UNIVERSITÀ DEGLI STUDI DI PARMA

PhD course in Molecular Biology and Pathology

Cycle XXIII

LOOKING FOR THE HOLY WELL OF TISSUE REGENERATION

ISOLATION, CHARACTERIZATION AND DIFFERENTIATION INTO HARD TISSUES OF MULTIPOTENT CELLS FROM HUMAN PLACENTA

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Introduction

Regenerative medicine

Regenerative medicine has fascinated mankind since the ancient world. With Prometheus' myth, Hesiod testified that ancient Greeks were aware of the fact that liver could renew, but such consciousness wasn't explored until the eighteenth century when the process of regeneration in amphibians was a matter of intense study. The ascendancy of epigenetic embryology widened the comprehension of regenerative mechanisms, but it was only in the twentieth century, with the advent of cell culture (the "biological revolution") and biotechnology that tissue engineering was identified as an autonomous discipline/subject. In particular, the first definition of tissue engineering was introduced by Robert Nerem on the occasion of a meeting held at Lake Tahoe, California, in 1988:

"Tissue engineering is the application of the principles and methods of engineering and the life sciences towards the fundamental understanding of structure/function relationship in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve functions."[1]

In the 1990s researches in this field accelerated and hit the headlines with Vacanti's mouse thanks to a BBC TV program that aroused sensation and horror.

However, by the middle of the first decade of the twenty-first century, tissue engineering had reached some biological limitations and the growing fields of stem cells and the upcoming cloning techniques and nanotechnology, lead the way to a widening of the concept of tissue engineering to that of "regenerative medicine". The term was used for the first time by Leland Kaiser in 1992 referring to "a new branch of medicine […] attempting to change the course of chronic disease and in many instances […] regenerate tired and failing organ systems". The expression gained more and more diffusion through the years until NIH adopted the following definition "regenerative medicine/tissue engineering is

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[1] Atala A, Lanza R, Thomson J, Nerem R *Principles of regenerative medicine* (2008) Academic press

a rapidly growing multidisciplinary field involving the life, physical and engineering sciences that seeks to develop functional cell, tissue, and organ substitutes to repair, replace or enhance biological function that has been lost due to congenital abnormalities, injury, diseases or aging".

The therapeutic use of growth factors and cytokines to stimulate the production and/or function of endogenous cells represents the area of regenerative medicine that arguably has shown the greatest clinical impact to date.

The extension to new areas, especially the development of neo-organs with complex three-dimensional structures, however, depends on complementary advances in biology, materials science, and engineering. A major limitation remains the ability to provide oxygen and nutrients to neo-tissues both in vitro and after implantation.

As far as cell sources are concerned, a primary issue remains the choice between using a patient's own cells, or those of a closely matched relative, versus those from an unrelated allergenic donor. Cells isolated from a patient's tissue have the great advantage of avoiding the risk of immune rejection, and guarantee an easier achievement of regulatory approval to reach the market. However, the ability to produce enough cells of the necessary types for bioengineered products depends on the presence of stem and progenitor cells in the corresponding adult tissues. This fairly limits the choice of autologous grafting, as the quantity and quality of cells that can be obtained are heavily dependent on the age and healthy state of the donor.

On the other hand, using cells from a donor implies serious issues about immune compatibility and safety. Whatever the source of cells, it's required to develop culture methods that both permit the expansion of precursors cells and allow enough differentiation for generation of the desired neo-tissue: this issue can be partially overtaken by cells with a high proliferation rate, such as stem cells.

Stem cells[2]

