/h. An inverted microscope (Olympus IX51, Japan) was used to monitor the flow within the microchannel. The product sample was collected from the reactor outlet (typically, 2 mL), and used for further analysis and size characterisation. DMSO was obtained by Sigma-Aldrich UK, and used as supplied. Polymer Pluronic® F127 was provided by BASF Chem Trade GmbH.

Following the preparation of PMs, water was added to the samples in order to have a comparable amount of solvent (3.2% v/v) in different samples. A nanoparticle. Tracking analysis (NTA) system NanoSight LM10 (NanoSight Ltd. Amesbury, UK) was used to measure the hydrodynamic particle diameter of the PMs. For each measurement, c.a. 300 μL of sample was loaded in the detection cell and five measurements were performed to give an average value using NanoSight NTA Analytical Software. For comparison with NTA system, also a classical Dynamic Light Scattering (DLS) was used to obtain the hydrodynamic particle diameter. Particle sizing was performed using dynamic light scattering with Zetasizer Nano ZS (Malvern Instruments Ltd., U.K.). For each measurement, c.a. 200 μL or more volume of the sample was loaded in a disposable low-volume cuvette. Three measurements were performed on each sample. Size distributions and Z-average sizes were obtained by averaging over three measurements.

For our experiments, specific PMs were prepared with the two aforementioned techniques by Prof. Claudio Nastruzzi at the Department of Pharmaceutical Science, University of Ferrara, Italy.

- Batch PMs (B-PMs):
    - 0.1 μM dexamethasone;

- 50 µg/mL L-ascorbic acid-2-phosphate;
- Working solution was 100 µL in 1 mL of culture MEM;

- Microfluidic PMs (M-PMs):
  - 0.1 µM dexamethasone;
  - 50 µg/mL L-ascorbic acid-2-phosphate,
  - Working solution was 100 µL in 1 mL of culture MEM.

When ostegenic differentiation of MSCs was performed with PMs, different culture condition was tested. The use of micelles allowed to include dexamethasone and ascorbic acid, while 10 mM of β-glycerophosphate addition in medium was still necessary in a traditional solution.

Osteogenic Differentiation medium:
- Dulbecco's Modified Essential Medium High Glucose
- 10% (v/v) fetal calf serum
- 1× L-glutamine
- 1× penicillin-streptomycin
- 10 mM β-glycerophosphate
- PMs (for both PMs preparations, working solution was 100 µL in 1 mL of culture MEM).

Statistical analysis

Data are presented as the mean ± SE from at least three independent experiments, where indicated. Statistical analysis was performed by one-way analysis of variance followed by the Student's t test. A P value <0.05 was considered statistically significant.

RESULTS

Characterization of human MSCs from Wharton's Jelly umbilical cord and from periodontal ligament

Isolation of MSCs from the WJ

All umbilical cord samples were collected by Professor Fortunato Vesce, Department of Biomedical Sciences and Advanced Therapies, Section of Obstetric and Gynaecological Clinic, Azienda Ospedaliero-Universitaria S. Anna, Ferrara, Italy. As soon as possible the umbilical cords samples were delivered to the laboratory, and within 3-4 hours all samples were processed as described above. During time elapse between collection and process in laboratory cords were manteined in sterile PBS at 4° C.

As previously reported [Secco et al. 2008] Wharton's Jelly was chosen instead of the whole cord (epithelium and vessels) to isolate a relatively homogeneous cell population, avoiding in this way any epithelial cell contamination.

Contrary to the majority of groups working on WJMSCs, we chose to process the WJ based essentially on the capacity of MSCs to adhere to a plastic surface, without enzymatic treatment or stripping of vessels (see **FIGURE 2**).

After section of 5 cm umbilical cord segments, each part was washed several times to eliminate clots and cord blood (see **FIGURE 2, A**).

With a sterile scalpel longitudinal incisions were performed on cord epithelium carefully, to expose the underneath matrix (see **FIGURE 2, B** and **C**).

While the presence of mesenchymal stem cells in umbilical cord blood is controversial, according to literature we were able to generate primary cultures from all cord samples with a 100% yield .

Among all samples processed in our lab, 60 samples of umbilical cord were collected, processed and analyzed for obstetric factors. For each mother and newborn, the clinical parameters reported in Table 1 were considered.

Sample	Mother's age (ys)	Weeks of pregnancy	Mode of delivery	Gender	Birth weight (Kg)
1	26	29	CS	F	1.25
2	31	32	CS	M	1.74
3	30	40	CS	F	3
4	30	32	CS	M	1.9
5	30	32	CS	M	1.91
6	37	40	SP	F	3.59
7	21	41	SP	F	3.75
8	35	40	SP	F	3.17
9	33	42	SP	M	3.05
10	31	40	SP	M	3.69
11	38	38	CS	F	3.32
12	38	38	CS	F	2.6
13	38	39	SP	M	3.45
14	36	40	SP	F	3.6
15	29	40	CS	F	3.22
16	33	38	SP	M	3.7
17	34	38	CS	F	3.45
18	35	35	CS	M	3.31
19	34	40	SP	M	3.21
20	35	40	SP	M	3.18
21	32	38	CS	F	3.6
22	33	35	CS	M	2.41
23	33	35	CS	M	2.53
24	40	40	SP	F	3.75
25	38	39	SP	M	3.36
26	29	37	CS	F	3.62

27	29	40	SP	M	3.4
28	39	39	CS	M	3.62
29	33	40	SP	F	3.12
30	32	35	SP	F	3.17
31	29	37	CS	M	2.14
32	37	39	CS	M	3.37
33	20	40	SP	F	3.02
34	41	39	CS	F	2.73
35	35	37	CS	F	2.77
36	34	38	CS	F	2.9
37	38	38	SP	F	3.05
38	34	40	SP	M	3.7
39	32	36	SP	M	2.93
40	27	38	SP	M	3.1
41	30	34	SP	M	2.6
42	31	41	SP	F	3.25
43	27	33	CS	M	1.915
44	27	33	CS	F	2.005
45	26	39	SP	M	3.35
46	34	42	CS	M	3.51
47	23	37	CS	F	2.95
48	30	39	CS	M	4.24
49	33	39	CS	F	3.3
50	37	39	CS	M	2.84
51	33	39	CS	M	3.27
52	32	40	CS	M	3.9
53	31	37	CS	F	1.98
54	35	40	SP	M	3.95
55	23	37	SP	M	3.11
56	40	38	CS	M	2.85
57	37	39	CS	F	3.3
58	25	35	CS	M	3.57
59	25	35	CS	F	2.38
60	27	29	CS	M	2.1
F (female) M (male) CS (caesarian delivery) SP (spontaneous delivery)					

Table 1: The population examined for clinical parameters influence consisted of 60 healthy pregnant women successively enrolled voluntarly between February 1, 2008 and March 1, 2009. The recorded clinical parameters are: mother's age, weeks of pregnancy, mode of delivery, newborn gender and birth weight.

Although differences between each mother and newborn clinical parameter, cords were all easy to chop and scrape, to obtain only the jelly mucoid connective tissue from each sample (see **FIGURE 2, D**).

As previously described we placed small pieces (2-3 mm^2) directly into 25-cm^2 flasks in 10% FCS D-MEM Low Glucose medium.

After 3 to 5 days cell colonies were observed adhering on plastic flask bottom. These colonies consisted of spindle-shape fibroblast-like cells. Sub confluent fibroblast-like cells were the only cell types growing under these conditions after 2 weeks of culture. ~50-60% confluence was reached within 2 weeks (see **FIGURE 3, B** and **C**).

After 2 weeks, the WJ tissue pieces were removed. At that time the adherent cells were expanded and the medium was renewed. These cells represented the so called “p 0”.

This method for cell isolation was easy, fast, and reliable with low contamination risk.

Isolation of MSCs populations from periodontal ligament

The human PDL connective tissue (see **FIGURE 4**) attached to tooth and alveolar bone from different donors was collected, as adult source of MSCs in so called “stem cell niches”, and processed within 4 hours.

All PDL samples came from bone impacted lower or upper third molars of healthy patients.

The protocol chosen (total impacted teeth; clorexidine mouthrinse before surgical procedures) avoided any contamination by oral bacteria. In addition, scalpel was

chosen to detach gently the tissue from the root, avoiding any contamination by dental papilla or dental pulp (see **FIGURE 5, B**).

Then the tissue was cut into small pieces (approximately 2 mm x 2 mm) and placed at the centre of 6-well culture dishes.

The small amount of tissue for each patient was considered, so 6-well culture plate condition was chosen instead of 25 cm^2 culture flasks to promote cells outgrowth and proliferation.

As done for UC samples, and contrary to different groups working on PDL tissue, we chose again to process the PDL based essentially on the capacity of MSCs to adhere to a plastic surface, without enzymatic treatment.

After 3 to 5 days outgrown cells were observed nearby tissue fragments. They had spindle and fibroblastic morphology (see **FIGURE 5, C**).

A total of 20 teeth (19 wisdom teeth) were obtained from 20 patients consecutively selected among those treated at Dental Section, University (19-34 years of age) and clinical data were recorded (see Table 2).

Sample	Age	Gender	Tooth	Colonies	Clinical Data	Drugs
1	26	M	18	+	-	-
2	23	M	48	-	-	-
3	21	F	28	+	asthma	steroids o.n.
4	33	F	48	+	-	-
5	31	F	48	-	-	-
6	19	M	38	+	-	-
7	27	M	48	+	-	-
8	33	F	18	-	-	-
9	21	F	18	+	-	-
10	19	M	28	+	-	-
11	31	F	48	-	-	-

12	28	F	38	+	-	Contracept.
13	24	M	38	+	-	-
14	34	M	28	+	-	-
15	23	F	48	+	-	-
16	24	M	38	+	-	-
17	26	M	48	+	-	-
18	23	F	38	-	-	Contracept.
19	34	M	38	+	-	-
20	22	M	48	+	-	-

Table 2: 20 healthy patients enrolled voluntarly between January 1, 2010 and 1, September 2010. All teeth were totally impacted.

15 primary human PDL cell populations were obtained on the basis of their ability to form well-defined colonies. All of them displayed a microscopically distinct spindle-shape fibroblast-like cells morphology (see **FIGURE 5, C**).

They grew rapidly and were subcultured at about day 14, with medium changes every 3 days, to avoid over-confluence, especially around explanted PDL tissue (see **FIGURE 5, D**).

The success rate of cell expansion, without collagenase/dispase or trypsin/EDTA was more than 75%.

Flow cytometric analysis

MSC characteristics were evaluated by the immunophenotypic profile of adherent cells from each culture by testing a panel of surface markers using flow cytometry. The expression of phenotypical markers was evaluated by the intensity of fluorescence of cells of the studied population.

Considering WJMSCs, samples were positive for mesenchymal cell markers including CD90 (Thy-1), CD73, CD105, CD146, CD44 (hyaluronan receptor),

CD29 (β-1 integrin), but negative for CD31 (endothelial cell marker), CD3, CD14, CD34, CD45 (hematopoietic cell markers). Also the expression of CD90, CD73 and CD105 (SH2, endoglin) was significanlty high, as for CD146 too (see **FIGURE 6**, panel **I**).

Also adherent cells derived from PDL did not express hematopoietic/endothelial markers (CD34, CD45) analyzed by flow cytometry. In addition, the majority of cells expressed high levels of mesenchymal and adhesion markers such as CD90 and CD105 (see **FIGURE 7,** panel **I**).

Adherent cells derived from PDL were also positive for STRO-1 expression which was analyzed by flow cytometric analysis and immunofluorescence (see **FIGURE 7** panel **I, A**).

Viability assay

Calcein-Propidium double stain was chosen as viability assay to show at the same time the presence of living or dead cells for all experiments. WJMSCs and PDLMSCs obtained with the described procedure appeared always viable: a representative experiment is reported in **FIGURE 6** panel **I, A** and **FIGURE 7** panel **II, A**.

Also microscopic observations in brightfield showed that cells owned the spindle-shape fibroblast-like cells morphology (see **FIGURE 6** panel **I, B** and **C** and see **FIGURE 7** panel **II, B**). These findings were recurrent during all experiments. This stainig allowed to follow during time cells viability also after encapsulation procedures, as mentioned successively.

Differentiation potential

The isolated WJMSCs and PDLMSCs were tested for their potential to differentiate into osteogenic or adipogenic cells in the presence of specific inducing factors. For this purpose the cells at the 3rd to 4th passage at least, were committed to differentiate in presence of the specific differentiation inducers. Osteogenic differentiation was induced for 3 weeks with DMEM-HG for both WJMSCs and PDLMSCs, supplemented with 10% FCS, 0.1 mM dexamethasone, 10 mM β-glycerophosphate and 50 μg/mL ascorbic acid. In both the control and differentiating cultures the medium was changed twice a week. Cells started to change morphologically as early as 5 days in an inducing culture medium; the cells lost their typical fibroblast appearance turning into wider polygonal cells. These changes became more evident with time and on the 21st day, when specific Alizarine Red staining was performed. The effectiveness of differentiation was assessed by this assay for the identification of Calcium deposition in extracellular space. Terminal differentiation of the cells appeared as intense red staining (see **FIGURE 8** panel **I, B** and panel **II, B**). Another change observed was that, upon differentiation, the osteogenic cells continued to grow as a monolayer while the cells in control cultures preserved their fibroblast-like morphology (see **FIGURE 8** panel **I, B** and panel **II, B**).

To assess adipogenic differentiation, WJMSCs and PDLMSCs were cultured in an adipogenic medium consisting of DMEM-LG or DMEM-HG for WJMSCs and PDLMSCs respectively, supplemented with 1 μM dexamethasone, 5 μg/mL bovine insulin, 0.5 mM IBMX, 60 μM indomethacin and 10% FCS for 21 days. After 14 days tiny intracytoplasmic droplets can be observed; most cells became larger and had a round shape. The effectiveness of differentiation was assessed by histochemical staining for the identification of neutral lipid vacuoles by Oil red O staining. In the adipogenic differentiated cells, red stained intracellular vacuoles (see **FIGURE 8** panel **I, A** and panel **II, A**) can be observed.

Scratch assay

The scratch test was chosen as in vitro wound model to investigate WJMSCs and PDLMSCs migration ability and its modulation after an artificially induced detachement within the cell monolayer in presence of a specific biological response modifier.

This set of experiments had the aim to investigate the effectiveness of wound assay in pointing out effect of cells phenotype modification, through the direct observation of different cells behaviour when comparing treated cells to control. The wound healing and the ability to migrate was tested in the cells depleted of a critical transcription factor such as Slug after siRNA interference. Slug is involved in a broad spectrum of biological functions, such as cell differentiation, motility, cell-cycle regulation and apoptosis.

Considering WJMSCs, cells were transfected, as described in the Material and Method section, for 3 days with siSlug or a nonrelevant siRNA as negative control. Knockdown of Slug expression was verified by Real-Time RT-PCR. RT-PCR results, after correction to GAPDH content, are expressed as siRNA/Slug2 over control ratio.

Slug-silenced or untreated WJMSCs were cultured until they reached 100% confluence. Monolayers were then scratch wounded for both conditions. siSlug treated cells exhibited a reduced capacity to cover the scratch if compared to untreated just after 24 hours (see **FIGURE 9** panel **I, B**). In fact, untreated WJMSCs exibited a rapid modification of wound margin, due to active proliferation and migration ability.

Considering PDLMSCs, the same Slug silencing protocol was performed to modulate cells behaviour in the same in vitro wound model created for WJMSCs. Slug expression was determined at mRNA level and knockdown of Slug expression was verified by Real-Time RT-PCR; expression of Slug was shown

significantly reduced, as reported (see **FIGURE 9** panel **II, A**). siSlug treated cells exhibited a reduced capacity to cover the scratch if compared to untreated (ctr). As done for WJMSCs, observations went on for more 24 hours, showing that PDLMSCs' Slug knockdown affected cells motility, avoiding any closure of the wound. The results confirm the role of Slug transcription factor in the regulation of cell migration ability. In addition, these data demonstrated that both experimental models are sensitive to depletion of an important transcription factor such as Slug, and at the same time are suitable to study the functional effect of a specific gene expression modulation.

Osteogenic potential of WJMSCs and variability between samples.

With the collaboration of Section of Obstetric and Gynaecological Clinic, Azienda Ospedaliero-Universitaria S, Anna, it was possible to obtain fresh samples of human umbilical cords, ready to be processed within few hours, as suggested by previous studies.

Among the collected samples, we selected the 20 most homogeneous ones considering the percentage of CD-90 presence and CD-45 absence (≥ 99%), and we analyzed for each one different obstetric parameters (see **FIGURE 10** table **A**).

We conducted analyses adjusting for two specific markers of osteoblast differentiation: the activity of Alkaline Phosphatase (ALP) and the expression levels of Runt-related transcription factor 2 (RUNX-2) which increases transcription of osteoblast specific genes. Focusing on basal levels of ALP and RUNX-2, it has been possible to demonstrate that these parameters can be predictive of osteoblastic potential of WJMSCs (see **FIGURE 10** panel **C**). In fact, the samples with high basal levels of RUNX-2 and ALP are more prone to deposit mineral matrix if compared to WJMSC with low levels of these two proteins.

Next we analyzed whether the basal levels of RUNX-2 and ALP correlate with the examined obstetrics factors (see **FIGURE 10** panel **C**). We found that the infant gender and mode of delivery didn't significantly correlate ($P > 0.05$) with basal RUNX-2 expression and ALP activity.

On the other hand, the age of the mother at delivery, has a significant impact on the basal ALP activity but doesn't affect RUNX-2 expression level (see **FIGURE 11** upper graph). Samples collected from mothers which were <32 years old give origin to WJMSCs with high ALP activity. Interestingly, birth weight of the infant was shown to significatively impact on RUNX-2 basal expression level which, (see **FIGURE 11** lower graph), decreases with the decreasing of the baby's weight. The same relationship was found for the duration of pregnancy (see **FIGURE 11** middle graph). In fact, it was found that WJMSCs from babies born before the 37 weeks of gestation express lower basal level of RUNX-2 than the full term borns. It is very likely that WJMSCs recovered from premature birth contain a high number of undifferentiated cells with high plasticity, a condition which is not actually required for osteoblast differentiation.

As a whole, these findings led us to focus on two parameters, weeks of pregnancy and consequently birth weight of the baby, and RUNX-2 basal levels, subdividing the collected samples in the two subgroups: subgroup I, premature birth with low levels of RUNX-2, and subgroup II, full term birth with high levels of RUNX-2 (see **FIGURE 10** table **A**). The ability of the samples belonging to these two subgroups to complete the event of cellular maturation, that is the deposition of mineralized matrix, was then compared. Two representative samples of the two subgroups (see **FIGURE 10** table **A**) demonstrate that, samples from subgroup I showed a null mineralization status also after 21 day of cell culture in osteogenic medium, whereas samples from subgroup II showed a high level of mineralization beginning from day 14. Therefore, these findings suggest that maximal WJMSCs

osteoblastic potential can be obtained by primary cultures with RUNX-2 high basal levels, selected from the heaviest term babies.

Another clinical observation that is important to do is that the cases below 37 weeks of gestation were all treated with 24 mg of bethametasone two hours before delivery, therapy routinely given to all the pregnant women delivering prematurely, in order to prevent respiratory distress syndrome in the newborns.

Absence of osteoblastogenesis could have been expected in the samples derived from women delivering prematurely. Since we observed a low or null mineralization status in WJMSCs from such patients (see **FIGURE 10** panel **B**), it could be possible to point out a correlation between the two events.

The analysis of the basal level of RUNX-2 and ALP activity allowed to quickly test an high number of mesenchymal precursors cultured in vitro and select the ones with probably higher osteogenic potential. Also our results suggest that it is preferred to recruit samples from full term borns without paying attention to mother's age.

The employment of Polymeric Micelles (PMs) to deliver osteogenic inducers.

After osteogenic differentiation potential was investigated for PDLMSC populations (see **FIGURE 8** panel **II**), we chose these cells to investigate an in vitro different approach to induce osteogenic differentiation.

Polymeric Micelles (PMs) designed and prepared by Prof. Claudio Nastruzzi at the Department of Pharmaceutical Science, University of Ferrara, Italy (see **FIGURE 12** panel **I**), were chosen to deliver in culture medium osteogenic inducers, instead of traditional solubilization with DMSO, which is already known to be cytotoxic.

For our experiments, two different kind of PMs were tested, differing for generation techniques: Batch technique for PMs (B-PMs), that is considered the traditional method, and Microfluidic technique for PMs (M-PMs) (see **FIGURE 12** panel **II**).

In order to investigate if PMs affect cell viability MTT test (see **FIGURE 13**) was performed. PDLMSCs cultured in adhesion conditions were exposed to different conditions for 72 hours. The presence of formazan salts, marker of the viable cells, was reported in the graph as percentage of viable cells respect to control culture condition (rated to 100%).

The use of PMs allowed to include dexamethasone and ascorbic acid, while 10 mM of β-glycerophosphate addition in medium was still necessary in a traditional solution. All different culture conditions exhibited no appreciable cytotoxicity (cells viability was higher than 75%) (see **FIGURE 13**). Comparing the results obtained with cells exposure to PMs produced in batch reactor (see **FIGURE 13,** grey column) or microreactors (see **FIGURE 13,** horizontal white stripes column), the effectiveness of the microfluidic technique to generate PMs instead of batch technique, in term of uniformity and no dispersion of solvents in culture medium, was observed. This characteristic resulted in lower cytotoxicity.

Afterwards, a set of in vitro experiments was performed to monitor effects of PMs for a 21-days period of time, in terms of cell viability and osteogenic differentiation (see **FIGURE 14**).

Microscopic observations, Viability Assay and Alizarin Red Staining were performed at 7, 14 and 21 days. PDLMSC cells viability wasn't affected by presence of PMs, both M-PMs and B-PMs at 7, 14 and 21 days (see **FIGURE 14, B-PMs** and **M-PMs columns** on the right, 2nd, 4th and 6th lines) .

At day 7, PMs conditions seemed to promote an earlier calcium deposition (see **FIGURE 14, ODM** and **B-PMs columns**, 1st line) when compared to traditional osteogenic condition (see **FIGURE 14, ODM, B-PMs** and **M-PMs columns**, 1st line).

After 14 or 21 days, Alizarin staining showed how this difference in promoting mineralization went on (see **FIGURE 14, ODM, B-PMs** and **M-PMs columns**, 3rd and 5th lines).

With the use of M-PMs the same amount of mineral deposition that was carried out with traditional osteogenic condition at day 21 was present at day 14 (see **FIGURE 14, ODM column** 5th line**, M-PMs column**, 3rd line respectively).

The results from this set of experiments confirmed the hypothesis that during in vitro experiments efforts to modify environment in terms of composition and soluble molecules could reveal the real osteogenic potential of each cell population, that sometimes is more affected by collateral events (i.e. culture medium flow inside the well, presence of higher concentration of toxic molecules than expected, like DMSO).

The use of the PMs here described to control in terms of space and time the release of inducers, like dexamethasone, avoided uncontrolled presence of DMSO and all its well known side effects, resulting in different speed of differentiation for PDLMSCs.

Encapsulation of WJMSCs and PDLMSCs in alginate microbeads

Alginate microbeads: preparation and characterization of beads

To overcome limits connected to the use of traditional 2D in vitro models, a first set of experiments was designed to study the behaviour of cells in three dimension conditions, considering WJMSCs as reliable cell population to set these conditions. Cells encapsulation was performed by Prof. Claudio Nastruzzi at the Department of Pharmaceutical Science, University of Ferrara, Italy.

WJMSCs were embedded into alginate microbeads by an encapsulation device based on a vibrating-nozzle (see **FIGURE 15**). The encapsulation procedure was relatively simple and consisted of a limited number of steps. In order to achieve complete biocompatibility, indispensable for mammalian cells, the encapsulation

procedure was conducted at room temperature under physiologic pH and tonicity using a pyrogen-free alginate solution.

A classical intuitive approach COST (Changing One Separate factor a Time) was employed to define the experimental set up, including the selection of the crucial experimental parameters. This approach was chosen in order to evaluate which factor(s) could mostly affect the morphology of the produced alginate microbeads in terms of general geometry, surface characteristics and dimensions. Following the above procedure, each experimental parameter was varied indipendently (see **FIGURE 16,** panel **I**), while all the others were maintained constant, to see direct effects on microbeads. Experiments were performed changing the following parameters: frequency of vibration (freq), amplitude of vibration (amp), polymer pumping rate (pump) and distance between the nozzle and the gelling bath (height). The frequency was calculated from a knowledge of the alginate solution viscosity for the nozzle diameter used, to obtain the desired microbeads size and morphology (mean diameter comprised between 550 and 650 μm with a spherical shape). The vibrating frequency of the nozzle was varied from 100.0 to 140.0 Hertz while the rate of polymer pumping was investigated in the range 7.5-9.5 (mL/min) (see **FIGURE 16,** panel **II**). The investigation of these parameters revealed that if the vibrating frequency was set too high (>200.0 Hertz) a progressive number of *coalescences* (formation of clusters of microbeads partially fused together) was detectable in the microbead population (data not shown). The amplitude of the vibration applied to the nozzle (varied in a range from 1.0 to 6.0 mm) had only a slight effect on the microbead characteristics such as size, sphericity and presence of coalescences. On the contrary, the distance between the nozzle and the gelling bath (range 100.0-140.0 mm) has a great effect on the microbead morphology. In fact, a distance of 100.0 or 120.0 mm caused the formation of microbead with an elliptic shape (formation of tails). Considering the results of this set of experiments (see **FIGURE 16,** panel **II**), among the 4 different

experimental variables, only the amplitude of the vibration has minor effect on the microbeads formation, while all the others exert significant morphological and dimensional changes on the alginate microbeads. The microbeads with the best morphology (see **FIGURE 17,** panel **I, A** and **B**) were produced by the following set up: a frequency of 150 Hz, an amplitude of 1.0 mm, a pump flow rate of 8.5 mL/min and an height of 140.0 mm.

Once the instrumental set up was achieved, we focused our attention on another important experimental parameter: the concentration of alginate solution. Different percentages were tested, ranging from 0.5 to 3.5% (w/v) and the effect on the production of microbeads was considered as well. As first observation, it should be mentioned that, using the automated vibrating nozzle instrument, we succeeded in obtaining microbeads using alginate concentration up to 2.0% (w/v). On the contrary, using higher concentrations of alginate (>2.0%, w/v), the resulting solution was too viscous to be efficiently converted in microdroplets. Samples of microbeads produced with an alginate concentration of 0.5, 1.0, 1.5 and 2.0% were analyzed for morphology, size and size distribution. The results of the above experiment are showed (see **FIGURE 16,** panel **II**). Microbeads produced with an alginate concentration comprised between 1.0-2.0% (w/v) were spherical in shape and characterized by a smooth surface (see **FIGURE 17,** panel **I, A** and **B**). Lowering the alginate concentration down to 0.5% (w/v) caused partial broke-up of microbeads resulting in particles with an irregular shape. This behaviour was attributed to the mechanical stress caused by the landing in the hardening barium chloride solution. Moreover, at the same concentration, some coalescences were detectable. We obtained alginate microbeads with a smaller mean diameter and homogenous dimensional distribution using a polymer concentration of 1.5% (w/v). Increasing the alginate concentration up to 2% (w/v), the size distribution became broader and the mean diameter increased from 630 (in the case of an alginate concentration of 1.5%) to 645 µm (see dimensional distribution plots reported in

Fig. 3 E, F). On the other hand, alginate microbeads prepared with lower polymer concentrations (0.5 and 1.0%, w/v) formed some *coalescences* (see **FIGURE 16,** panel **II**), although the size distribution remained acceptably narrow.

Encapsulation of WJMSCs in alginate microbeads:a DoE approach

After performing the COST study, a “design of the experiments” (DoE) optimisation and screening of the experimental parameters were also conducted. The DoE reduces the number of experiments and provides statistical information about the effects of different variables and their possible interactions.

The parameters frequency, pump and height were chosen as variables and tested at three levels by Prof. Nastruzzi and his groups.

After investigation of the factors influence, the validity and the significance of the model was estimated by analysis of variance (ANOVA). All the data obtained fitted well the model determining a good reproducibility of the studied model.

Viability and proliferation of encapsulated WJMSCs

A crucial issue that we investigated at the beginnig of embedding/seeding protocol for cell scaffolding, was the effect of the encapsulation procedure on the growth and the activity of cells.

With the aforementioned protocol set by Prof. Nastruzzi and his group, the resulting barium alginate microbeads were elastic, size uniform and transparent, facilitating the microscopic observation of the WJMSCs viability and morphology during the in vitro studies (see **FIGURE 17,** panel **I, A-F**). The hardening of alginate solution was accomplished by an ionic gelation procedure based on barium chloride. The use of barium ions (instead of more often used calcium ions)

resulted in the formation of mechanically stable microbeads preserving the in vitro and in vivo viability of the embedded cells (see **FIGURE 17,** panel **I, C** and **D**).

The viability of WJMSCs encapsulated in alginate microbeads was determined by the live/dead test. The observation of the fluorescent images recorded immediately after the encapsulation procedure at the typical excitation wavelengths, (see **FIGURE 17,** panel **II, A** and **B**) indicated that the cells were highly viable (>95%). In order to strengthen these data, the cell viability was determined after different length of culture time (up to 6 days) by using two alternative procedures, namely, the double staining with a Calcein-AM cell viability assay kit (see **FIGURE 17,** panel **II, C** and **D**) and the MTT test (see **FIGURE 17,** panel **II, F**). About one hundred of alginate microbeads were incubated with thyazolyl blue, and the presence of formazan salts, marker of the viable cells, was reported in the graph as percentage of viable cells respect to day “0” (rated to 100%). At each day of measurement, we observed a steady but not significant ($p > 0.05$) decrease in formazan absorption during the first 10 days (see **FIGURE 17,** panel **II, F**).

Secretive profile: WJMSCs in alginate microbeads vs adherent WJMSCs

The secretive profile of both adherent (free) and alginate-encapsulated WJMSCs was analyzed by multiplex bead-based sandwich immunoassay (shortly Bio-Plex®). The panel of analyzed factors includes member of the family of interleukins, chemokines, growth factors and soluble form of adhesion molecules (see **FIGURE 18**). All the proteins analyzed had a molecular weight lower of the molecular weight cut off of the microbead membrane. Most of the protein analyzed were secreted both by the free and encapsulated cells, even if in a different extent. The levels of IFN-α, IL-2Rα, TRAIL, M-CSF, IL-12, ICAM-1, SCGF-β, CTACK, VCAM, SDF-1α, were higher in the supernatant from free cells. In particular, IFN-

α, IL-2Rα, TRAIL, M-CSF, IL-12, ICAM-1, SCGF-β, were significantly higher in the supernatant from free cells ($p<0.05$). On the contrary, MCP3, GRO-α, MIF and HGF were found more abundantly present in the supernatant from the encapsulated cells. In particular, MCP3 and GRO-α were significantly higher in the supernatant from from the encapsulated cells ($p<0.05$) when compared to free ones. Finally, some factors (IL-1α, IL-18, β-NGF, SCF, TNF-β LIF, MIG) were not detectable or detected at very low levels (IL-3, IL-16) in both situations.

After encapsulation treatment, WJMSCs within alginate interacted with the surrounding environemet in an open-two-ways pattern of modifications.

Alginate microbeads encapsulation of WJMSCs and PDLMSCs: induction of osteogenic differentiation

After all sets of experiments were succesfull for encapsulation of WJMSCs, also PDLMSCs were embedded in alginate microbeads.

Since WJMSCs have been proven, as well as PDLMSCs, to be able to differentiate in osteogenic lineage, the ability of the WJMSCs and PDLMSCs entrapped in alginate to differentiate in osteoblasts was assessed at day 21 of osteogenic induction by a number of classical criteria and compared with WJMSCs and PDLMSCs entrapped in alginate in absence of osteogenic medium respectively.

To describe different culture conditions for cells encapsulated in alginate microbeads, combinations of some different abbreviations were chosen:

- To indicate WJMSCs in alginate microbeads: WJABs
- To indicate WJABs cultured in 6 well-plate (We):
    - “control” culture medium (CCM): WJABs/We/CCM
    - osteogenic differentiation medium (ODM): WJABs/We/ODM
- To indicate PDLMSCs in alginate microbeads: PDLABs

- To indicate PDLABs cultured in 6 well-plate (We):
    - "control" culture medium (CCM): PDLABs/We/CCM
    - osteogenic differentiation medium (ODM): PDLABs/We/ODM

The experimental protocol was the same for both cell population.

Considering WJABs, WJABs/We/CCM cells after 21 days of culture in control medium didn't show any change in beads morphology, size or shape. WJABs were still transparent and easy to observe in brightfield (see **FIGURE 19, A**). On the other hand observation of WJABs/We/ODM revealed that beads in osteogenic medium turned darker and seemed less transparent (see **FIGURE 19, B**). The Calcein AM/Propidium iodide double staining assay at day 21 showed that cells were vital in both conditions (see **FIGURE 19, C** and **D**).

The mineral deposition within beads was measured by Alizarin Red staining assay, that showed unstained WJABs in control conditions (see **FIGURE 19, E**), as well as red color was seen within WJABs after 21 days of osteogenic differentiation (see **FIGURE 19, F**).

Considering PDLABs, PDLABs/We/CCM cells after 21 days of culture in control medium didn't show any change in beads morphology, size or shape, as well as for WJABs/We/CCM. Also PDLABs appeared transparent and easy to observe in brightfield (see **FIGURE 20, A**). Parallel observation of PDLABs/We/ODM revealed that beads in osteogenic medium turned darker and seemed less transparent (see **FIGURE 20, B**). A sort of brown shading characterized aspects of all PDLABs/We/ODM. The Calcein AM/Propidium iodide double staining assay at day 21 showed that cells were vital in both conditions (see **FIGURE 20, C** and **D**), as well as for WJABs.

The mineral deposition within beads was measured by Alizarin Red staining assay, that showed unstained PDLABs in control conditions (see **FIGURE 20, E**), while intense red color was seen within PDLABs after 21 days of osteogenic differentiation (see **FIGURE 20, F**).

Comparing results for WJABs and PDLABs in osteogenic conditions (see **FIGURE 19, F** and **20, F** respectively), both cell populations were able to differentiate in osteogenic lineage, with an increased mineralization for PDLABs samples. In 6-well plate conditions, cells underwent osteogenic differentiation despite encapsulation.

Considering osteoblastic markers, WJABs and PDLABs showed an appreciable increase of ALP activity, an early marker for osteoblast differentiation, after 21 days in osteogenic condition (see **FIGURE 21,** upper graph). WJABs and PDLABs showed also an increase of the expression of the bone-specific gene such as Runx2 analyzed by quantitative RT-PCR (see **FIGURE 21,** lower graph).

In both cases, PDLABs osteoblastic markers showed higher increase if compared to WJABs, as well as RUNX2 modification wasn't significant ($p > 0.05$).

Therefore, even if further investigations are needed, these data suggest that the alginate microbeads here described provide a valuable support to osteogenic differentiation.

Alginate microbeads encapsulation of WJMSCs and PDLMSCs induced to osteogenic differentiation: effect of Rotary Cell Culture System™ (RCCS-4™ bioreactor) with High Aspect Ratio Vessel (HARV™)

Bioreactors have been proven to be able to provide cell a new tool for the study of three-dimensional (3-D) cell growth and differentiation.

With the aim to verify if differentiation could be enhanced through the improvement of cells exchanges with culture environement, a set of experiments in different culture conditions (like the continuing culture medium flow, the enhanced gas exchange, or the gravity modification) were performed in Rotary Cell Culture System™ (RCCS-4™ bioreactor, Synthecon™, Inc., Houston, TX, U.S.A.) (see

FIGURE 22 panel **I**), with High Aspect Ratio Vessel (HARV™) (see **FIGURE 22** panel **II**).

For all experiments 10 ml HARV™ vessels were used. For all experimental period (21 days) 10 ml HARV™ vessels were manteined in incubator, providing costant rotation speed (25 rpm) at 5% CO^2 and 37°C (see **FIGURE 22** panel **III**).

As previously done, to describe different culture conditions for cells encapsulated in alginate microbeads, combinations of some different abbreviations were choosen:

- To indicate WJABs cultured in rotating HARV bioreactor 10 ml vessels (HARV):
    - "control" culture medium (CCM): WJABs/HARV/CCM
    - osteogenic differentiation medium (ODM): WJABs/HARV/ODM
- To indicate PDLABs cultured in rotating HARV bioreactor 10 ml vessels (HARV):
    - "control" culture medium (CCM): PDLABs/HARV/CCM
    - osteogenic differentiation medium (ODM): PDLABs/HARV/ODM

During this first set of experiments, culture conditions were maintained the same for 4 different samples: 2 WJMSCs populations and 2 PDLMSCs populations.

Representative experiments are shown in **FIGURE 23** to **28**.

We successfully cultured RCCS environment, and under the observation of phase contrast microscope, WJABs and PDLABs appeared vital also after 21 days of culture (see **FIGURE 23, B, D, H, J** and **FIGURE 26, B, D, H, J**).

Considering WJABs, WJABs/We/CCM, as well as WJABs/HARV/CCM cells after 21 days of culture in control medium didn't show any change in beads morphology, size or shape. WJABs were still transparent and easy to observe in brightfield (see **FIGURE 23, A, C**).

As previously observed, WJABs/We/ODM turned darker and seemed less transparent (see **FIGURE 23, G**) in osteogenic medium. This change in color and

transparency was higher when WJABs/HARV/ODM were observed (see **FIGURE 23, I**). The Calcein AM/Propidium iodide double staining assay at day 21 showed that cells were vital in all conditions (see **FIGURE 23, B, D, H, J**), but darkfield aspects of beads were different considering fluorescence amount. Despite the same number of PBS rinses after Calcein AM/Propidium iodide staining, it appeared like aspecific staining wasn't able to be washed out from WJABs/HARV/ODM sample (see **FIGURE 23, J**). Cells were all vital, and merging images didn't evidence the presence of any dead cell in beads.

The mineral deposition within beads was measured by Alizarin Red staining assay, that showed unstained WJABs in control conditions (see **FIGURE 23, E, F**), as well as red color was seen within WJABs after 21 days of osteogenic differentiation (see **FIGURE 23, K, L**), with more intense red coloration for WJABs/HARV/ODM (see **FIGURE 23, L**).

RUNX2 expression level was evaluated after 21 days of control and osteogenic conditions for WJABs in 4 conditions. This analysis was done for 2 samples and results are shown (see **FIGURE 24, samples 1** and **2**). Samples showed an higher RUNX2 level in osteogenic conditions (see **FIGURE 24, samples 1** and **2, green columns**). On the other hand Col I expression levels seemed to vary more than RUNX2, despite of osteogenic differentiation (see **FIGURE 25, samples 1** and **2, yellow columns**).

Considering PDLABs, PDLABs/We/CCM, as well as PDLABs/HARV/CCM cells after 21 days of culture in control medium didn't show any change in beads morphology, size or shape. PDLABs were still transparent and easy to observe in brightfield (see **FIGURE 26, A, C**).

As previously observed, also this experiments resulted in turning dark for both PDLABs/We/ODM and PDLABs/HARV/ODM in osteogenic medium. They seemed less transparent too (see **FIGURE 26, G, I**). This change in color and transparency

was higher when PDLABs/HARV/ODM were observed (see **FIGURE 26, I**). The Calcein AM/Propidium iodide double staining assay at day 21 showed that cells were vital in all conditions (see **FIGURE 26, B, D, H, J**). As happened for WJABs/ HARV/ODM, darkfield aspects of beads was different considering fluorescence amount. PDLABs/HARV/ODM appeared like aspecific staining wasn't able to be washed out from WJABs/HARV/ODM sample (see **FIGURE 23, J**). Cells were all vital, and merging images didn't evidence the presence of any dead cell in beads.

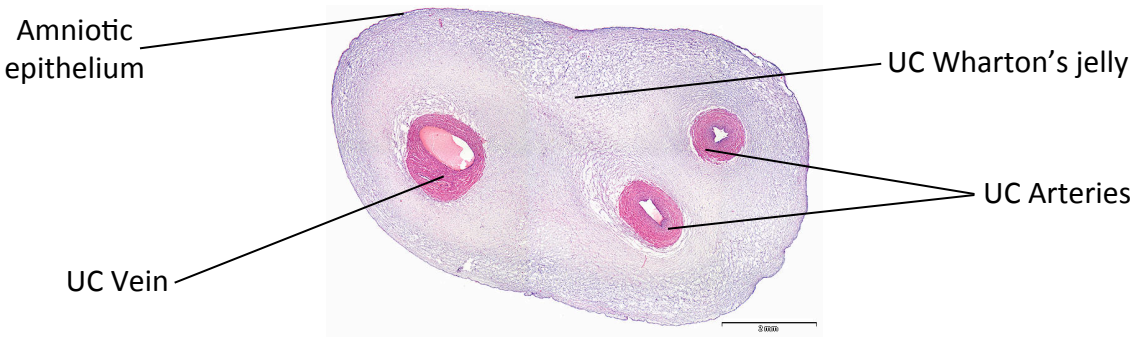
The mineral deposition within beads was measured by Alizarin Red staining assay, that showed unstained PDLABs in control conditions (see **FIGURE 26, E, F**), as well as red color was seen within PDLABs after 21 days of osteogenic differentiation (see **FIGURE 26, K, L**), with more intense red coloration for PDLABs/HARV/ODM (see **FIGURE 26, L**).

Comparing results for WJABs and PDLABs in osteogenic conditions (see **FIGURE 23, K** and **L,** and see **FIGURE 26, K** and **L**), both cell populations were able to differentiate in osteogenic lineage, with an increased mineralization for PDLABs samples. Therefore, the presence of continuous medium flow in HARV™ vessels conditions provided a different environement where both WJABs and PDLABs underwent a stronger mineral deposition (see (see **FIGURE 23, L** and **FIGURE 26 L**, respectively).

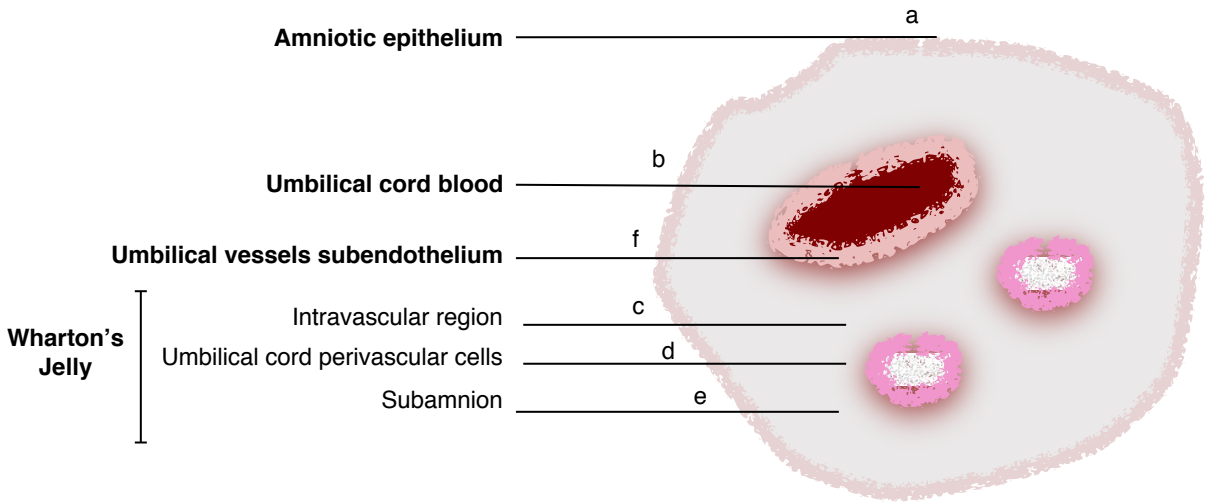
RUNX2 expression level was evaluated after 21 days of control and osteogenic conditions for PDLABs in 4 conditions. This analysis was done for 2 samples and results are shown (see **FIGURE 27, samples 1** and **2**). Samples showed an higher RUNX2 level in osteogenic conditions (see **FIGURE 27, samples 1** and **2, green column**). On the other hand Col I expression levels seemed to vary more than RUNX2, despite of osteogenic differentiation (see **FIGURE 28, samples 1** and **2, yellow columns**).