Stem cells are a quite recent discovery of biology: it was 1981 when Evans and Kaufman isolated stem cells for the first time from a mouse embryo. The interest about these special cells has grown with time, together with the comprehension of the mechanisms that lie under their biological behaviour. Actually stem cells can be identified by some specific cues that are self-renewal and plasticity.[3] Self-renewal refers to the faculty of a stem cell, during division, of giving rise to one or two daughter cells that maintain the same phenotype as the mother. Self-renewal is sustained by the balance between symmetric and asymmetric divisions[4], which determines the appropriate numbers of stem cells and differentiated daughters. Cells generated by a symmetric division have the same fate, being it differentiated or not; on the other hand, asymmetric division produces a daughter cell that keeps the characteristics of stemness, while the other differentiates into a more specialized cell type. This last division mechanism is driven by intrinsic and extrinsic determinants, such as cell polarity factors, cell fate determinants and signals. Self-renewal is strongly linked to the concept of plasticity, which refers to the ability of a stem cell to differentiate into multiple cell types and is also known as developmental potential. Zygote is the cell with the greatest developmental potential, being it able to give rise to every cell type of an organism, including extra-embryonic tissues, and is, for this reason, defined as totipotent. As the number of cell cycles increases, cells derived from the zygote are no longer under control of factors that maintain the undifferentiated phenotype and lose more and more their potential, becoming multipotent cells, also known as progenitor or precursor cells or transit amplifying cells. As differentiation potential decreases, cells can no more differentiate into cells of all germ layers, but become restricted to one germ layer and finally unipotent, that is they can differentiate into a single cell type.

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[2] Van Blitterswijk C. *Tissue engineering*, Academic Press Series in Biomedical Engineering, 2008

[3] Morrison SJ, Shah NM, Anderson DJ. Regulatory mechanisms in stem cell biology. *Cell* (1997) **88**: 287-298

[4] Morrison SJ, Kimble J. Asymmetric and symmetric stem-cell divisions in development and cancer. *Nature* (2006) **441**: 1068-74

Multipotent stem cells derived from embryo are the most promising instruments for tissue regeneration. In fact, their origin ensures a greatest self-renewal capacity: human embryonic stem cells have been reported to proliferate for years and to go through hundreds of population doublings. However, at present, the use of embryonic stem cells arise some technical and ethical issues. The first one is evidenced by the in vivo method for determining the pluripotency of embryonic stem cells (ESC): the test consist in the evaluation of the ability of ESC to form teratomas in immunodeficient mice: in fact, in such conditions ESC give rise to a benign tumour in which tissue types from all three germ layers can be identified. The reason why such characteristic could turn in a disadvantage for this kind of cells is manifest, as present skills in governing ESC differentiation are not yet sufficient in prospect of implantation into patients. Moreover, ESC maintenance is still dependent on the use of feeder-layer cell lines and this method is time-consuming and labour-intensive, preventing a large-scale propagation of these cells. For such reason, a feeder-free growth technique, potentially without animal products would be a huge step forward. Another challenge will be the purification of the cell type of interest out of heterogeneous populations and the demonstration of treatment efficacy in large animal models. Moreover immune-rejection can be expected from the heterologous use of human ESC, which would force patients to a lifelong treatment with immunosuppressive drugs. In the hope that all such technical limits will be overtaken, serious ethical controversies persist. ESC isolation, in fact, implies the destruction of an embryo, which means for someone destroying a life. Without delving into this subject, it is a matter of fact that the search for alternative sources of stem cells is a quite pressing need.

Since their identification, adult stem cells seemed a good alternative to embryonic stem cells and the search for more and more tissue stem cells brought satisfactory results, leading to the discovery of stem cell niches even in those tissues, such as the central nervous system, whose limited turnover suggested their absence[5]. The main role of adult stem cells is actually the maintenance of tissue homeostasis, by replacing lost cells due to normal tissue turnover, injury or

[5] Wagers AJ, Weissman IL. Plasticity of adult stem cells. *Cell* (2004) **116**: 639-648

disease. Such cells are considered as precursors committed toward differentiation along a specific cellular pathway.

Historically, the first adult stem cells identified were haematopoietic stem cells (HSCs), which were isolated from bone marrow. Those cells could reconstitute all haemato-lymphoid cell types of an animal, leading the way for therapies against leukaemia. It was only in 1988 that Owen and Friedenstein[6] found in bone marrow a second population of stem cells, that would be later named "mesenchymal stem cells", in order to clearly distinguish it from the haematopoietic population. Those cells can give rise to all differentiated cell types in the stromal system, meaning in essence all the connective tissues of an organism.