GRAPHIC CONTENTS

FIGURE 1
UMBILICAL CORD: OVERVIEW



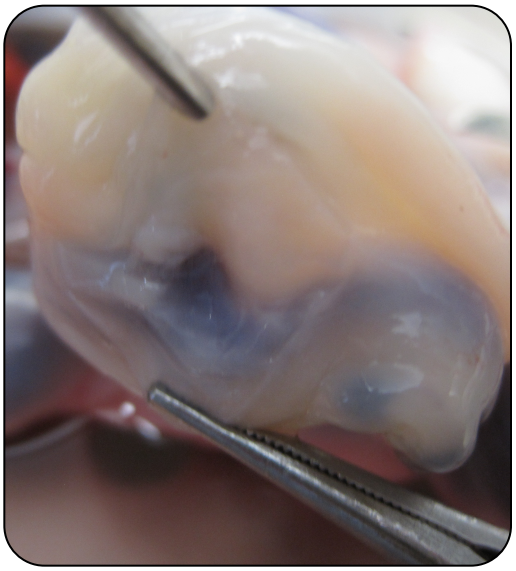
The umbilical cord (UC) in cross section. The three round structures are vessels. The two smallest are the umbilical arteries, and the largest is the umbilical vein. the wall of the vein is much thicker than the walls that you find in other veins. The material surrounding the vessels is so-called mucoid connective tissue, better known as Wharton's jelly. (modified from http://www.med.uio.no/dlo/mikro/Images/img03748.jpg)



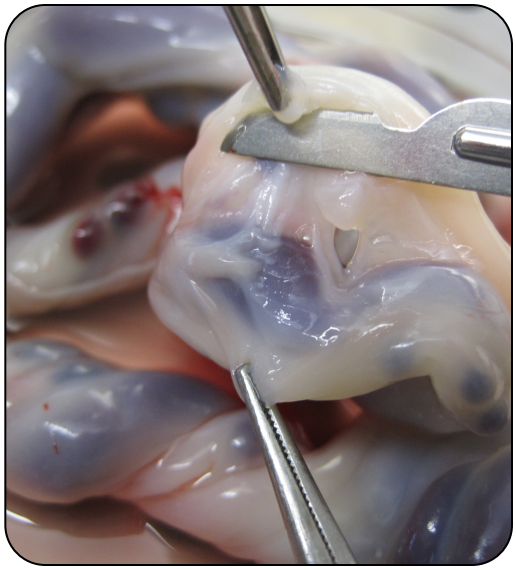
At least six distinctive zones are now recognized based on the structural and functional studies: (a) surface epithelium (amniotic epithelium, UC epithelium), (b) UC blood, (c) subamniotic stroma, (d) intervascular stroma , (e) perivascular stroma, ((c), (d) and (e) recognized as Wharton's jelly) and (f) vessels.

FIGURE 2
NON ENZYMATIC ISOLATION OF WHARTON'S JELLY (WJ) MSCs

A



B

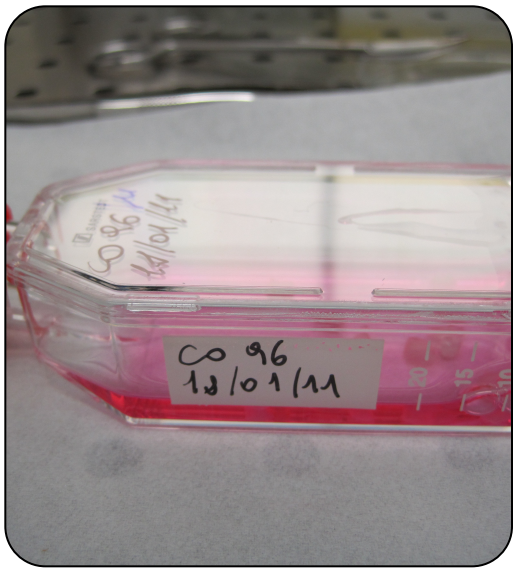


C

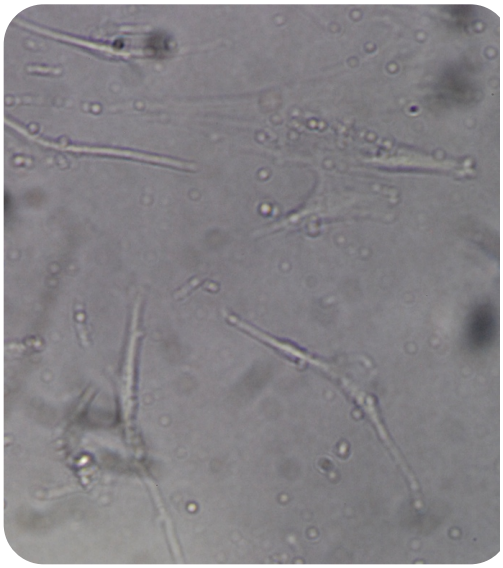
D

The umbilical cord (A), cut longitudinally (B), was incised with a scalpel (C). WJ small pieces were washed several time before direct culture into flasks (D). WJ, Wharton's jelly.

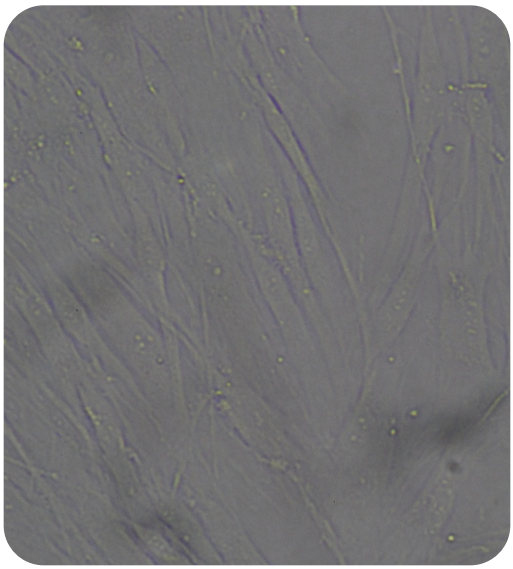
FIGURE 3
NON ENZYMATIC ISOLATION OF WHARTON'S JELLY (WJ) MSCs



A



B

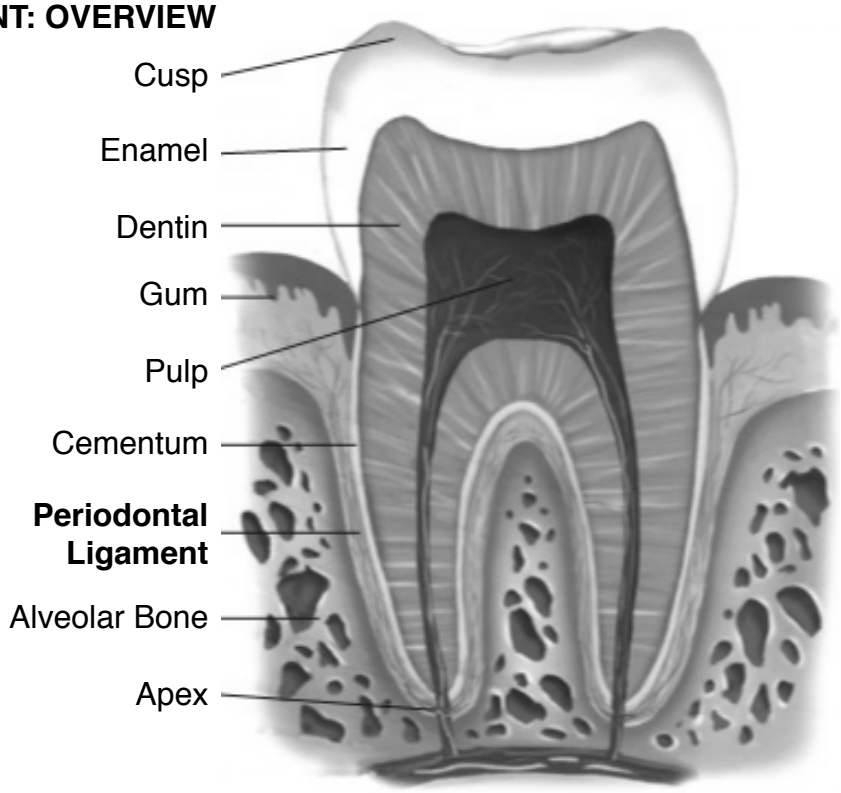


C

After 5 to 7 days of direct culture in tissue flasks (A), the outgrowth of spindle shape fibroblast-like cells was observed (B). After 2 weeks WJ pieces were removed, and medium was changed, allowing colonies expansion and subconfluence (C). WJ, Wharton's jelly.

FIGURE 4
PERIODONTAL LIGAMENT: OVERVIEW

The periodontium, which is the supporting structure of a tooth, consists of the cementum, periodontal ligaments, gingiva, and alveolar bone. Cementum is the only one of these that is a part of a tooth. Alveolar bone surrounds the roots of teeth to provide support and creates what is commonly called a "socket". Periodontal ligaments connect the alveolar bone to the cementum, and the gingiva is the surrounding tissue visible in the mouth.



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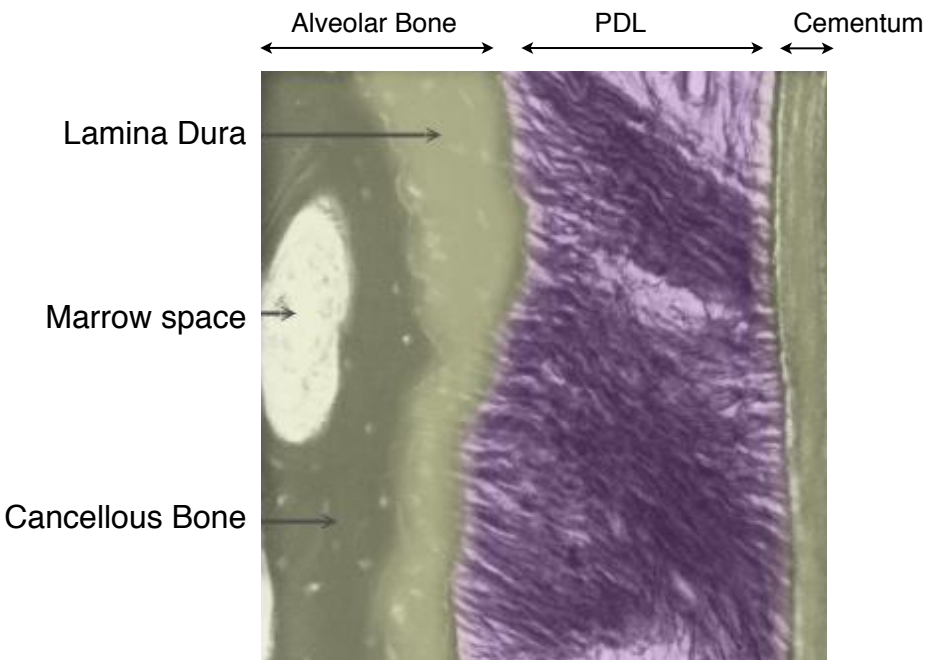
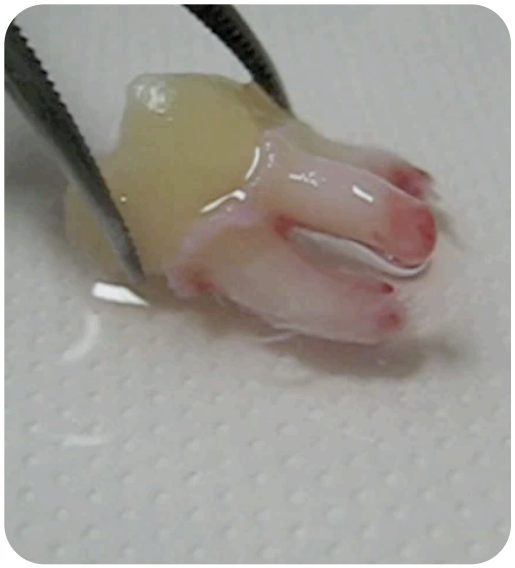


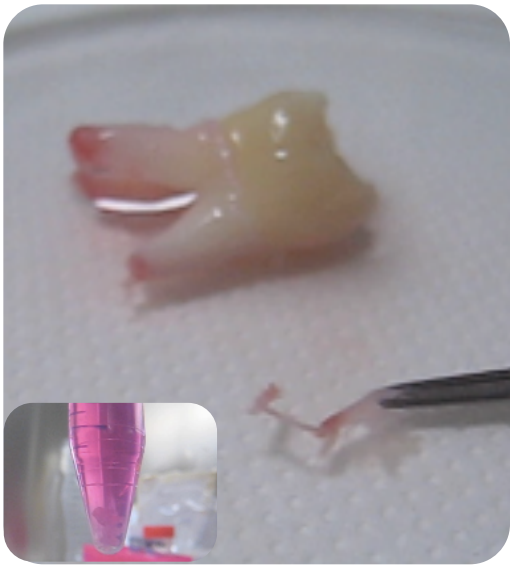
modifed by: Ten Cate, A. R. [1980] (1998). Oral Histology: development, structure, and function, 5th ed., St. Louis: Mosby. ISBN 0-8151-2952-1.

Cells from the dental follicle give rise to the periodontal ligament (PDL). These fibroblasts secrete collagen, which interacts with fibers on the surfaces of adjacent bone and cementum. The periodontal ligament is a group of specialized connective tissue fibers that essentially attach a tooth to the alveolar bone within which it sits. These fibers help the tooth withstand the naturally substantial compressive forces which occur during chewing and remain embedded in the bone.

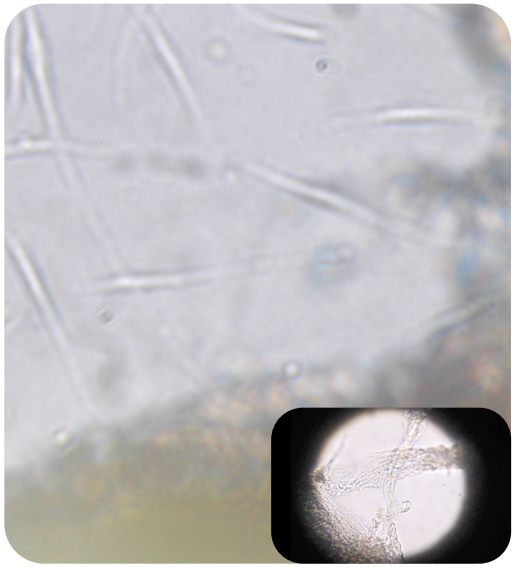
FIGURE 5
NON ENZYMATIC ISOLATION OF PERIODONTAL LIGAMENT (PDL) CELLS



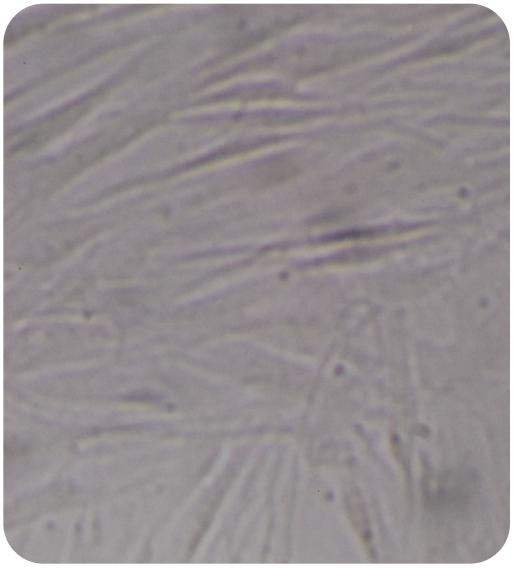
A



B



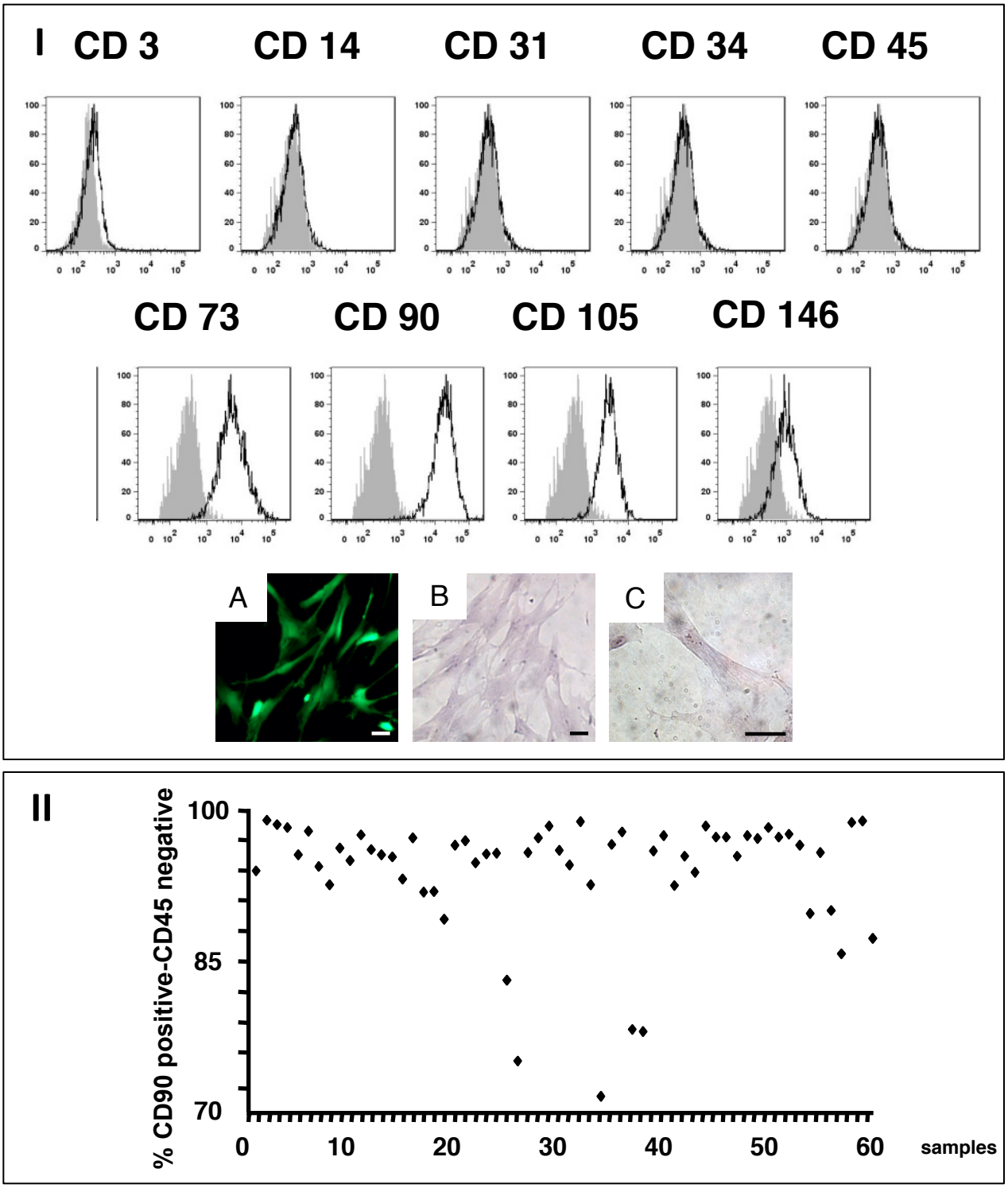
C



D

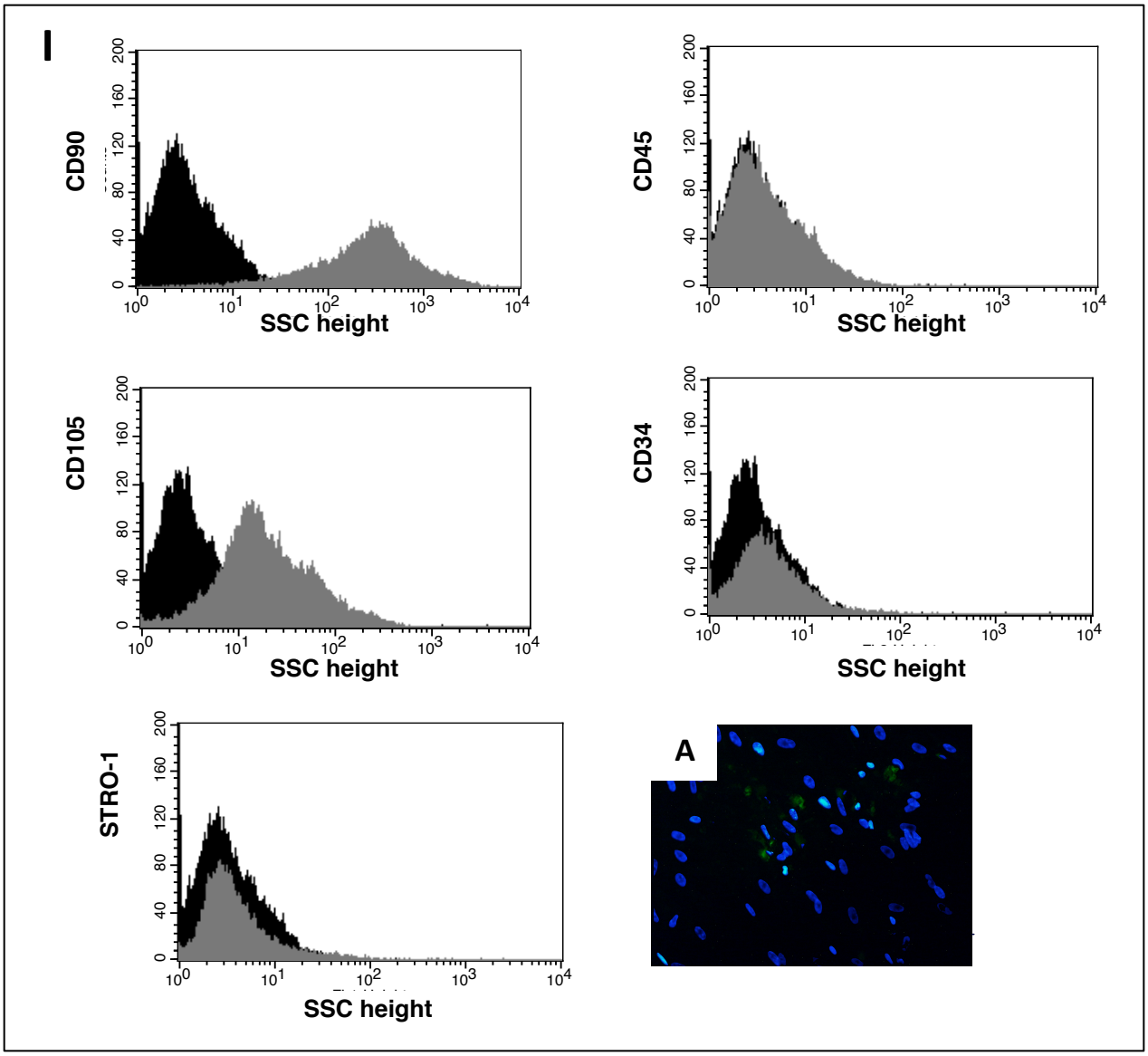
Gently PDL was separated from the middle third of the tooth root surface using forceps and a size 15 surgical blade (A), then washed and placed direclty in 6-well plate with culture medium (B). After 4 to 6 days cells outgrowth was observed next to tissue pieces (C). Subconfluence was generally reached within 2 weeks (D).