Adult stem cells were at first considered less powerful than embryonic stem cells, owing this belief to the fact that many of these cells were restricted to a single germ layer: at present this notion is challenged by some works showing the transdifferentiation potential of such cells[7,8]. This gives a new stimulus to their study, which is further amplified by results concerning the ability of MSC to modulate the immune response. Mechanisms underlying this observation are still partially unclear but are very promising even in the perspective of a heterologous clinical outcome. Despite the numerous advantages that those cells bring along, the procedures for their isolation are still quite invasive and are avoided as far as it's possible. Moreover, the number of cells that can be isolated is often not enough for regenerative purposes, seriously limiting this strategy. In this perspective, other sources of multipotent stem cells are coming to the fore, that is cells derived from foetal annexes. The work of this PhD thesis is focused on the isolation, characterization and differentiation of multipotent cells isolated from human placenta.

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[6] Owen M, Friedenstein AJ. Stromal stem cells: marrow-derived osteogenic precursors *Ciba Found Symp* (1988) 136:42-60

[7] Anderson DJ, Gage FH, Weissman IL. Can stem cells cross lineage boundaries? *Nature Medicine* (2001) **7**(4): 393-5

[8] Joshi CV, Enver T. Plasticity revisited. *Current Opinion in Cell Biology* (2002) **14**: 749-755

Placenta as a source of stem cells

Placenta is the organ of pregnancy that, together with foetal membranes and amnion, supports growth and development of the foetus. It is composed of a mix of maternal and foetal tissues that are strongly intermingled and is heavily modified during pregnancy.[9]

Placenta formation starts right after implantation of blastocyst: the ectodermic layer of trophoblast proliferates and gives origin to an internal cytotrophoblastic layer, composed of cubic cells, and to an external multinucleated mass called syncytiotrophoblast. This invasive tissue penetrates the endometrium and allows the formation of an intervillous space and a lacunar network in which maternal blood circulates and bathes the villi to allow the exchange of oxygen and nourishment with the foetus. After successful implantation and initiation of placentation, trophoblast cells undergo extensive proliferation and differentiation. There are two main pathways by which trophoblast differentiation may occur, that is, villous and extravillous. By days 13 to 14 of pregnancy, cytotrophoblast cells penetrate the layer of syncytiotrophoblast surrounding the early conceptus to form columns of extravillous cytotrophoblast cells. Extravillous trophoblast cells invade the decidua and migrate so that they penetrate and remodel maternal blood vessels in the uterine decidua (endovascular trophoblast). This process produces dilated, compliant uterine arterioles that are unresponsive to maternal vasomotor control. Thus, the maternal blood supply to the placenta is promoted by this process and is, by term, about 30% of the mother's cardiac output, which itself has increased by 30–40%. Extravillous cytotrophoblast cells also invade interstitially (interstitial trophoblast). These invasive cells promote the circumferential expansion of the placental site and recruitment of maternal arterioles. The full thickness of the uterine mucosa to the decidual–myometrial border has been extensively colonised by 8 weeks of pregnancy. Interstitial trophoblast cells become multinucleated and more rounded and form placental bed giant cells as they move deeper into the decidua. Villous (non-migratory) cytotrophoblast cells

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[9] Gude NM, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. *Thrombosis Research* (2004) **114**: 397-407

proliferate, differentiate and fuse to form the outer epithelial layer of the chorionic villi, the syncytiotrophobast. The primary villi are formed by evaginations of syncytiotrophoblast with a cytotrophoblast core. Foetal mesenchyme grows into the cytotrophoblast to form the secondary villi and by the third week of gestation foetal capillaries develop within the villous mesenchyme forming the tertiary villi. Initially, chorionic villi are found on the surface of the entire chorion but, as the conceptus grows, the decidua on the uterine luminal pole (decidua capsularis) atrophies, as does the villi apposed to it. This leaves the definitive placental villi in a discoid region. As gestation progresses the chorionic villi grow and arborise. Exchange takes place in the most part through the terminal villi that project into the intervillous space.

By the end of pregnancy, placenta is a discoidal organ of about 22 cm diameter and 3 cm thick. Foetal and maternal side are clearly distinguishable.

The foetal side, also known as chorionic or amniotic side has a bright aspect, thanks to the amniotic epithelial layer. The umbilical cord sprouts from the centre of the dish, connecting the placenta with the foetus. At delivery it can reach a length of about 50 cm and it shows two arteries and a vein. It is covered by an ectodermic layer that is continuous with amnion that wraps the cord and a special viscous tissue called Wharton's jelly. The maternal side of placenta is opaque and spongious, divided by a series of grooves that define 10-40 lobes or cotyledons, which correspond to internal septa supplied by as many arborisations of placental vessels.