FIGURE 6
CHARACTERIZATION OF WHARTON'S JELLY (WJ) MSCs



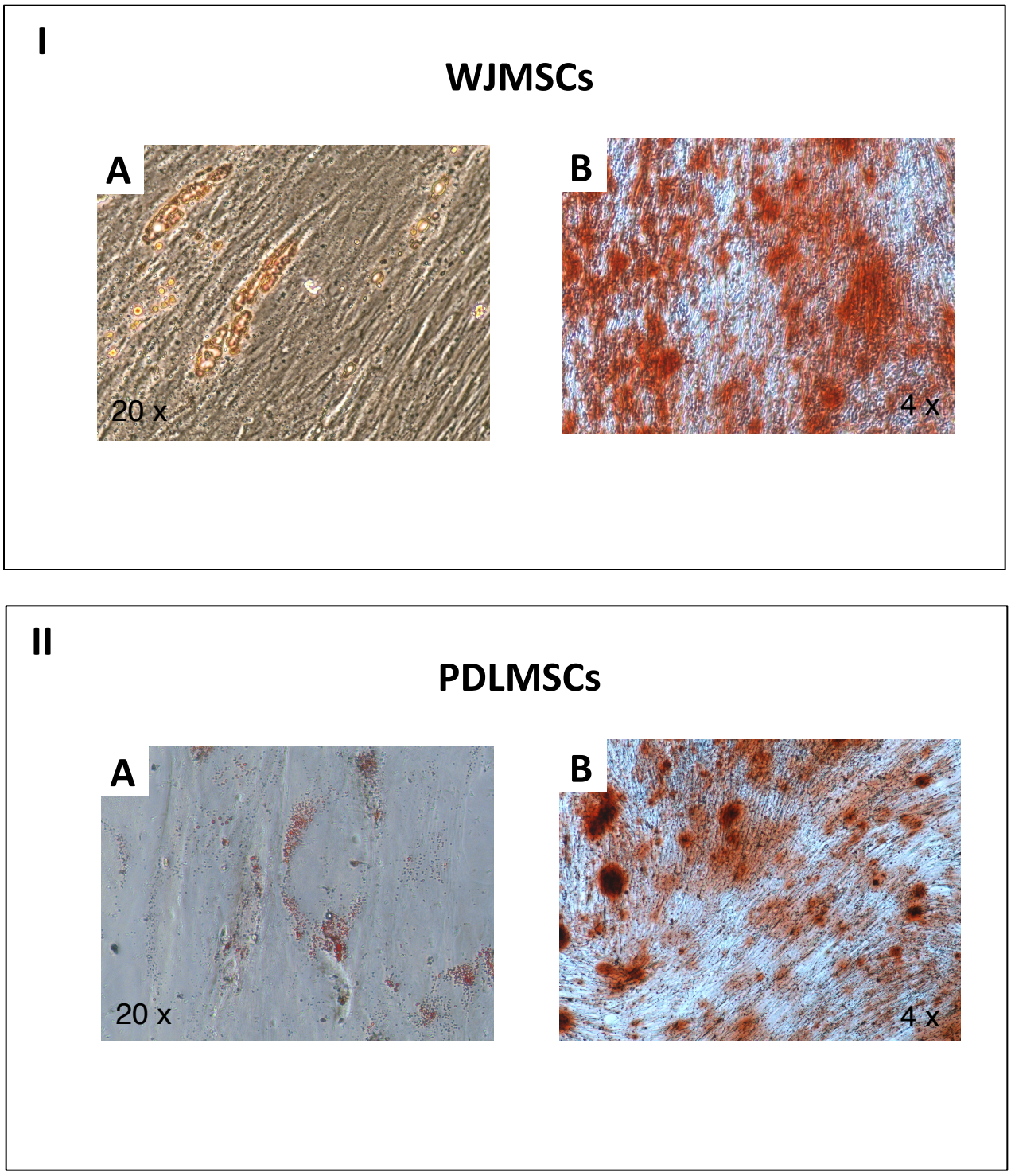
Phenotype characterization of WJMSCs. I) Flow cytometric histograms showing the immunophenotype of WJMSCs (representative experiment). Open histograms represent the isotype control antibody, dotted histograms represent anti-CD3, -CD14, -CD31, -CD34, -CD45, -CD73, -CD90, -CD105, -CD146 antibodies. X-axis, mean fluorescent channel; Y-axis, number of events. Lower part of panel I: (A) representative fluorescence photomicrograph of WJMSCs monolayer after Calcein-AM staining (magnification: 20x); (B, C) optical micrographs of hematoxylin counterstained WJMSCs (after 2 weeks of culture), showing a fibroblast-like morphology (Magnification: 20x and 40x, in panel B and C respectively). Bar corresponds to 20 μm. II) Schematical distribution of the cell surface parameters of the samples analyzed.

FIGURE 7
CHARACTERIZATION OF PERIODONTAL LIGAMENT (PDL) CELLS



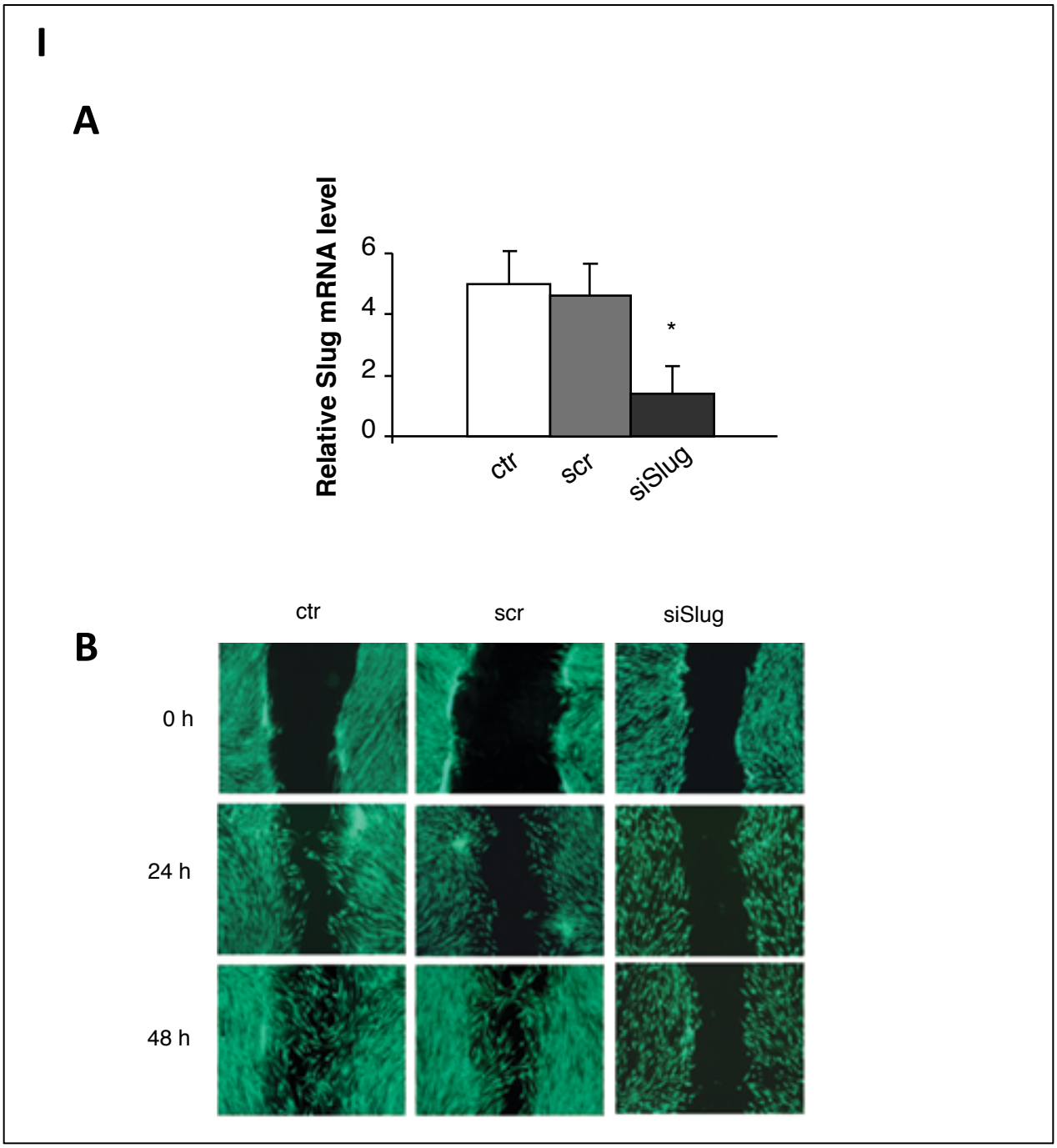
Phenotype characterization of PDLMSCs. I) Flow cytometric histograms showing the immunophenotype of PDLMSCs. The gated cells were negative for the hematopoietic line marker CD45 and CD34, partially positive for CD105 and STRO-1, and positive for the mesenchymal stem cells marker CD90. (A) Periodontal ligament cells STRO-1 expression. Binding of STRO-1 antigen to a cytospin preparation was detected by using FITC-conjugated secondary antibody on human PDLMSCs cultured for 7 days in standard medium. II) A,B: Representative fluorescence and brightfield photomicrographs of PDLMSCs monolayer after Calcein-AM staining (magnification 10x).

FIGURE 8
WJMSCs AND PDLMSCs DIFFERENTIATION POTENTIAL



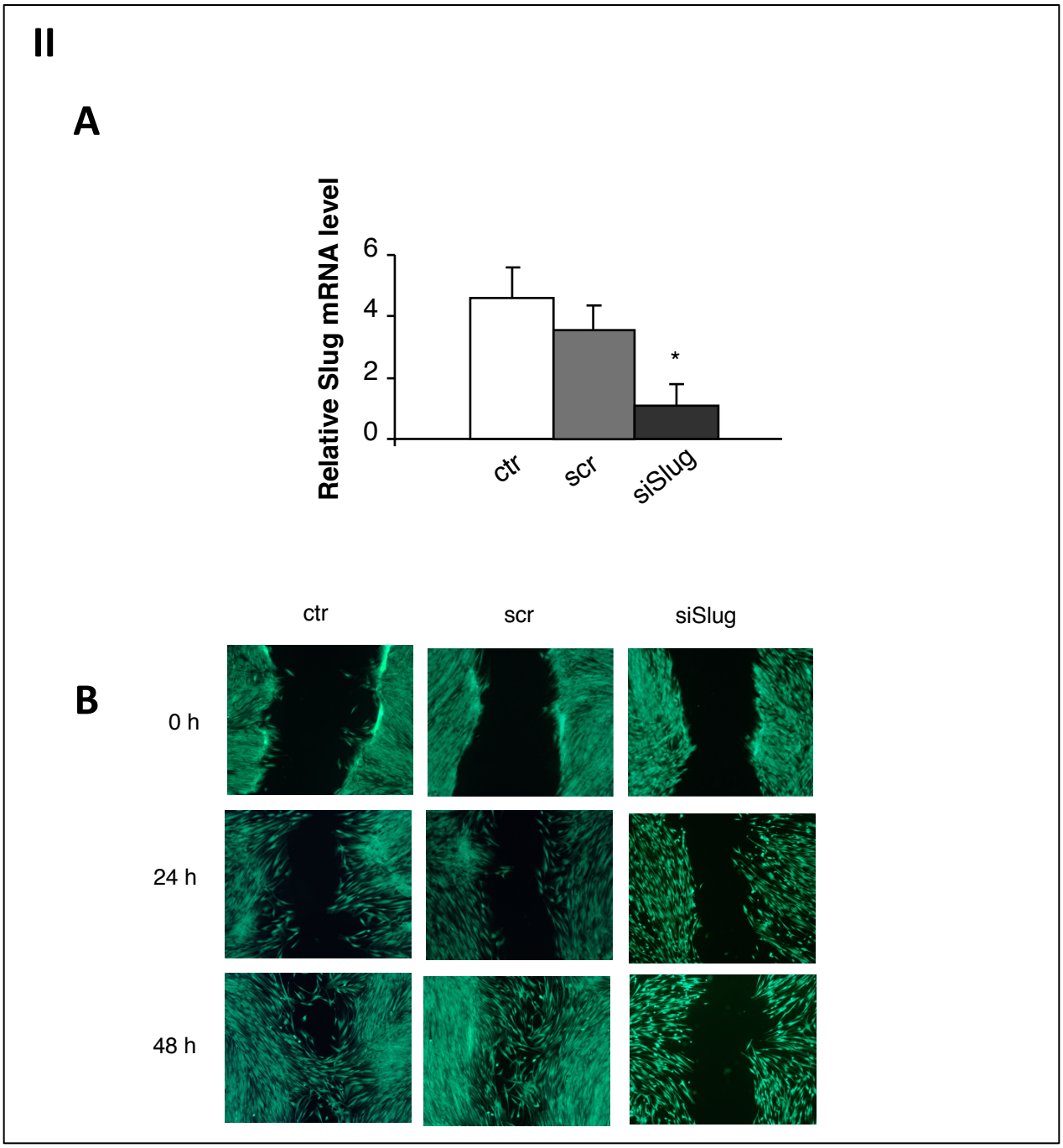
Adipogenic and osteogenic differentiation potential. The capacity of mesenchymal stem cells to generate stromal tissues including those similar to which they were derived from is recognized as a hallmark feature of these cells. The ability of WJMSCs and PDLMSCs to differentiate into osteblastic cell lineages in vitro can be investigated by culturing under inductive conditions. After 21 days of specific induction WJMSCs (see panel I) and PDLMSCs (see panel II) exhibited lipidic (A) and osteogenic (B) phenotype (magnification: 20x and 4x, in panel A and B respectively).

FIGURE 9
SCRATCH WOUND MIGRATION ASSAY: siRNA SILENCING STRATEGY FOR WJMSCs AND PDLMSCs PHENOTYPE MODULATION



Analysis of WJMSCs migration by in vitro scratch assay.
(A) WJMSCs were transfected with 30 nM siSlug or a nonrelevant siRNA (scr) as negative control. Slug expression was determined at mRNA level. Knockdown of Slug expression was verified by Real-Time RT-PCR.
(B) 72h after silencing treatment, WJMSCs monolayers were scratch wounded with a pipet tip (0h), and observed over the indicated time periods, 0h, 24h, and 48h (4X magnification). Images show siSlug treated cells exhibited a reduced capacity to cover the scratch if compared to untreated (ctr).
This set of experiments had the aim to investigate the effectiveness of wound assay in pointing out effect of cells phenotype modification, through the direct observation of different cells behaviour when comparing siSlug treated cells to control.
WJMSCs exibited a rapid modification of wound margin, due to active proliferation and migration ability.

FIGURE 9 (continued)
SCRATCH WOUND MIGRATION ASSAY: siRNA SILENCING STRATEGY FOR WJMSCs AND PDLMSCs PHENOTYPE MODULATION



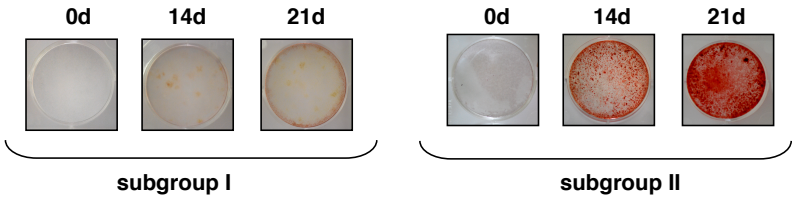
Analysis of PDLMSCs migration by in vitro scratch assay.
(A) PDLMSCs were transfected with 30 nM siSlug or a nonrelevant siRNA (scr) as negative control. Slug expression was determined at mRNA level. Knockdown of Slug expression was verified by Real-Time RT-PCR.
(B) 72h after silencing treatment, WJMSCs monolayers were scratch wounded with a pipet tip (0h), and observed over the indicated time periods, 0h, 24h, and 48h (4X magnification). Images show siSlug treated cells exhibited a reduced capacity to cover the scratch if compared to untreated (ctr).
as done for WJMSCs, observations went on for more 24 hours, showing that cells' Slug knockdown affected cells motility, avoiding any closure of the wound. These findings could lead to the speculation of an important role of SLUG also during tissue reparation and/or tissue regeneration, as well as for PDL and PDLMSCs.

FIGURE 10
OSTEOGENIC POTENTIAL OF WJMSCS: VARIABILITY BETWEEN SAMPLES

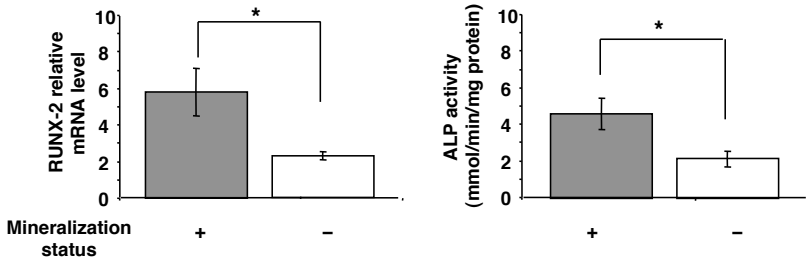
A

	Sample	Mother's age (ys)	Weeks of pregnancy	Mode of delivery	Gender	Birth weight (Kg)	RUNX-2	ALP
subgroup I	1	26	29	CS	F	1.25	3.76±0.2	3.52±0.2
	2	31	32	CS	M	1.74	1.92±0.1	8.105±01
	22	33	35	CS	M	2.41	2.55±0.2	1.34±0.3
	23	33	35	CS	M	2.53	1	2.33±0.2
	30	32	35	SP	F	3.17	1.55±0.2	2.18±0.15
	35	35	37	CS	F	2.77	4.12±0.1	1.72±0.1
	41	30	34	SP	M	2.6	2.59±0.1	5.23±0.3
	43	27	33	CS	M	1.915	2.85±0.1	3.4±0.3
	44	27	33	CS	F	2.005	2.22±0.1	2.18±0.1
	53	31	37	CS	F	1.98	6.03±0.1	6.69±0.4
	60	27	29	CS	M	2.1	1.15±0.12	6.08±0.2
subgroup II	12	38	38	CS	F	2.6	1.67±0.2	3.68±0.1
	13	38	39	SP	M	3.45	2.06±0.01	7.38±0.2
	14	36	40	SP	F	3.6	12.85±0.1	2.43±0.2
	20	35	40	SP	M	3.18	7.28±0.2	2.62±0.1
	27	29	40	SP	M	3.4	9.94±0.01	4.8±0.15
	28	39	39	CS	M	3.62	9.8±0.1	4.06±0.2
	33	20	40	SP	F	3.02	2.85±0.2	2.23±0.2
	37	38	38	SP	F	3.05	10.31±0.2	4.6±0.1
	51	33	39	CS	M	3.27	2.14±0.2	5.75±0.1

B



C

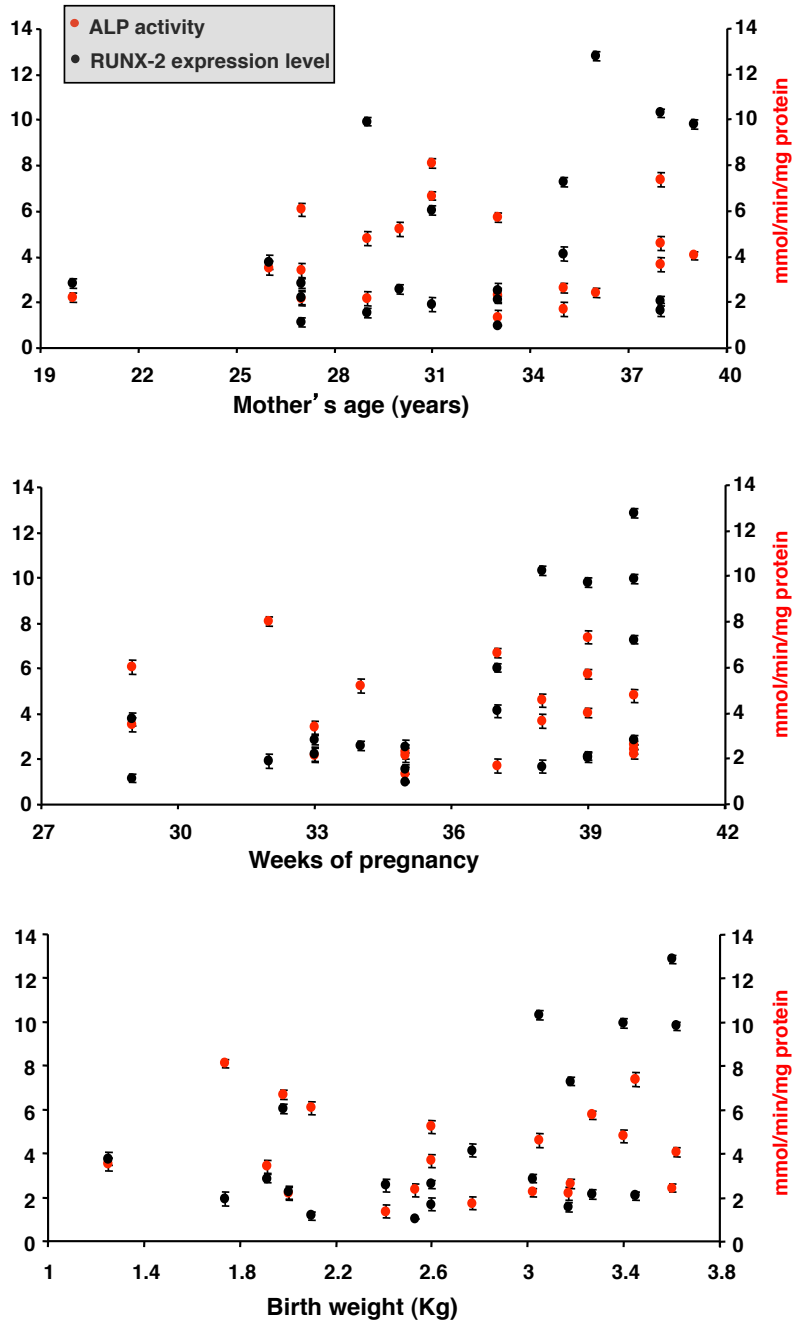


In the table (A) clinical parameters, RUNX-2 expression and Alkaline Phosphatase activity (ALP) basal level (mmol/min/mg protein) are reported. As indicated, the samples were divided in two subgroups: subgroup I, premature birth; subgroup II, full term birth. F(female), M(male), CS(Caesarian delivery), SP(spontaneous delivery).

(B)The advance of the mineralization status (0d, 14d, 21d) for one representative sample of each subgroup is reported. The two samples mineralized differently, according to the clinical parameters.

(C) The analysis of the relationship between the mineralization status (+, -) of the selected WJMSCs samples and, respectively, the basal level (at day "0") of RUNX-2 expression (on the left) and ALP activity (on the right) is reported. WJMSCs mineralize after 21 days in osteogenic conditions preferentially with high basal level of RUNX-2 and ALP activity (* = $P < 0.05$).

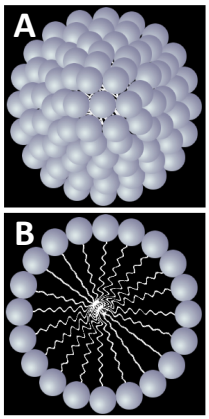
FIGURE 11
RELATIONSHIP BETWEEN MOLECULAR AND OSTETRIC PARAMETERS IN WJMSCs



Relationship between molecular and obstetric parameters. Basal levels of RUNX-2 expression and ALP activity were related to mother's age, weeks of pregnancy and birth weight in 20 WJMSC samples.
Mother's age at delivery had a significant impact on the basal ALP activity but didn't affect RUNX-2 expression level. Interestingly, birth weight of the infant was shown to significatively impact on RUNX-2 basal expression level which decreases with the decreasing of the baby's weight. The same relationship was found for the duration of pregnancy. In fact, it was found that WJMSCs from babies born before the 37 weeks of gestation express lower basal level of RUNX-2 than the full term borns.

FIGURE 12
POLYMERIC MICELLES (PMs)

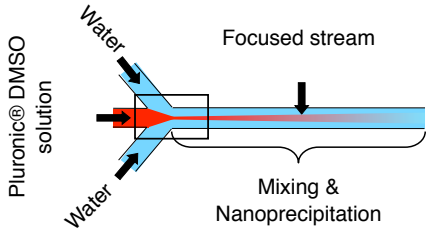
I



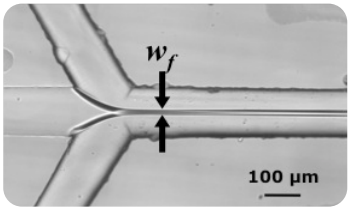
MICELLES

-Aggregate of surfactant molecules (unimers)
-Spontaneous self-assembling (CMC, hydrophobic effect)
-Hydrophilic block generally PEG
-Hydrophobic block (poly-ester, poly L-aminoacid, poly-ethers)
-Spherically shaped core–shell structures
-Size between 10 and 200 nm (freely circulate in the blood stream)

II



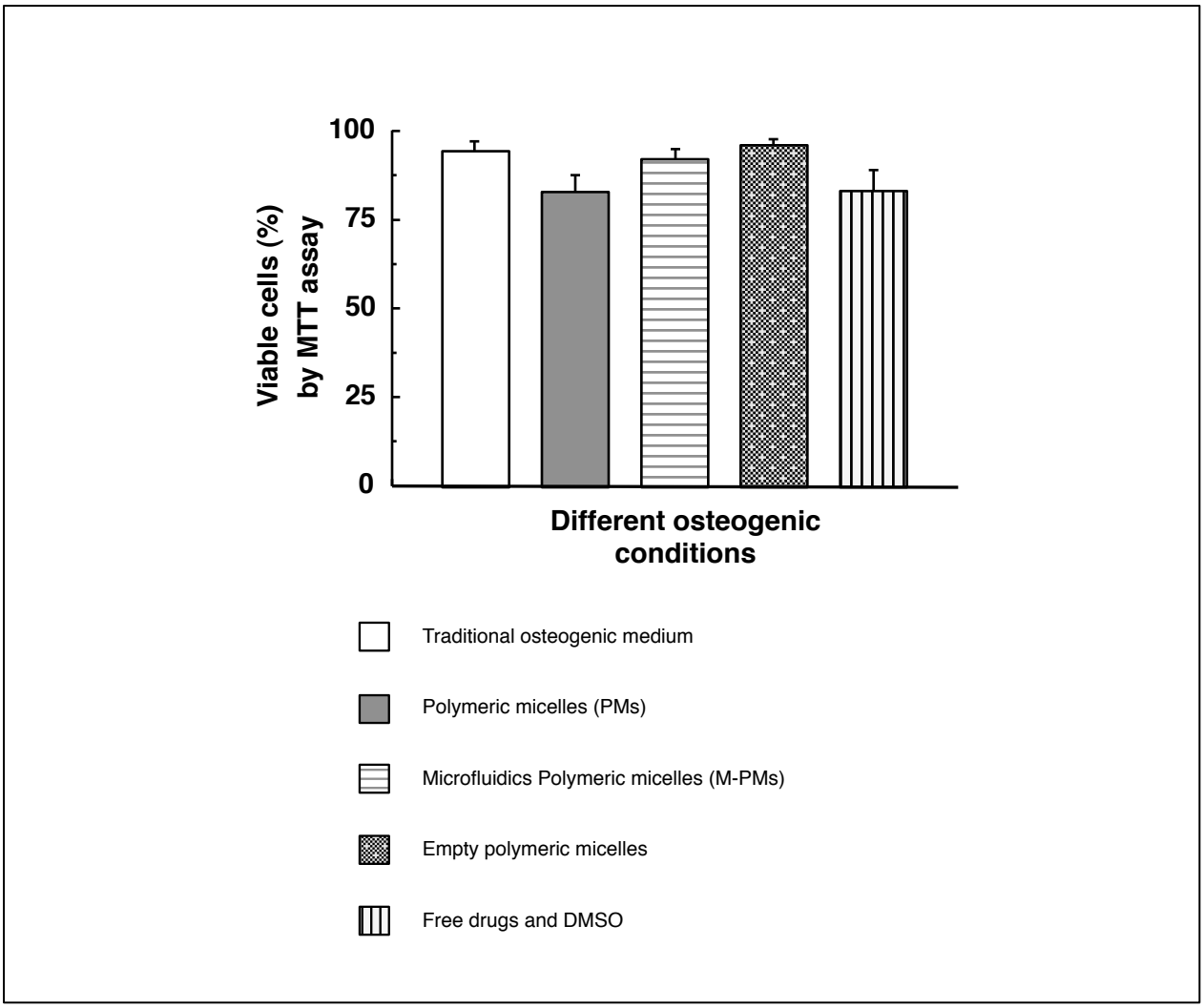
A



B

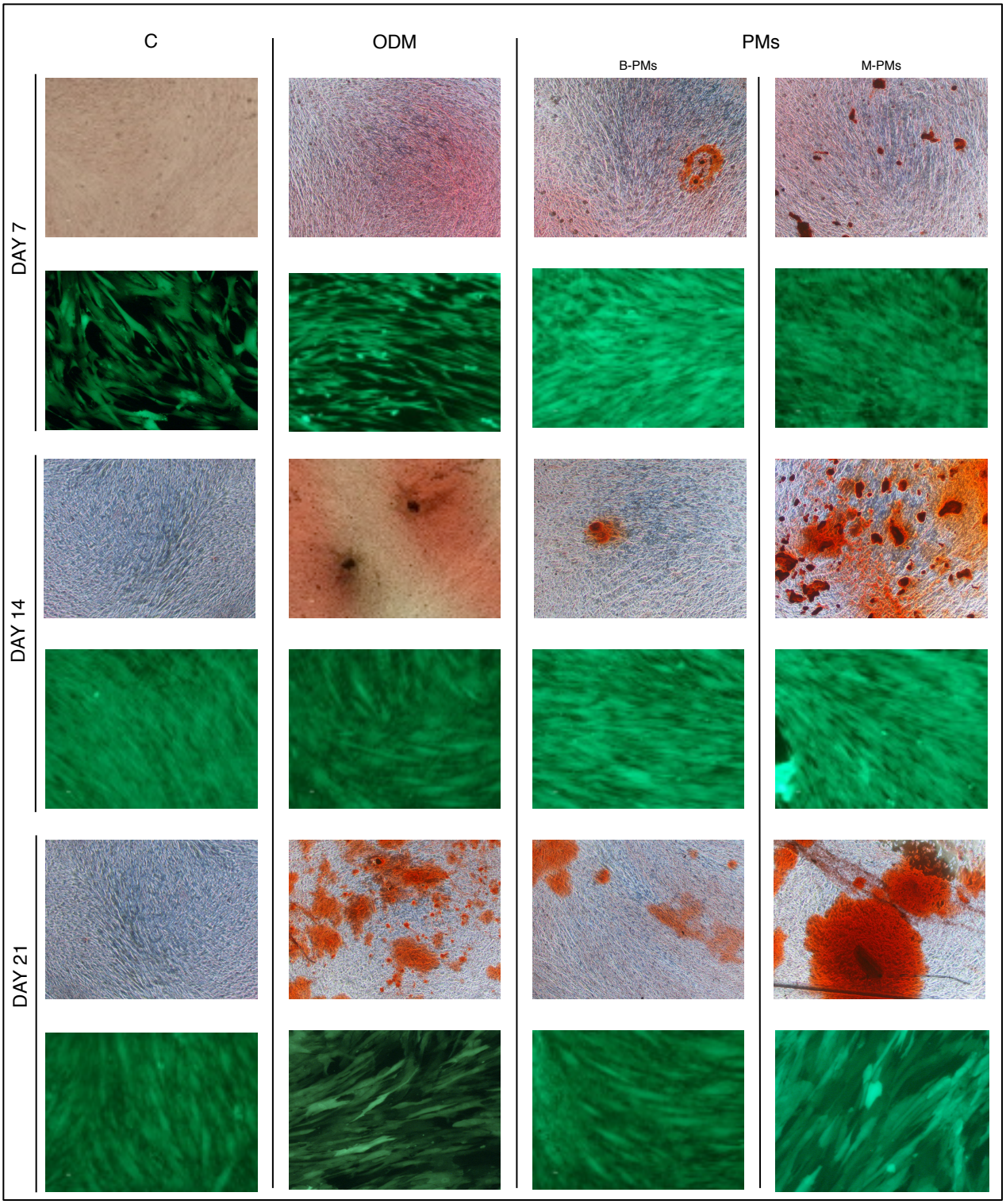
Panel I) A micelle (A) is an aggregate of surfactant molecules dispersed in a liquid colloid. A typical micelle in aqueous solution forms an aggregate with the hydrophilic "head" regions in contact with surrounding solvent, sequestering the hydrophobic single tail regions in the micelle centre (B).
Panel II) (A) Scheme of microfluidic reactor. instead of traditional batch procedure to generate PMs, microfluidic reactor was chosen, with three inlets creating a hydrodynamic flow, focusing where the central stream is polymer Pluronic solution in DMSO. (B) The width of the focusing central stream, wf, is controlled by varying the volumetric flow rates of the three inlets.

FIGURE 13
CITOTOXICITY OF PMs ON PDLMSCs: MTT ASSAY



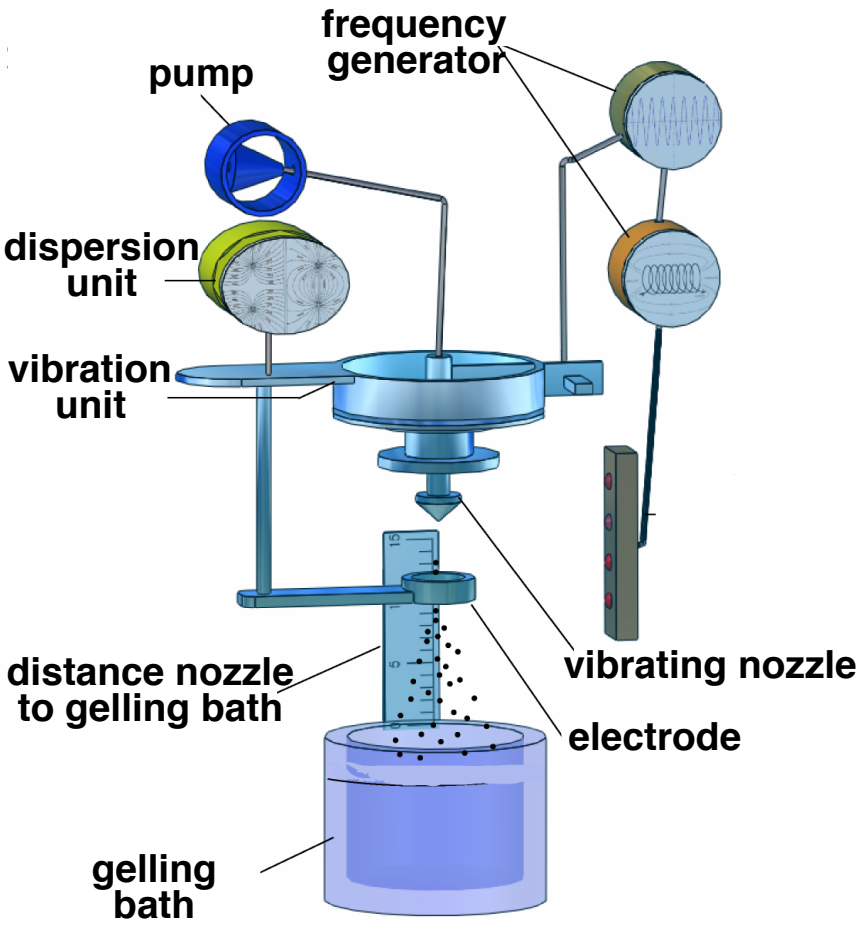
MTT analysis was performed after 72 hours on cells exposed to the different PMs. Values are expressed as percentage of viable cells respect culture condition without osteogenic induction (DMEM High Glucose supplemented with 10% FCS). Data represent the average of 3 independent experiments, conducted in triplicate. Empty PMs were used as negative control.
Proliferation rate and viability of the PDLMSCs were compared. As shown, all different culture conditions exhibited no appreciable cytotoxicity (cells viability was higher than 75%).
Instead of traditional batch method for PMs, microfluidic synthesis of M-PMs was carried out.

FIGURE 14
EFFECT OF PMs-MEDIATED OSTEOGENIC DIFFERENTIATION ON PDLMSCs: VIABILITY AND MINERALIZATION.



Cells were cultured for 21 days in different conditions: (C) control medium (DMEM HG supplemented with 10% FCS); (ODM) osteogenic differentiation medium was supplemented with inducers (0.1 µM dexamethasone; 10 mM β-glycerophosphate; 50 µg/mL L-ascorbic acid-2-phosphate); (PMs) PMs medium (10% FCS DMEM HG) supplemented with 10 mM β-glycerophosphate and PMs containing 0.1 µM dexamethasone and 50 µg/mL L-ascorbic acid-2-phosphate (working solution 100 µL/1 mL). PMs were realized with batch technique (B-PMs) or microfluidics (M-PMs). Mineralization status and viability (with Alizarin Red Staining Assay and double staining with Calcein-AM and propidium bromide respectively) were tested at day 7, 14 and 21. Cells viability wasn't affected by PMs, that provided osteogenic differentiation of PDLMSCs either. Pattern of mineralization was different for each condition.

FIGURE 15
ALGINATE MICROBEADS: ENCAPSULATION DEVICE



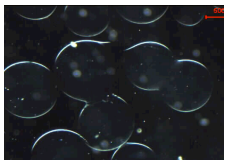
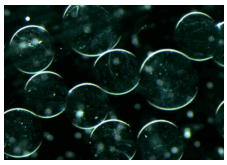
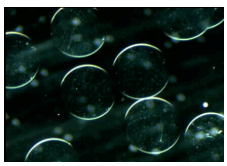
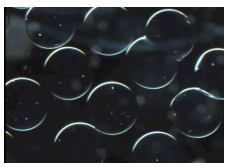
Schematic representation of the encapsulation device. Monodisperse alginate beads containing WJMSCs were prepared using an encapsulation device which is based on a vibrating-nozzle (Encapsulator Research Inotech, Dottikon Switzerland) according to the experimental procedure previously described.33,34 The encapsulator is composed by a 2-liter glass reaction vessel with stainless steel top and bottom plates. The top plate contains a feed-line connected to a syringe and a vibrating nozzle. A nozzle with an internal diameter of 300 µm was used. The flow of alginate to the nozzle is achieved by a precision syringe pump.

FIGURE 16
ALGINATE MICROBEADS GENERATION

I

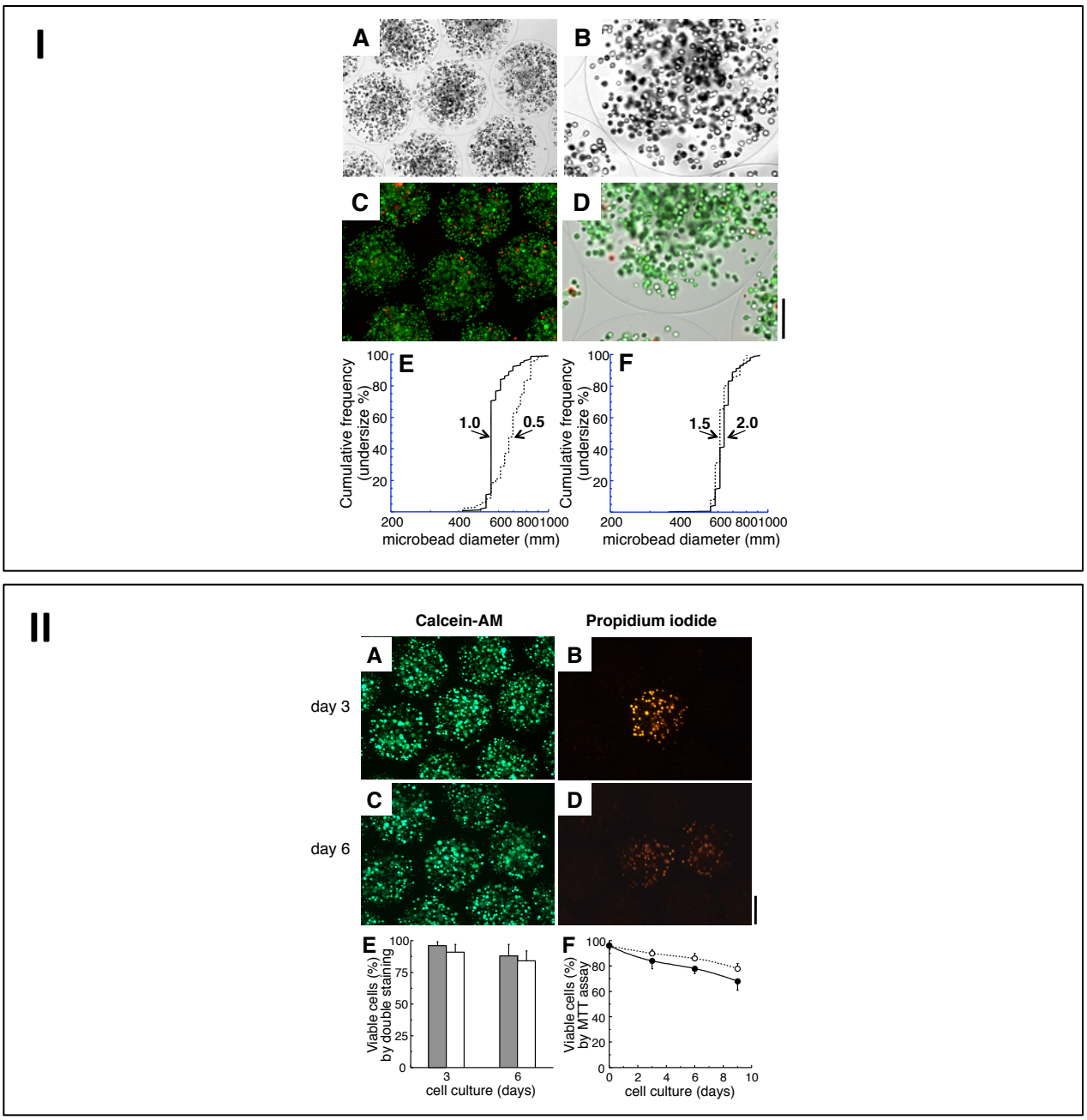
Parameter	*Abbreviation*	*Meaning*	*Range*
frequency	freq	frequency of the vibration of the nozzle	100.0-140.0 hz
amplitude	amp	amplitude of vibration of the nozzle	1.0-6.0 mm
pump	pump	polymer pumping rate	7.5-9.5 mL/min
height	height	distance from nozzle to surface of gelling bath	100.0-140.0 mm

II

Alginate Conc. (%, w/v)	*Mean diameter ±SD (mm)*	*Skewness*	*Kurtosis*	*Max. Production Rate*(g/s)*	*Photo*
0.5	685.3±114.7	0.09	0.20	0.5	
1.0	586.4±84.4	2.84	10.70	1.7	
1.5	630.4±63.7	1.40	1.06	2.8	
2.0	645.8±66.5	1.28	4.53	4.2	

Panel I): The production of alginate microbeads was optimized by changing different experimental parameters.
Panel II): Effect on alginate microbeads concentration of the dimensional and production rate of Ba-alginate microbeads. (left column) Samples of microbeads produced with an alginate concentration of 0.5, 1.0, 1.5 and 2.0% were analyzed for morphology, size and size distribution. Table showes that microbeads produced with an alginate concentration comprised between 1.0-2.0% (w/v) were spherical in shape and characterized by a smooth surface. Lowering the alginate concentration down to 0.5% (w/v) caused partial broke-up of beads resulting in particles with an irregular shape. This behaviour was attributed to the mechanical stress caused by the landing in the hardening barium chloride solution.

FIGURE 17
ALGINATE MICROBEADS ENCAPSULATION OF WJMSCs



Panel I): Optical (A, B) and fluorescence photomicrographs (C,D) of freshly prepared alginate microbeads containing WJMSCs. Dark field (C) photomicrographs were taken after double staining with Calcein-AM and propidium bromide. Cumulative size distribution analysis (E, F) of microbeads prepared for experiments.

Panel II): Viability of WJMSCs encapsulated in alginate microbeads (WJMSCs alginate beads, **WJABs**) after determination with the double staining assay with Calcein-AM and propidium bromide (panels A-D). Bar corresponds to 200 µm. (E) The percentage of viable cells, by Calcein AM/Propidium iodide double staining, at "3"and "6" day is reported in the graph (**WJABs** were cultured in in 6 well-plate (We) "control" culture medium (CCM) (**WJABs/We/CCM**). Adherent WJMCs: dashed bars; **WJABs**: white bars. (F) Determination by MTT assay of the viability of adherent WJMCs (dotted line) and encapsulated WJMCs (unbroken line) after 0, 3, 6 and 9 days of culture. Values are expressed as percentage of viable cells respect to day "0" (rated to 100%). Data represent the mean of two independent samples in quadruplicate ±SD.