Placenta has not only the aforementioned nourishing functions, but has an important endocrine and xenoprotective role, too. Moreover, a haematopoietic function of placenta was recently discovered, as HSC niches were found in this organ[10]. At delivery, placenta still preserves mesenchymal and embryonic cells[11,12,13] that can be isolated and grown in vitro without any invasive procedure.

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[10] Gekas C, Dieterlen-Lièvre F, Orkin SH, Mikkola HKA. The placenta is a niche for hematopoietic stem cells. *Developmental Cell* (2005) **8**: 365-75

[11] Fauza D. Amniotic fluid and placental stem cells. *Best Practice and Research Clinical Obstetrics and Gynaecology* (2004) **18**: 877

Given the foetal origin of those cells it is conceivable that they retain some of the "special" characteristics of the organ they come from, especially in terms of resistance to xenobiotics and immunomodulation, which could be very useful in the perspective of a clinical application[14,15,16]. Actually many works refer about the engraftment potential of placenta-derived stromal cells, that possess immunomodulatory characteristics and good survival after transplantation in model animals.

The use of such source of stem cells is devoid of ethical concerns, as placenta would be otherwise discarded after delivery, thus making this organ a very promising alternative for stem cell isolation.

Mesenchymal stem cells

Even though mesenchymal cell biology is an evolving field, a univocal definition of those cells remains elusive, due to a lack of exclusive markers. According to the International Society for Cellular Therapy17 "a logical proposal for the definition of a marrow mesenchymal stem cell would be, the putative marrow cell that can self renew and give rise to a one or more mesenchymal tissues." However, such definition is quite constraining as this excludes the ability of such cells to give rise to tissues other than the mesodermal one. In any case,

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[12] In'T Anker PS, Scherjon SA, Kleijburg-van der Keur C, De Groot-Swings GMJS, Claas FHJ, Fibbe WE, Kanhai HHH. Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. *Stem cells* (2004) **22**: 1338-1345

[13] Fukuchi Y, Nakajima H, sugiyama D, Hirose I, Kitamura T, Tsuji K. Human placenta-derived cells have mesenchymal stem/progenitor cell potential. *Stem cells* (2004) **22**: 649-658

[14] Soncini M, Vertua E, Gibelli L, Zorzi F, Denegri M, Albertini A, Wengler GS, Parolini O. Isolation and characterization of mesenchymal cells from human fetal membranes. *Journal of Tissue engineering and regenerative medicine.* (2007) **1** (4): 296-305

[15] Evangelista M, Soncini M, Parolini O Placenta-derived stem cells: new hope for cell therapy?. *Cytotechnology* (2008) **58**: 33-42

[16] Bailo M, Soncini M, Vertua E, Signoroni PB, Sanzone S, Lombardi G, Arienti D, Calamani F, Zatti D, Paul P, Albertini A, Zorzi F, Cavagnini A, Candotti F, Wengler GS, Parolini O. Engraftment potential of human amnion and chorion cells derived from term placenta. *Transplantation* (2004) **78** (10): 1439-48

[17] Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* (2006) **8**:315-317.

this definition is by itself quite useless from a practical point of view, as the identification and isolation of mesenchymal cells need more specific characteristics to be pointed out. Actually, unlike haematopoietic stem cells, which are identified by the presence of CD34, a universal antigenic definition of mesenchymal stem cells is missing, thus rendering very hard the comparison of cell lines isolated from different sources. What is widely accepted so far about mesenchymal stem cells is their ability to adhere to plastic surfaces thus acquiring a spindle, fibroblastic shape; moreover this cells can generate colony forming units and can be differentiated at least into the osteogenic, chondrogenic or adipogenic lineage. From an antigenic point of view, minimal criteria that should be satisfied by a mesenchymal stem cell are the presence of CD105 and CD73 (originally identified as SH2 and SH3), CD29, CD44, CD90, STRO-1. These minimal criteria have been taken into consideration for the characterization of cells isolated from placenta.