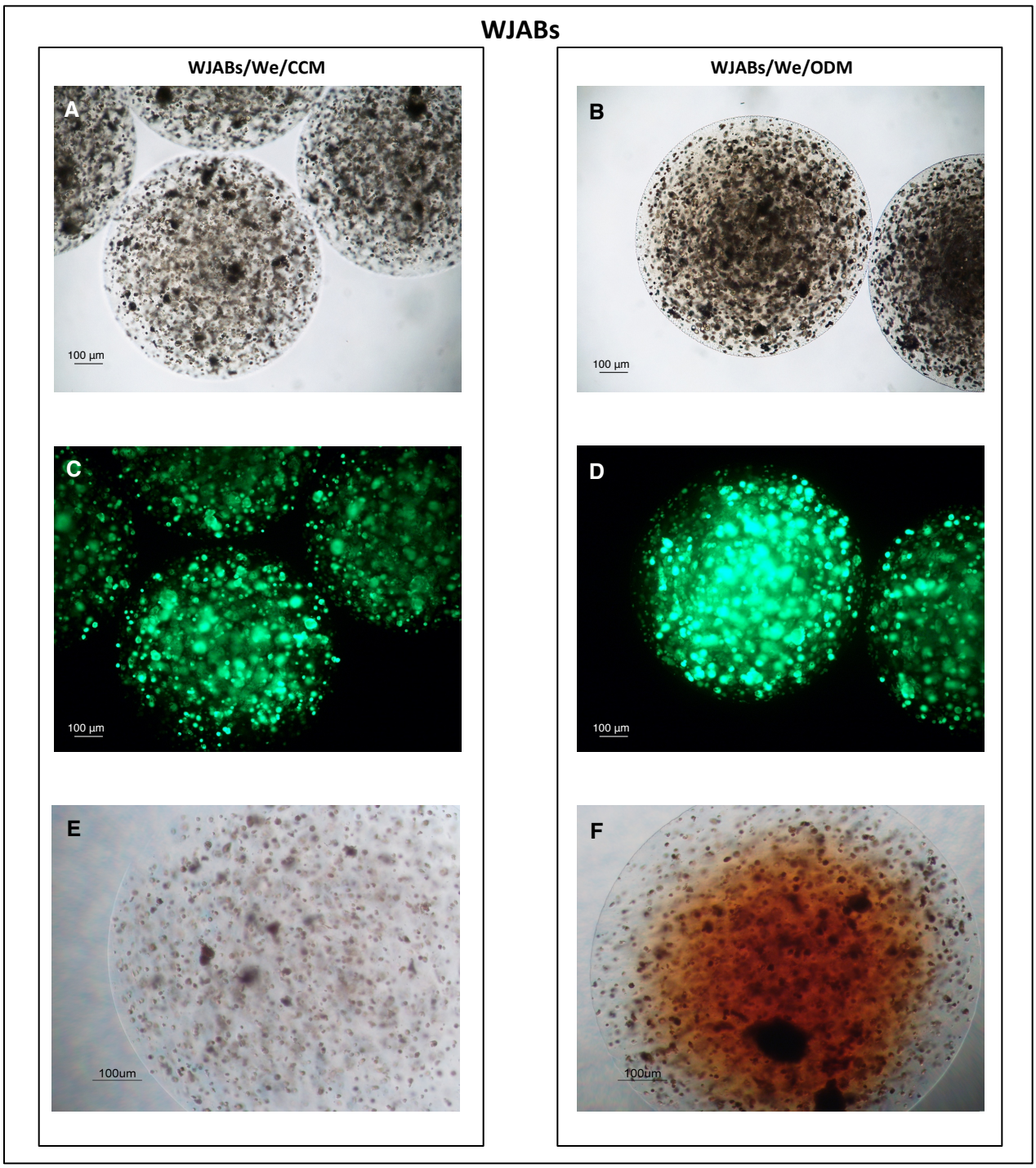
FIGURE 18
MSCs SECRETIVE PROFILE BEFORE AND AFTER ENCAPSULATION: BIOPLEX® ANALYSIS FOR WJMSCs

Secreted protein	Protein amount (pg/ml/mg ± S.D.)	
	Adherent WJMSCs	Encapsulated WJMSCs
IL-1α	n.d.	n.d.
MIG	n.d.	n.d.
IL-18	n.d.	n.d.
β-NGF	n.d.	n.d.
SCF	n.d.	n.d.
TNF-β	n.d.	n.d.
LIF	n.d.	n.d.
IFN-α	4.83 ± 0.67	1.78 ±0.30
IL-2Rα	1.78 ± 0.73	0.75 ±0.32
CTACK	4.90 ±1.65	1.86 ±0.28
IL-16	0.83 ± 0.36	n.d.
VCAM	1.80 ± 0.40	0.80 ±0.30
IL-3	0.96 ± 0.41	n.d.
TRAIL	6.70 ±1.20	2.60 ±1.40
M-CSF	12.70 ±2.50	3.90 ±0.60
SDF1α	5.80 ±1.50	3.80 ±1.20
IL-12	11.20 ±2.16	4.60 ±0.95
ICAM-1	29.50 ±5.60	13.10 ±3.70
SCGF-β	959.00 ±209.00	289.60 ±98.20
GRO-α	4.20 ±1.70	25.70 ±8.60
MCP3	6.10 ±2.00	19.60 ±5.00
HGF	46.70 ±10.90	78.60 ±18.60
MIF	13.00 ±2.80	13.50 ±3.60

Data represent the average of four independent experiments conducted in triplicate.
WJMSCs, Wharton's jelly mesenchymal stem cells; IL-1a, interleukin- 1alpha; n.d., not detectable; b-NGF, beta-nerve growth factor; SCF, stem cell factor; TNF-b, tumor necrosis factor-beta; IFN-a, interferon-alpha; SCGF-b, stem cell growth factor-beta; HGF, hepatocyte growth factor; MIG, monokine induced by IFN-Gamma; LIF, leukemia inhibitory factor; CTACK, cutaneous T-cell-attracting chemokine; VCAM-1, vascular cell adhesion molecule-1; TRAIL, tumor-necrosis-factor related apoptosis inducing ligand; M-CSF, macrophage colony stimulating factor; SDF-1a, stromal cell-derived factor 1a; ICAM-1, intercellular adhesion molecule-1; GRO-a, growth regulated oncogene-alpha; MCP3, monocyte chemotactic protein-3; MIF, macrophage migration inhibitory factor.

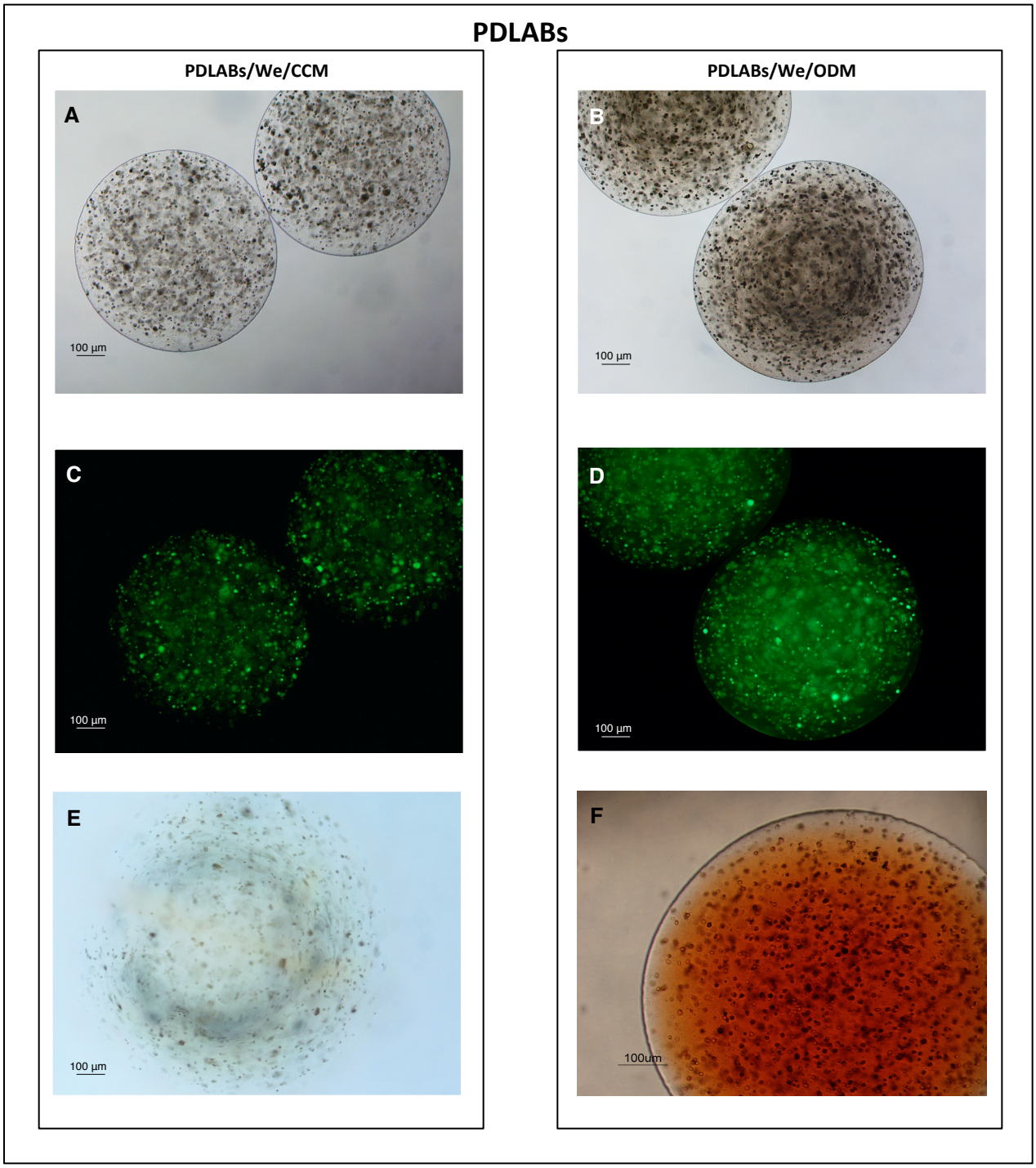
Adherent WJMSCs and alginate-encapsulated WJMSCs secretions were subjected to Bio-Plex analysis. Both secretive profiles are sumarized. Data is presented as normalization of the amount of factors (in pg/ml) released in culture medium for the cellular protein content. The panel of analyzed factors includes members of interleukins and chemokines families, growth factors and adhesion molecules soluble form. All the proteins analyzed have a molecular weight lower of the molecular weight cut off of the microbead membrane.

FIGURE 19
ALGINATE MICROBEADS ENCAPSULATION OF WJMSCs INDUCED TO OSTEOGENIC DIFFERENTIATION



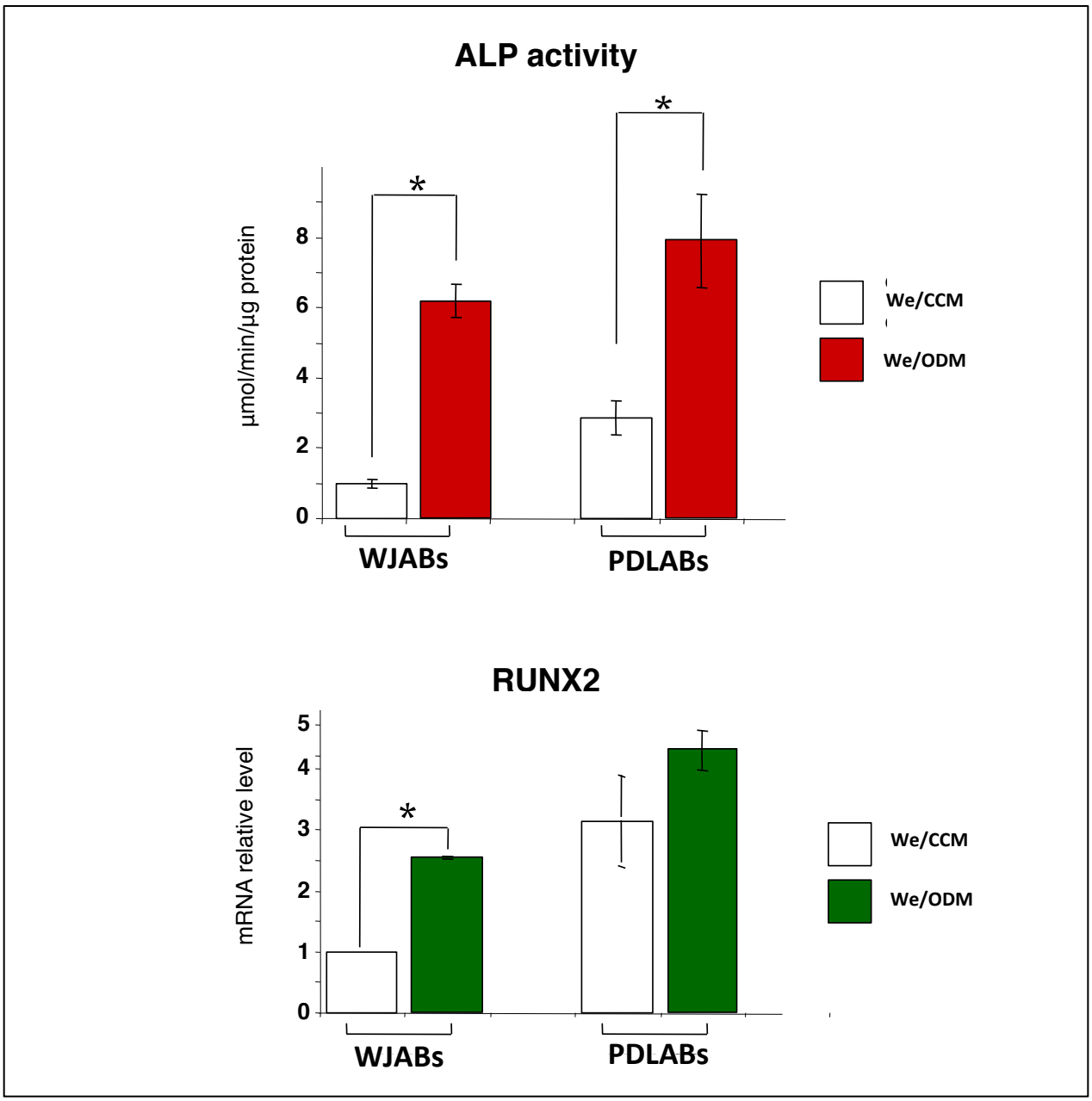
WJMSCs in alginate beads (**WJABs**) after 21-day experimental period of osteogenic induction. Medium was changed every three days. **WJABs** were cultured in 6-well (We) plate in osteogenic differentiation medium (ODM) (**WJABs/We/ODM**). 10%FCS supplemented DMEM LG was chosen as "control" culture medium (CCM) condition (**WJABs/We/CCM**).
Optical (A, B) photomicrographs of **WJABs** after 21 days of culture. Beads morphology was still regular and shape wasn't affected. Beads in osteogenic medium (B) seemed opaque, if compared to control ones (A). The Calcein AM/Propidium iodide double staining assay at day 21 showed that cells were vital in both conditions (C, D).The mineral deposition within beads was measured by Alizarin Red staining assay (E, F). (magnification 4x)

FIGURE 20
ALGINATE MICROBEADS ENCAPSULATION OF PDLMSCs INDUCED TO OSTEOGENIC DIFFERENTIATION



PDLMSCs in alginate beads (**PDLABs**) after 21-day experimental period of osteogenic induction. Medium was changed every three days. **PDLABs** were cultured in 6-well (We) plate in osteogenic differentiation medium (ODM) (**PDLABs/We/ODM**). 10%FCS supplemented DMEM LG was chosen as "control" culture medium (CCM) condition (**PDLABs/We/CCM**).
Optical (A, B) photomicrographs of **PDLABs** after 21 days of culture. Beads morphology was still regular and shape wasn't affected. Beads in control medium (A) appeared brighter if compared to the differentiated ones (B). Brown shade were recognized inside the beads, without affecting beads morphology or size. The Calcein AM/Propidium iodide double staining assay at day 21 showed that cells were vital in both conditions (C, D). The mineral deposition within beads was measured by Alizarin Red staining assay (E, F). (magnification 4x)

FIGURE 21
ALGINATE MICROBEADS ENCAPSULATION OF WJMSCs AND PDLMSCs INDUCED TO OSTEOGENIC DIFFERENTIATION: OSTEOBLASTIC MARKERS

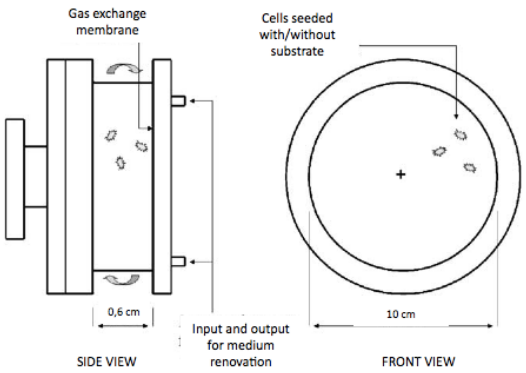


ALP activity and RUNX-2 expression level were evaluated after 21 days of cuture of alginate microbeads (WJABs and PDLABs) in control (We/CCM) and osteogenic conditions (We/ODM). Samples showed an increase of ALP and RUNX-2 levels after osteogenic induction.
the increase of markers was significant (* $p < 0.05$) except for RUNX2 in PDLABs.

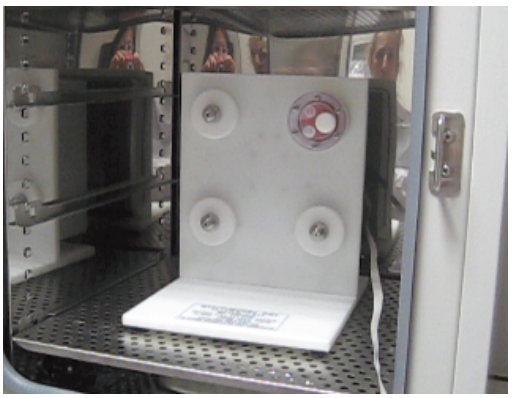
FIGURE 22
ROTARY CELL CULTURE SYSTEM™ (RCCS-4™ BIOREACTOR) WITH HIGH ASPECT RATIO VESSEL (HARV™)

I

II



III



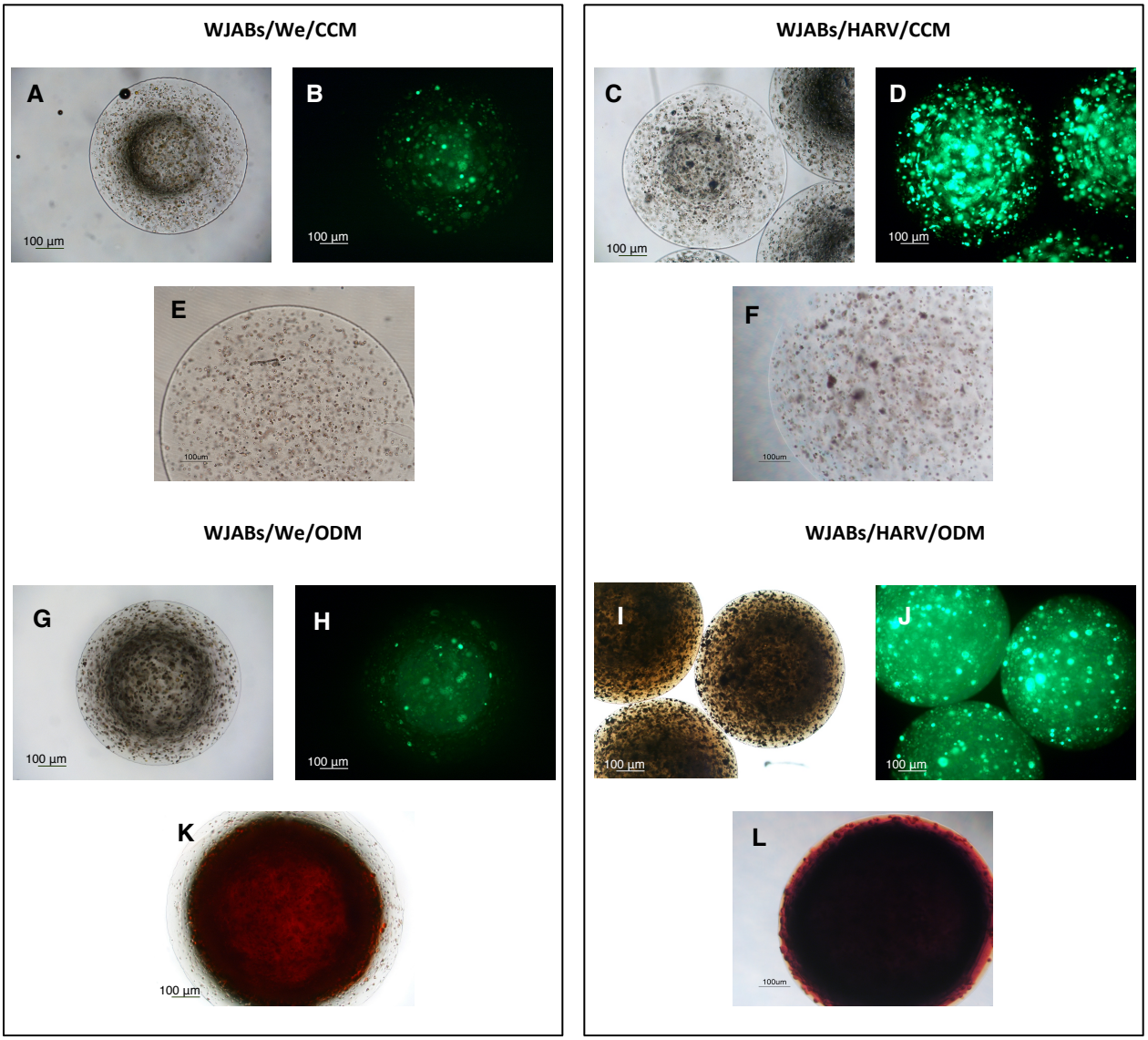
Panel I) Rotary Cell Culture System™ . A cell suspension bioreactor is a closed stirred vessel where it is possible to inline monitor and control some culture parameters. One approach to maintain homogeneous environment with low stress, is the use of rotating wall vessels (RWV). The National Aeronautics and Space Administration (NASA) developed the Rotary Cell Culture System™ (RCCS-4™ bioreactor, Synthecon™, Inc., Houston, TX, U.S.A.), with 2 different systems: High Aspect Ratio Vessel (HARV™).

Panel II) High aspect ratio vessel: side view (left) and front view (right). With HARV™ system gas exchange is provided by a 31.4 cm2 gas exchange membrane at the inner concentric cylinder. On Earth, this reactor is often rotated at a speed of approximately 15–30 rpm as the tissues grow, so the constructs in the reactor are maintained ‘‘stationary’’ in a state of continuous free-fall. Solid body rotation of the constructs at higher rotating speeds is also reported in space and Earth experiments. Both the inner and outer cylinders can be independently rotated [Martin & Vermette 2005].

The high aspect ratio vessel (HARV) allows high level of gas exchange. It is enhanced by the presence of a large gas exchange membrane at one end of the vessel.

Panel III) RCCS-4™ bioreactor with 10 mL HARV™ vessel inside incubator. Cells culture can be easy maintained at 5% CO_2 and 37°C.

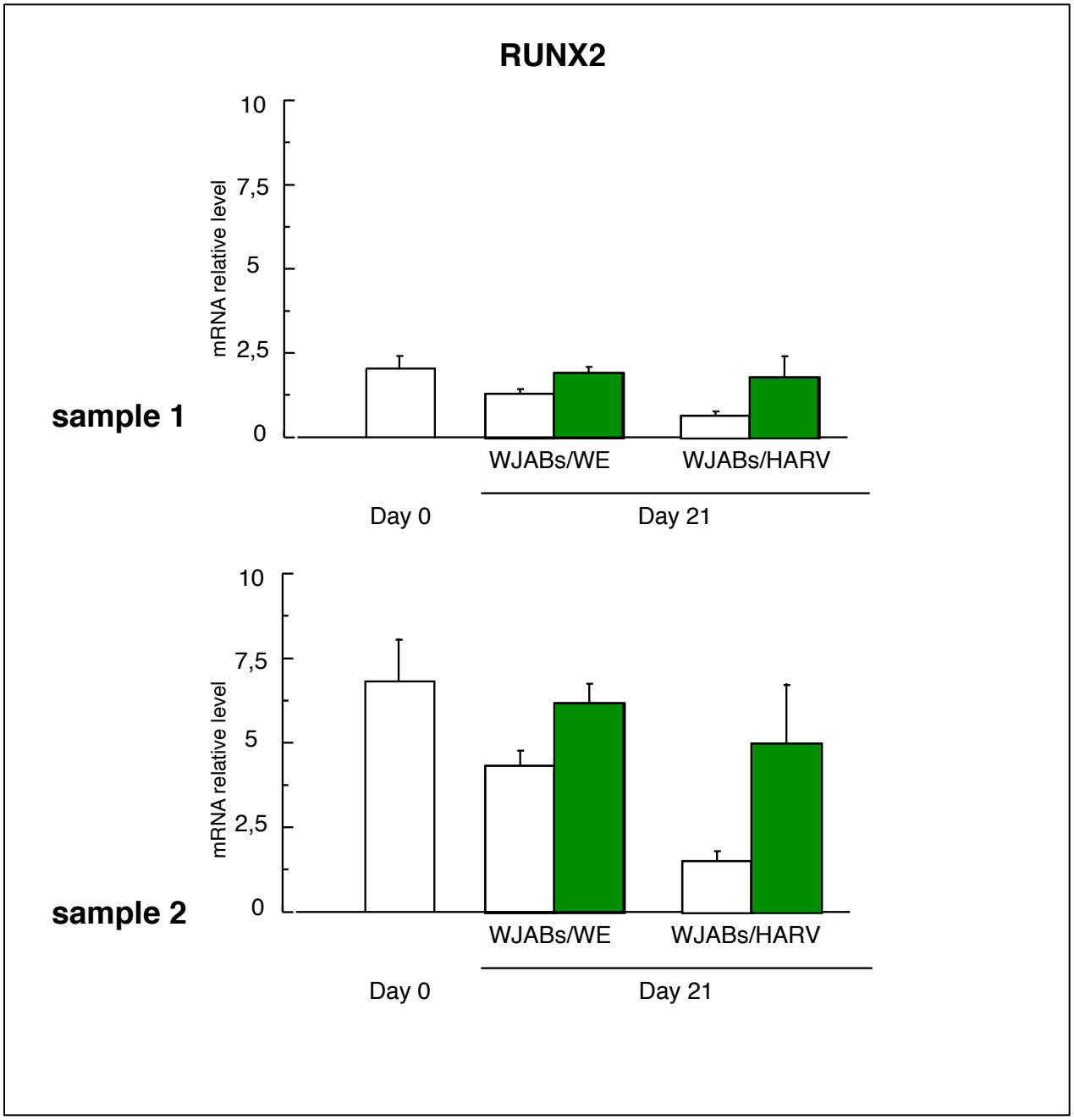
FIGURE 23
ALGINATE MICROBEADS ENCAPSULATION OF WJMSCs INDUCED TO OSTEOGENIC DIFFERENTIATION: EFFECT OF ROTARY CELL CULTURE SYSTEM™ (RCCS-4™ BIOREACTOR) WITH HIGH ASPECT RATIO VESSEL (HARV™).



WJMSCs in alginate beads (**WJABs**) after 21-day of osteogenic induction. **WJABs** were cultured in 6-well (We) plate in osteogenic differentiation medium (ODM) (**WJABs/We/ODM**),and in "control" culture medium (CCM) condition (**WJABs/We/CCM**). **WJABs** were also cultured in Rotary Cell Culture System™ (RCCS-4™ bioreactor) with High Aspect Ratio Vessel (HARV™), in osteogenic differentiation medium (ODM) (**WJABs/HARV/ODM**). 10%FCS supplemented DMEM LG was chosen as "control" culture medium (CCM) condition (**WJABs/HARV/CCM**).

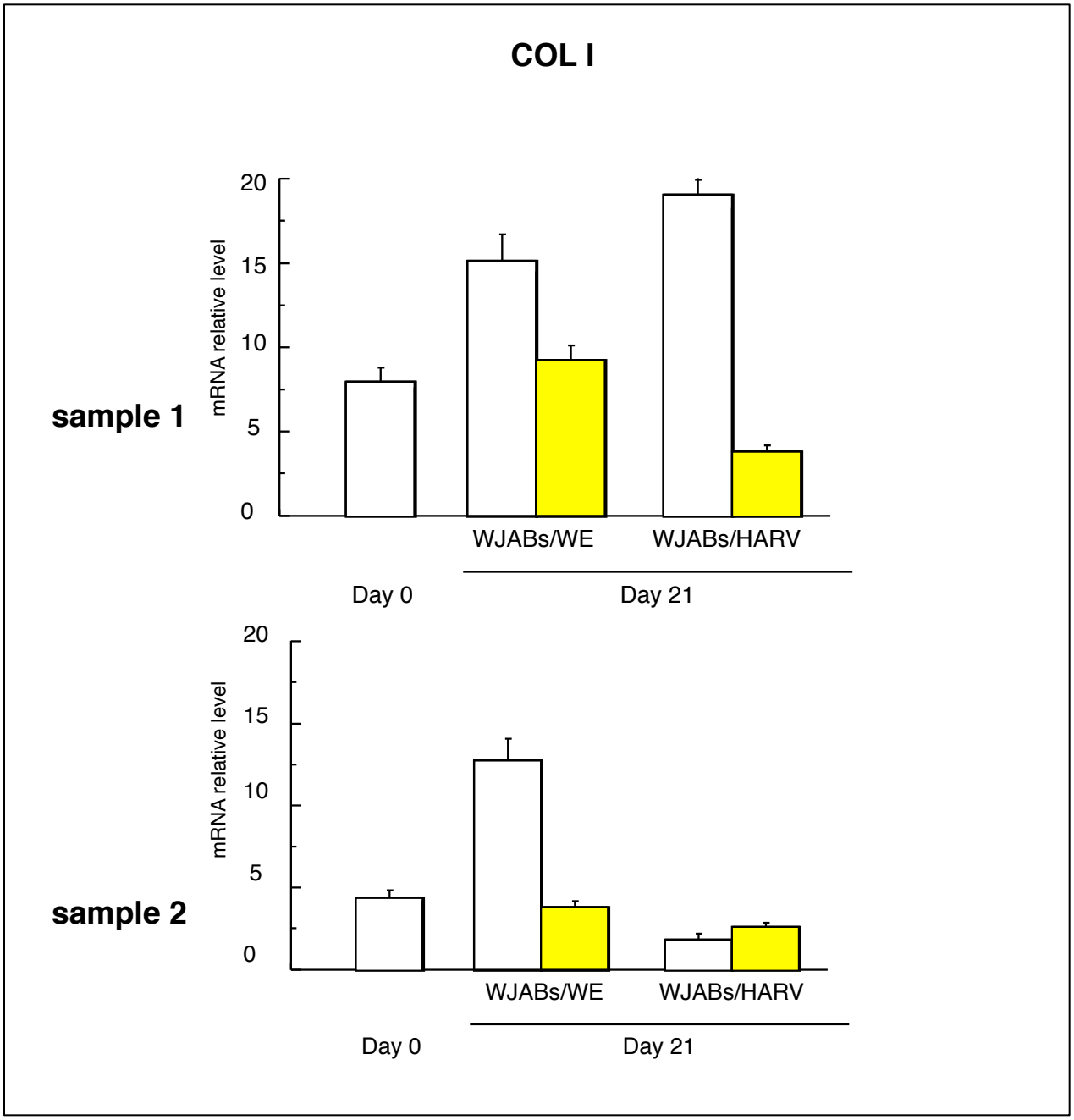
Optical (A, C, G, I) photomicrographs of **WJABs** after 21 days of culture. Beads morphology was still regular and shape wasn't affected. Beads in osteogenic medium (G, I) seemed opaque, if compared to control ones (A, C). The Calcein AM/Propidium iodide double staining assay at day 21 showed that cells were vital in 4 conditions (B, D, H, J).T The mineral deposition within beads was measured by Alizarin Red staining assay. It showed unstained cells (E, F) in both control conditions, and intense red color fot both osteigenic conditions, within and out of HARV™ vessel (K, L), suggesting that the cells underwent osteogenic differentiation despite encapsulation .

FIGURE 24
ALGINATE MICROBEADS ENCAPSULATION OF WJMSCs INDUCED TO OSTEOGENIC DIFFERENTIATION: EFFECT OF ROTARY CELL CULTURE SYSTEM™ WITH HARV™ VESSEL ON RUNX2 EXPRESSION.



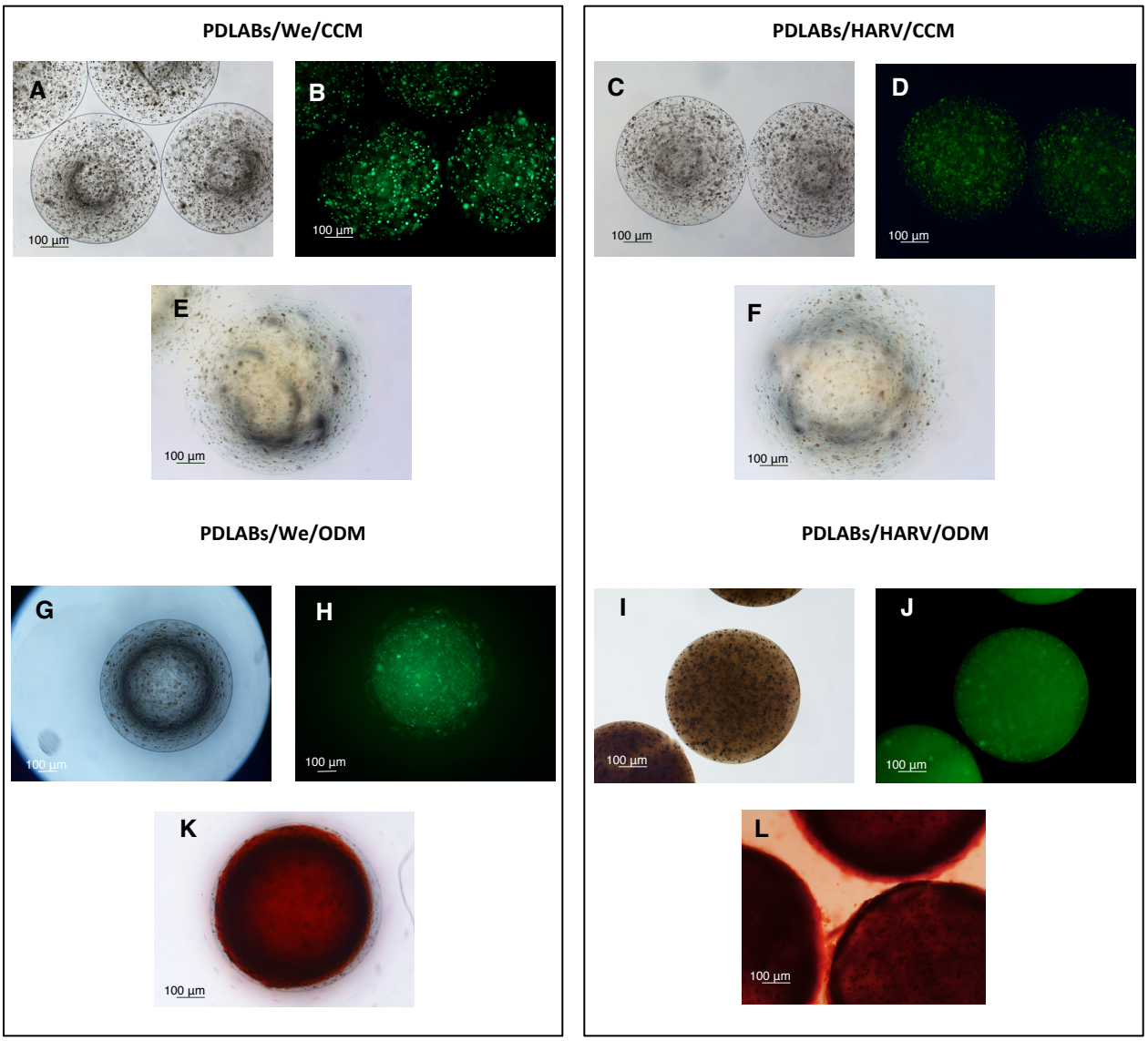
RUNX2 expression level was evaluated after 21 days of cuture of WJMSCs encapsulated in alginate microbeads (WJABs). Levels were compared before osteogenic induction (Day 0) and after 21 days of culture in both conditions (6-well plate culture, WE, and HARV^{TM} vessels, HARV) in control (We/CCM and HARV/CCM, white columns) and osteogenic conditions (We/ODM and HARV/ODM, green columns). Samples showed an higher RUNX2 level in osteogenic conditions.

FIGURE 25
ALGINATE MICROBEADS ENCAPSULATION OF WJMSCs INDUCED TO OSTEOGENIC DIFFERENTIATION: EFFECT OF ROTARY CELL CULTURE SYSTEM™ WITH HARV™ VESSEL ON COL I EXPRESSION



COL I expression level was evaluated after 21 days of cuture of WJMSCs encapsulated in alginate microbeads (WJABs). Levels were compared before osteogenic induction (Day 0) and after 21 days of culture in both conditions (6-well plate culture, WE, and HARV^{TM} vessels, HARV) in control (We/CCM and HARV/CCM, white columns) and osteogenic conditions (We/ODM and HARV/ODM, yellow columns). Samples showed an higher Col I level in control conditions.

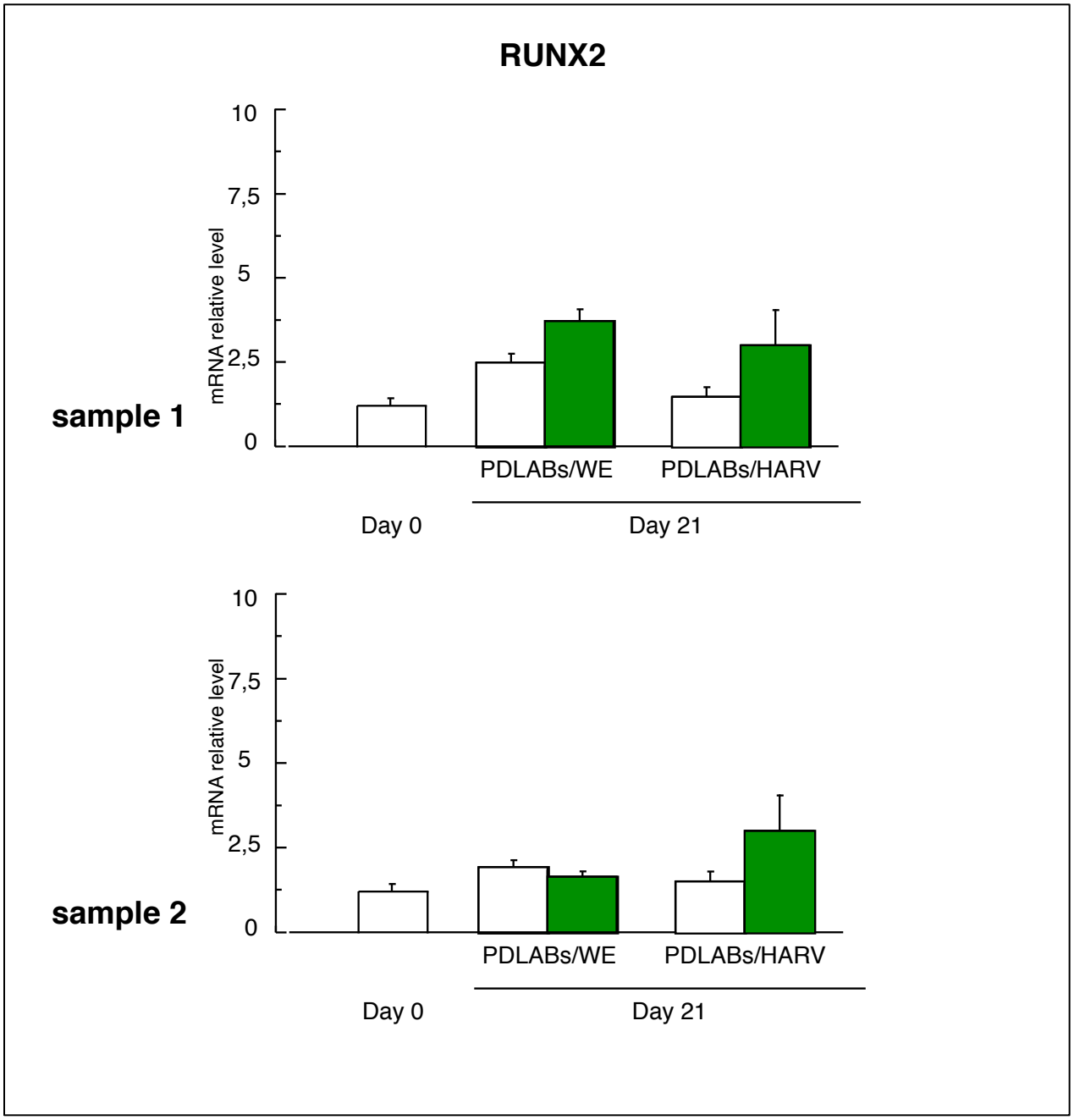
FIGURE 26
ALGINATE MICROBEADS ENCAPSULATION OF PDLMSCs INDUCED TO OSTEOGENIC DIFFERENTIATION: EFFECT OF ROTARY CELL CULTURE SYSTEM™ (RCCS-4™ BIOREACTOR) WITH HIGH ASPECT RATIO VESSEL (HARV™).



PDLMSCs in alginate beads (**PDLABs**) after 21-day experimental period of osteogenic induction. Medium was changed every three days. **PDLABs** were cultured in 6-well (We) plate in osteogenic differentiation medium (ODM) (**PDLABs/We/ODM**). 10%FCS supplemented DMEM LG was chosen as "control" culture medium (CCM) condition (**PDLABs/We/CCM**). **PDLABs** were also cultured in Rotary Cell Culture System™ (RCCS-4™ bioreactor) with High Aspect Ratio Vessel (HARV™), in osteogenic differentiation medium (ODM) (**PDLABs/HARV/ODM**). 10%FCS supplemented DMEM LG was chosen as "control" culture medium (CCM) condition (**PDLABs/HARV/CCM**).

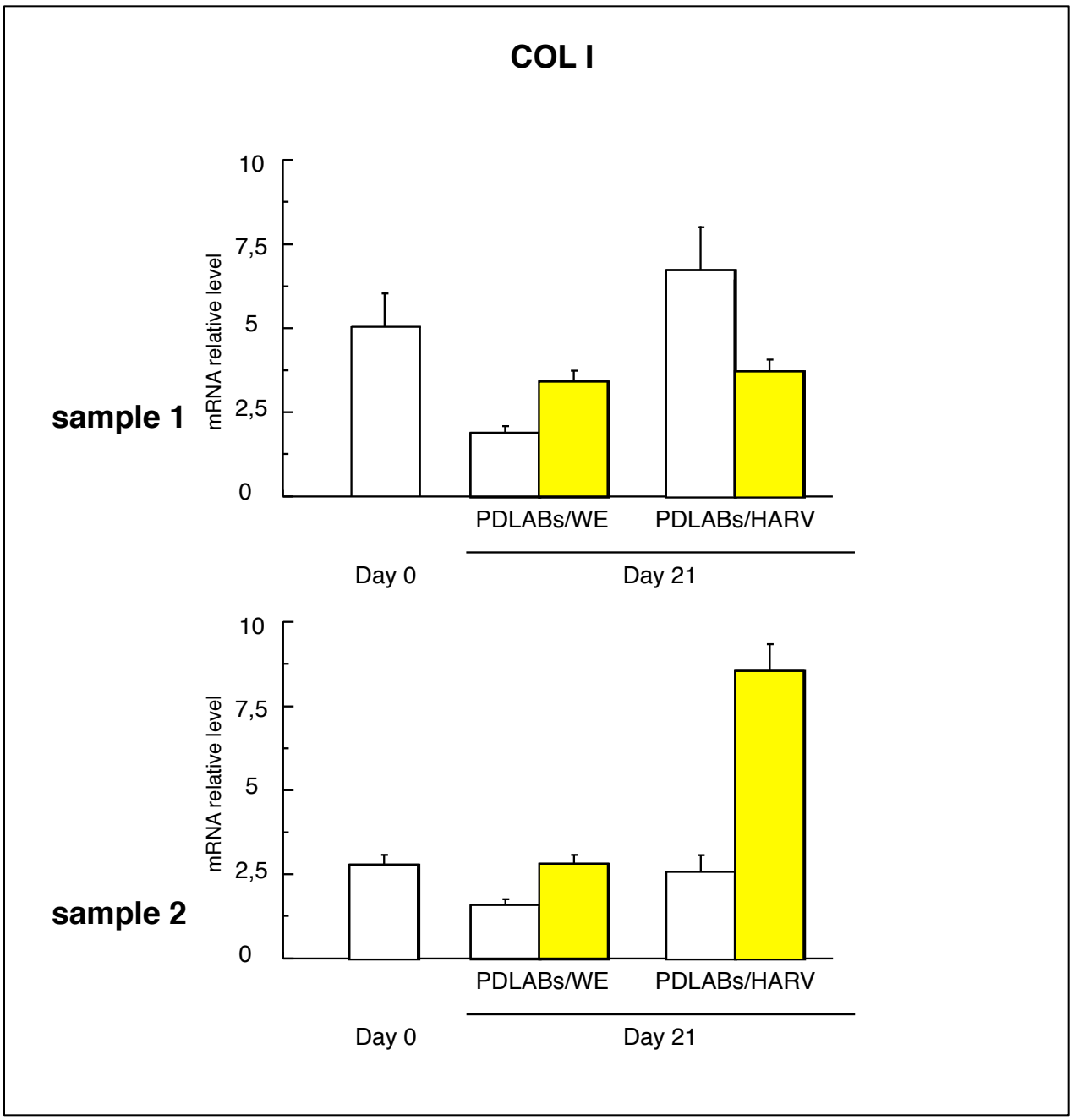
Optical (A, C, G, I) photomicrographs of **PDLABs** after 21 days of culture. Beads morphology was still regular and shape wasn't affected. Beads in osteogenic medium (G, I) seemed opaque, if compared to control ones (A, C). The Calcein AM/Propidium iodide double staining assay at day 21 showed that cells were vital in 4 conditions (B, D, H, J).T The mineral deposition within beads was measured by Alizarin Red staining assay. It showed unstained cells (E, F) in both control conditions, and intense red color fot both osteigenic conditions, within and out of HARV™ vessel (K, L), suggesting that the cells underwent osteogenic differentiation despite encapsulation .