Aim of the study

The aim of work reported in this PhD thesis was the investigation of the regenerative potential nested in placenta, the isolation and expansion of resident placental progenitor cells and their differentiation towards classical mesenchymal lineages in view of a possible clinical use of such cells in tissue regeneration. For this reason cells were isolated from human placenta and submitted to an antigenic and molecular characterization. We detected niche-like structures and we developed a straightforward technique to isolate human multipotent chorionic mesenchymal stromal cells (hCMSCs), devoid of EPC contaminants and endowed with a high degree of plasticity. In fact cells were induced to differentiate into bone, cartilage and fat tissue, thus supporting their ascription to the mesenchymal lineage.

As work progressed, it was more and more focused on chondroblastic and osteoblastic lineages. In particular cell differentiation into cartilage was dedicated to the identification of a suitable culture condition that could allow good cell survival and three-dimensional differentiation into cartilage. As for osteogenic differentiation, we focused our attention on the role of microenvironment,

meaning with this the sum of a suitable medium and a commercially available hydroxyapatite bone substitute of bovine origin, in inducing osteoblastic differentiation.

Hence multipotent cells isolated from placenta, once properly addressed, may provide an interesting new resource for the treatment of osteochondral lesions characterized by loss of substances difficult to fix.

Experimental procedures

Cells sources

Placental multipotent cells

Isolations of multipotent cells were performed on term human placentas (n= 17) that were collected by caesarean sections from the Hospital of Parma. Donors were healthy women between 30 and 45, with uncomplicated pregnancies, who underwrote an informed consent.

Placentas were quickly transferred to a sterile vessel, kept at room temperature and processed within 4 hours from delivery under a laminar flow cabinet. All experiments were approved by the University of Parma Ethical Committee.

Tissue sampling

Samples from placenta were fixed in formalin and embedded in paraffin. Five-micron sections were stained with H&E to examine histomorphology. For antigen retrieval, sections were treated with 10 mmol/L citrate pH 6.0 in a 750-W microwave oven for three cycles of 5 minutes. For immunofluorescence, primary antibodies used were mouse anti-CK18 (DAKO S.p.a., Milan, Italy, 1:100 dilution), rabbit anti-C-Kit (Santa Cruz Biotechnology Inc., 1:20 dilution), mouse anti-CD34 (NeoMarkers, 1:50 dilution), mouse anti-ABCG2 (Alexis Biochemicals, Lausen, Switzerland, 1:20 dilution), rat anti-CD44 (Santa Cruz Biotechnology Inc., USA, 1:20 dilution), mouse anti-Vimentin (DAKO S.p.a., Milan, Italy, 1:100 dilution). The following secondary antibodies were used according to manufacturers' guides: anti-mouse–FITC and anti-rabbit–TRITC (both from Sigma-Aldrich, Italy 1:300 dilution). Nuclei were stained with DAPI (Invitrogen s.r.l, Milan, Italy) or propidium iodide.

After several washes with phosphate buffer saline (PBS), samples of amniotic leaves were snap frozen in liquid nitrogen, powdered and total RNA was isolated using TRIZOL (Invitrogen s.r.l, Milan, Italy), following manufacturer's protocol, as will be resumed later. After amniotic membrane detachment, lucent decidua

parietalis was carefully removed and samples of chorionic villi were rinsed in PBS, frozen in liquid nitrogen and total RNA was isolated.

Cell isolation

In order to detect the foetal side of placenta, after the removal of amniotic membrane, the decidua's leaf was carefully dissected as well as the chorionic plate. From each placenta, about 10 gr of placental tissue were isolated and extensively washed with PBS, in order to remove red blood cells and damaged cells. The sample was then cut into small pieces with scissors or scalpel, washed again with Ca-Mg free PBS and digested with 0.2% w/v Dispase II (Roche Applied Science, Indianapolis, USA) solution in PBS for 2 hours at 37°C in continuous agitation. Digested tissue was then passed through a 30 μm mesh filter: what was retained by the filter was subjected to a second digestion for 1 hour while single cells in the percolate were centrifuged ad 100 x g for 10' then layered onto a Lympholyte density gradient (1.077 g/ml, Cedarlane Laboratories). This step was useful to remove red blood cells from the suspension.