FIGURE 27
ALGINATE MICROBEADS ENCAPSULATION OF PDLMSCs INDUCED TO OSTEOGENIC DIFFERENTIATION: EFFECT OF ROTARY CELL CULTURE SYSTEM™ WITH HARV™ VESSEL ON RUNX2 EXPRESSION.



RUNX2 expression level was evaluated after 21 days of cuture of PDLMSCs encapsulated in alginate microbeads (PDLABs). Levels were compared before osteogenic induction (Day 0) and after 21 days of culture in both conditions (6-well plate culture, WE, and HARV™ vessels, HARV) in control (We/CCM and HARV/CCM, white columns) and osteogenic conditions (We/ODM and HARV/ODM, green columns). Samples showed an higher RUNX2 level in osteogenic conditions.

FIGURE 28
ALGINATE MICROBEADS ENCAPSULATION OF PDLMSCs INDUCED TO OSTEOGENIC DIFFERENTIATION: EFFECT OF ROTARY CELL CULTURE SYSTEM™ WITH HARV™ VESSEL ON COL I EXPRESSION



COL I expression level was evaluated after 21 days of cuture of PDLMSCs encapsulated in alginate microbeads (PDLABs) Levels were compared before osteogenic induction (Day 0) and after 21 days of culture in both conditions (6-well plate culture, WE, and HARV^{TM} vessels, HARV) in control (We/CCM and HARV/CCM, white columns) and osteogenic conditions (We/ODM and HARV/ODM, yellow columns).

DISCUSSION

Better quality of life ask, together with progressive population aging, are increasing bone tissue transplant cases and needings. Bone defects repair, with attention to replacement with funcitional healthy bone, is a significant question in everyday clinical work.

Autogeneous bone is still the most effective bone graft substitution material (“gold standard”), fulfilling all essential physico-chemical and biological properties, despite its inherent limitations (availability, morbility, postoperative pain).

The most common alternatives to the auto-graft material are allografts (human) or xenografts (animal, e.g., bovine). Allografts have the disadvantages of limited supply and potential infectivity, while the use of xenografts could lead to unfavorable immune response and also infectivity.

Considering problem list connected to few donors availability, possible infections and rejection of heterologous tissue implanted, bone tissue engineering is having in these years particolar impulse.

Tissue-engineered bones with excellent biocompatibility are common alternatives to autogeneous bone, xenograft, or allograft materials.

In this scenario, scientific community is paying attention (i) to cellular and molecular research fields, to discover new factors that potentially can improve or stimulate the production of bone within the defect, and (ii) to biomaterials field, to create effective “combined systems” (combinations of cells/biomolecules/ biomaterials).

In vitro cultures of osteoblasts and chondrocytes progenitors (mesenchymal stem cells, MSCs) and adult cells (osteoblasts and chondrocytes) together in vivo animal models are traditionally used to study cell physiology and development. In the last years the employment of different strategies of differentiation induction are designed giving to researchers useful information in field of regenerative medicine

too. Particularly, a lot of studies appraised the interactions between progenitors or mature cells and biomaterials for the development of orthopedic devices, with the aim of treating bone defects that can affect skeletal system as well as dental root support tissues.

In vitro studies have been dedicated to screening new scaffolds for use in vivo, with particular attention to their ability in supporting infiltration of host cells (osteoconductive) and enhance bone regeneration (osteoinductive).

More recently, tissue-engineered bone constructs based on scaffold-cells combinations benefit from the use of dynamic cell culture system represented by bioreactors. The benefits from these technological innovations in the form of “multidimensional systems” implantable in vivo to form bone are numerous. In fact, biodegradable polymer scaffolds permit cells to maintain or differentiate their phenotype, provide a three-dimensional framework for tissue regeneration; bioreactors provide control over the conditions of cell seeding and tissue cultivation and affected construct structures and compositions [Jin et al. 2010].

In situ engineered constructs containing in vitro expanded autologous cells could be used to regenerate tissue within the defect. The key technique for bioengineering tissue passes necessarily through the developing of in vitro strategies to achieve plenty of functional seeded cells. A theoretical way to achieve this goal would be to provide a “stimulatory’’ environment for cells to expedite tissue engineering.

Bone tissue engineering using MSCs is one of the most promising alternatives to estabilish treatment for bony defects. This approach has all of the advantages of an autograft, but is not subject to supply limitations due to its self-renewal capacity [Jin et al. 2010].

All these considerations are subjected to several matters. Bone tissue regeneration and repair obtained through cell-based therapy requires special attention to at least four major issues:

- the definition of optimal osteoprogenitor sources;
- the definition of innovative strategies for the commitment of human cultured MSCs towards osteogenic lineage;

- the search for new suitable scaffolds, in consideration of which defect has to be repaired;
- the knowledge of the molecular networks implicated in osteogenic differentiation, and, at the same time, the identification of the role of specific transcription factors in mediating the fate and maturation of human MSCs.

During last years many different efforts have been and still are being made to solve and overcome problems concerning these major issues. Unfortunately, current therapies, including tissue regeneration strategies and their combination with various bone substitutes, do not always yield a satisfactory clinical outcome. It is therefore important to develop new strategies that can increase the reliability and effectiveness of tissue regeneration.

Recently, biological approaches have emerged as interesting alternatives to existing treatments for the repair and regeneration of the lost bone, or periodontium too [Froum et al. 2002; Elangovan et al. 2009]. Such approaches have included protein-based, cell-based, and gene-based therapies, as well as the local application of biocompatible scaffolds containing selected growth factors. Each approach has its own advantages and disadvantages, and all of them are suitable only for some defects, laking of universal characteristics and hallmark that can make the suggested models useful for any bone defect, in any anatomic district.

This thesis focused on developing the concept borrowed from updated literature on engineered tissue regeneration and the use of human MSCs for in vitro and in vivo studies. The main aims were (i) to understand, control and influence all processes and biomolecular interactions between cells and environment, (ii) to modulate most useful cell phenotype characteristics during tissue regeneration, (iii) to contribute to treat tissue defect, particulary periodontal or bone defects, with appropriate MSCs transplant.

This can be achieved only through several pre clinical experiments, considering and investigating factors that can affect cell differentiation and proliferation, mimicking in vitro, as accurately as possible, conditions of in vivo environment.

Considering the four major issues previously cited, this thesis work focused on the development of experiments and strategies useful for model applicable in tissue engineering and regenerative medicine field, through the employment of two different human cell populations, Wharton's jelly mesenchymal stem cells (*WJMSCs*) and periodontal ligament mesenchymal stem cells (*PDLMSCs*).

Along with the ease of accessibility, lack of ethical concerns, and abundant cell number, WJMSCs was shown to be an attractive, alternative *source of progenitor or stem cells for basic research*. On the other hand periodontal ligament has been recognized as a reliable adult source of MSCs.

Due to this consideration, the chance to extract MSCs from an easy to reach tissue was considered by our group, with the aim to investigate characteristics of this cell population and compare them to WJMSCs.

In both cases, our findings demonstrated the successful isolation and characterization of undifferentiated progenitor cells from both examined tissues, the umbilical cord Wharton's Jelly and the periodontal ligament of extracted teeth: these cells can be expanded in vitro, providing a unique reservoir of stem cells obtained with minimally invasive procedures from otherwise discared tissues.

Therefore, these cells displayed both important hallmarks for our research purposes:

- easy samples recruitment;
- lack of ethical controversies;
- easy culture and ex vivo expansion;
- reliable and consistent osteogenic potential and differentiation
- relatively homogeneous populations and superimposable characteristics.

The way we chose to explore these features and answer to major issues is reported.

Because of *WJMSCs* properties, umbilical cord was confirmed to be a *noncontroversial and feasible source* of mesenchymal stem cells, with high potential in term of viability, adhesion, proliferation before and after cryopreservation. This study showed that the WJMSCs could be easily isolated and expanded without morphological and characteristic changes.

The data on WJMSCs are promising, and there may be important therapeutic uses for these cells. Even if further studies are required to better understand the precise nature of umbilical cord matrix-derived cells and to explore their potential clinical applications, nevertheless, along with the ease of accessibility, lack of ethical concerns, and abundant cell number, WJMSCs may be an attractive, alternative source of progenitor or stem cells for basic research.

Within all WJMSCs samples recruited, *we characterized WJMSCs* from different donors for their *osteogenic potential*, trying to establish predictable elements to discriminate the cells with most promising osteoprogenitor cell potential.
The comparative study between the presence of *osteoblastic markers* and different *obstetric parameters*, including baby's gender and birth weight, mother's age at delivey, gestational stage at parturition and mode of delivery was performed. We found that osteoblastic potential was not influenced by baby's gender, mother's age at delivey, and mode of delivery. On the contrary, the *highest degree of osteoblastic potential has been shown by WJMSCs with RUNX-2 transcription factor high basal levels*, selected from umbilical cords of the heaviest term babies.
Even if further evaluation is required, our hypothesis is that our findings may help in *selecting the optimal umbilical cord donors* and in collecting high potential Wharton's jelly-derived osteoprogenitors efficiently. The analysis of the basal level of RUNX-2 and ALP activity could allow to quickly test an high number of mesenchymal precursors cultured in vitro and select the more suitable to potentially use for bone tissue engineering application. In addition, for the same aim, our results suggest that it is preferred to recruit the samples from full term borns without paying attention to mother's age.
These observations suggested that it may be possible to *discriminate among different WJMSC samples those will have a positive outcome towards osteoblastic differentiation*.

Our findings also demonstrated the *successful isolation* and characterization of *undifferentiated progenitor* cells contained in *human PDL*, that can be expanded in

vitro, providing a unique reservoir of stem cells obtained with minimally invasive procedures from discared tissue.

When MSCs hallmarks are investigated, the in vitro-obtained data are overly dependent on culture conditions for isolation and expansion of MSC populations and, therefore, are unlikely to be extrapolated to the native cells. Moreover the optimal combination and concentration of soluble and molecular factors to treat MSCs, to preserve the stem state, or optimize cells differentiation, has not been yet established.

To overcome these problems, i.e. the cytotoxic effects of some molecules necessary to dissolve inducers in medium, we developed *alternative strategies for osteogenic differentiation*.

A first set of experiments considered PDLMSCs useful to analyze and observe effects of entrapped inducers, like dexamethasone and ascorbic acid, in *polymeric micelles* (*PMs*), reducing the use of DMSO to dissove them in culture medium for osteogenic differentiation.

During in vitro experiments efforts to modify environment in terms of composition and soluble molecules revealed a different osteogenic potential for PDLMSCs population tested than what expected, confirming that collateral events (i.e. culture medium flow inside the well, presence of higher concentration of toxic molecules than expected, like DMSO) cas affect cells characteristics in term of proliferation and differentiation. The use of the *PMs* as *delivery systems* increased the *osteogenic potential* of the cells, allowing to control in terms of space and time the release of inducers, avoiding uncontrolled presence of DMSO and all its well known side effects.

It is important to underline that delivery system of bioactive factors to target cells or tissues is a very critical aspect in tissue engineering [Richardson et al. 2001]. Since tissue repair or regeneration is a complicated cascade of the multiple events in which a large number of growth factors or hormones are associated, a finely controlled release system, like the one investigated by our group, based on PMs, of specific growth factors or drugs is one of the pivotal strategies in achieving the goal of tissue engineering.

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 identified influencing these processes. To date, there have been several successful approaches to direct osteogenic differentiation of MSCs from different sources as well as success in the fabrication of bone-like tissue using 3-D supporting matrixes and culture systems.

This approach has the potential advantage of transplanting a preformed functional "combined systems". However, cells tend to retain their differentiated phenotype in vitro only if cultured under conditions that resemble their natural in vivo environment.

Both cell attachment and incorporation in vitro, as well as subsequent tissue maturation during in situ regeneration, are crucial features of tissue engineering. Therefore, the supporting 3-D structure plays a crucial role that need to be taken into consideration when designing tissue-engineering scaffolds [Boyan et al. 1996].

These considerations drove our group to develop and set of experiments aimed at defining cells behaviuor after modification of environment from a traditional 2-D culture condition to a 3-D one. The *3-D systems* tested were: *alginate microbeads* and cell culture in *bioreactor*.

The produced barium-alginate microbeads were characterized by excellent morphological characteristics as well as a very narrow size distribution. The microencapsulation procedure did not alter the morphology and viability of the embedded WJMSCs. The described *encapsulation procedure* represents a promising strategy to use WJMSCs for possible in vivo applications in tissue engineering and biomedicine.

The same protocol for encapsulation was chosen for WJMSCs and PDLMSCs, due to similar cells characteristics. The use of barium ions (instead of more often used calcium ions) resulted in the formation of mechanically stable microbeads

with an extremely high biocompatibility, preserving the in vitro and in vivo viability of the embedded cells.
When induced to osteogenic differetiation, embedded cells showed also an *increase of the expression of osteoblastic markers* such as Runx2, and ALP activity. Presence of mineralization was detected, and these results suggested that *despite encapsulation* the *cells* underwent *osteogenic differentiation*.

In addition, we investigated the functional properties, in term of *secretive profiles* of both free and encapsulated WJMSCs. The analyzed factors were members of the family of interleukins, chemokines, growth factors and soluble form of adhesion molecules These experiments showed that upon encapsulation, most of the *proteins* analyzed *were secreted both by the free and encapsulated cells*, even if in a *different extent*.
Without forcing the speculations on these data, nevertheless some considerations may be done. The decrease of SCGF-β production by encapsulated WJMSCs may be correlated with their cytostaticity. In fact, SCGF-β is a cytokine of the C-type lectin family acting on hematopoietic stem/progenitor cells to support their proliferation. In addition, an appreciable decrease in the production of factors strictly associated with the immune response including IFN-α (-63%), IL-12 (-60%) 51,52 was observed in encapsulated cells. IL-3 and IL-16 became undetectable after encapsulation. This should be interpreted as a benefit in consideration of an effective role of alginate in the protection of the cells from the host's immune response. In this respect it should be underlined that the optimized microbeads have shown an excellent biocompatibility and immunoprotection skills, as demonstrated by in vivo studies conducted up to 8 months of transplantation in the peritoneal cavity of NOD mice [Luca et al. 2007]. The microbeads hardened with barium chloride were freely floating in the peritoneal cavity and morphologically intact, with the majority of the microbeads free of fibrotic tissue overgrowth. In addition at 8 months of transplantation, the encapsulated cells were extraordinarily viable [Luca et al. 2007].
At present time, we don't know the *correlation between the functionality of encapsulated WJMSCs and the over-production of* two important *chemokines*

(GRO-α and MCP3) involved in mesenchymal stem cell chemotaxis and a pleyotropic cytokine of mesenchymal origin (HGF, hepatocyte growth factor) promoting migration and survival of MSCs. Nevertheless it is interesting to underline that HGF is one of the factors with therapeutic potential in regenerative medicine. In addition, many researchers are looking for the best approach to maintain the therapeutic level of HGF at the repair site for endogenous stem cell recruitment [Neuss et al. 2004].

These results pointed out that the *extracellular matrix can control stem cell fate*, inducing seeded MSCs towards osteogenesis depending on its physical properties. These findings opened up avenues for the regulation of MSCs differentiation using physical factors, combining these conditions with the use of biomolecules too. Consequently, biological features of cells in different conditions, in term of proliferation and differentiation rate, gene expression and sensitivity to specific biological response modifier, were strongly modify.

Bioreactors have been proven to be able to provide cell a new tool for the study of three-dimensional (3-D) cell growth and differentiation.

With the aim to verify if differentiation could be enhanced through the improvement of cells exchanges with culture environement, we performed experiments in Rotary Cell Culture System™ (RCCS-4™ bioreactor, Synthecon™, Inc., Houston, TX, U.S.A.) with High Aspect Ratio Vessel (HARV™)

The *rotational motion* of the system chosen prevented sedimentation, creating an optimized suspension culture able of *supporting living cells in alginate microbeads*. The HARVs used in the RCCS-4™ provide two essential components of the optimized suspension cell culture system:

- the solid body rotation (minimal shear stress and mechanical damage to the microbeads)
- diffusion-mediated oxygenation (oxygenation occurs as a result of a silicon membrane that allows appropriate diffusion of gases preventing hypoxic conditions and turbulence inducing air space or bubbles.

In the preliminary study reported in this thesis, the microgravity hydrodynamic conditions during in vitro cultivation of alginate microbeads was costant, with use of rotating vessels.

Fluid flow and mixing markedly affected the aspect and composition (in term of presence of Alizarine-positive mineral deposits) of *alginate microbeads* containing MSCs. In the rotating vessels, the associated fluctuations in fluid velocity appeared to permit the MSCs to maintain their differentiated phenotype and to depose mineral extracellular components.

Despite different results and findings coming from literature (due to the big amount of differences and experimental designs for each kind of mammalian cells examined), this dynamic system provided a *useful in vitro model* to understand and control the effects on proliferation, differentiation, and MSCs osteogenic potential in hydrodynamic conditions. Regulation of these parameters in bioreactors could modulate the composition, morphology, and function of "combined systems" (combinations of cells/biomolecules/biomaterials).

In particular, enhanced rates of mass transfer and hydrodynamic effects associated with dynamic laminar flow patterns in rotating vessels were expected to stimulate MSCs to differentiate and regenerate a functional extracellular matrix.

Based on findings obtained, new studies in dentistry could be designed to identify and characterize dental-derived stem cells populations, aiming at developing more predictable regenerative approaches for lost tissues as consequence of disease and/or trauma. Considering oral defects, the bone loss around teeth is due to a combined loss of hard and soft tissues, that all together form the periodontal ligament complex. Thus regeneration of all these tissues is the ultimate goal of clinical periodontal therapy [Yang et al. 2010].

CONCLUSIONS AND FUTURE WORK

Our study encourages the development of alternative strategies to induce efficient differentiation for clinical use of hMSCs in bone and cartilage tissue engineering and repair.

Considering present knowledge, reseachers efforts and developing technologies, there are several main objectives that need to be addressed for the development of effective cellular-based therapies for regenerative medicine:

- Development of new strategies to select most potential cells donors/sites could be helpful in expanding and obtaining larger cell population, overcoming the problem connected to the small number of stem cells in adult tissue stem cell niche;
- In vitro manipulation of MSCs could allow the maintenance of their proliferative phenotype, meant as “characterized by stemness”. On the other hand, strategies to prevent and avoid MSCs transformation have to be developed, in order to ensure safe use in short and long term terapies with these cells;
- Upregulation or downregulation of proper gene expression, could modify permanently or not cells characteristics, easing their use for both investigation of cell-based therapy.

All these efforts and increasing knowledge around MSCs during basic research experiments will be of considerable clinical significance, especially in terms of developing novel mechanisms of achieving tissue regeneration.

Within regenerative medicine field, the idea to realize, through the smart combination of biomolecules and cells in a system useful also "in vivo" for bone disease therapy, it is tightly linked to availability of proper biomaterials and scaffolding strategies.

Considering the specific biomaterials field, recently, a variety of systems has been proposed for matrix and membrane with immunoisolation devices with different

physio-chemical properties and geometries, including vascular perfusion devices, avascular diffusion chambers, macrocapsules, microcapsules and conformal coating.

The needing for vascular surgery, and associated risks of surface-induced thrombosis, has largely limited the development of vascular perfusion devices. Although notable exceptions exist, clinical application of macrocapsules and avascular diffusion chambers has been hampered because of insufficient oxygen and nutrient transport to cells in the center of such devices. Consequently, largest part of immunoisolation strategies employed microcapsules, consisting of cells or cell clusters entrapped within a spherical semi-permeable membrane, an inherently favourable geometry for diffusive nutrient transport, that can be implanted with minor surgery. Cell encapsulation/entrapment represents a technique for the entrapment of viable cells within a semi-permeable polymeric membrane or 3-D scaffolds.

These matrices are usually formulated to allow small molecules, such as nutrients, growth factors and pro-differentiating agents, to freely permeate through microcapsules, while blocking larger molecules (antibodies) and cells. Encapsulation allows both (i) the transplant of xenogenic or allogenic cells into a host to repair or replace damaged or diseased tissue without the need of immunosupressive regimens as well as (ii) to co-cultivate cells of different origin and/or histotype without the needing of a physical separation between the cells. Most cell immobilization matrix are usually composed of biopolymers such as alginate, carrageenans, collagen, chitosan, cellulose and agar/agarose.

Despite technological advancements, just described, it must be underlined that the use of immunoisolated cells for transplantation is rather limited because of the scarce viability, functionality and tissue-integration of the transplanted cells.

All these aspects, could be improved through the use of developed biomaterials, using new concept of "multicompartimental scaffolds".

The biomaterials for cell transplantation would be improved, including inside stubs polymeric molecules able (i) to avoid immune system answer toward installed cells, (ii) to dispatch a function of "scavenger", to inhibit or to metabolize cytotoxic molecules, (iii) to influence the proliferation and differentiation through gene

expression modulation (i.e. RNA interference) and finally (iv) to promote cells adhesion of polymeric scaffold to damaged tissue.

A further application of the concept of multicompartimental scaffolds is the realization of new scaffold with different matrix and functions.

Thus, alginate or agarose in combination with adhesion proteins such as fibronectin, vitronectin and laminin could be proposed to promote cell anchorage and interaction of cells within the multicompartimentali scaffold.

The smart choice of biomaterials has to consider also the characteristics of the defect, in terms of anatomic shape and size. An interesting approach to combine these efforts could be given by MSCs embedded in alginate microbeads and transplanted in vivo. Considering that stem cells exhibit the ability to sense and move in the direction of a chemoattractant, the elective secretion of pleyotropic cytokine of mesenchymal origin, promoting migration and survival of MSCs, could be useful for cells homing strategies, i.e. through the implant of beads in the defect area.

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APPENDIX: PUBLICATIONS AND ABSTRACTS

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Foreword

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With love

Renata