Two volumes of cell suspension were layered on a volume of Lympholyte, taking care not to mix the fluids, in a 15 ml tube. Tubes were then spun at 800 x g for 20 min with brakes off. After centrifugation, red blood cells were stacked on the bottom of the tube, while the ring forming cells at the interface of the two liquids were collected and washed by spinning in PBS at 100 x g for 10'. An aliquot was characterized by FACScan analysis and by immunocytochemistry while the majority of purified mononuclear cells were plated into plastic Petri dishes in Iscove's modified Dulbecco's medium (IMDM) with 10% foetal bovine serum (FBS) and allowed to recover overnight. Adherent cells (mainly macrophages) were discharged, while floating cells were recovered and selected by magnetic activated cell sorting to isolate the CD34- negative population.

Magnetic activated cell sorting

A maximum of 10^8 nucleated cells were collected by centrifugation in PBS at 100 x g for 10' then treated according to Miltenyi's protocol for magnetic activated cell sorting. After centrifugation the cell pellet was resuspended in 300 μl of pre-cold separation buffer, composed of PBS plus 2 mM EDTA and 0,5%

BSA (pH 7.2). 100 μl of FcR Blocking Reagent were added and mixed, followed by 100 μl of anti-CD34 paramagnetic beads solution (Miltenyi Biotec, Italy). After 30' incubation at 4°C cells were collected and washed in 20 volumes of separation buffer then centrifuged at 300 x g for 10'. The pellet was then resuspended in 500 μl of separation buffer and loaded on a MS column that was previously set on a magnetic support and rinsed with 500 μl of separation buffer. The suspension was allowed to drop into a collection tube then the column was washed 4 times with 500 μl of separation buffer. The column was then dislodged from the magnetic field and placed into a collection tube. After this step, the column was loaded with 1 ml of separation buffer and a plunger was applied to flush out the fraction containing the magnetic labelled cells. After magnetic-activated cell sorting, CD34 immuno-depleted cells were collected, plated onto an uncoated plastic dish and cultured in complete growth medium (CM, see below) at 37°C and 5% CO_2. Medium was changed every 3-4 days and, when cultures reached 80% of confluence, cells were recovered by 0.25% trypsin-1 mM EDTA treatment for 2 minutes at 37°C and finally plated into 75-cm^2 flasks. Phenotype of the negatively sorted cell population was characterized by FACS analysis and Real Time PCR (RT-PCR). For cryopreservation, 50% DMEM (Dulbecco's Modified Eagle's Medium), 40% FBS and 10% DMSO was used as freezing medium.

Human osteoblasts isolation

Human osteoblasts (hOBs) were isolated from mandibular bone chips aseptically explanted during oral surgery. Bone chips were digested in 0.1% w/v Collagenase II (295 units/mg) in Ca-Mg free PBS solution for 2 hours at 37°C in continuous agitation. Undigested bone chips were collected by gravity precipitation then seeded on plastic dishes to allow spontaneous migration, while isolated cells were recovered from the solution by centrifugation at 100 x g for 10'. Both isolated cells and bone chips were expanded in DMEM with 10% FBS.

Cloning assay on placental multipotent stem cells

Passage 1 (P1) placental cells were counted by an automatic Coulter Counter then seeded in CM in 96 wells plates at a density of 1-2 cells per well. Single-cell deposition was confirmed microscopically and wells containing more than two cells were excluded. To evaluate the formation of colonies, wells were examined twice a week. After 2 weeks, growing colonies were sub-cloned in fresh CM. Newly developed colonies were expanded and characterized.

RNA isolation and quantification

Isolation of RNA was performed with Trizol® (Invitrogen s.r.l, Milan, Italy), or RNeasy Mini Kit® (Qiagen S.p.a., Milano, Italy) depending on the size of sample.

Tissue samples taken from whole placenta were treated with Trizol, following manufacturer's instructions. Briefly, tissue samples were homogenized in 1 mL of TRIZOL Reagent and incubated for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Subsequently, 0.2 mL of chloroform per 1 mL of TRIZOL were added and capped sample tubes were shaken vigorously by hand for 15 seconds then incubated at 15 to 30°C for 2 to 3 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remained exclusively in the aqueous phase, which was transferred to a fresh tube for precipitation. The aqueous phase was mixed with 0.5 ml of isopropyl alcohol and incubated at 15 to 30°C for 10 minutes and then centrifuged at 12,000 x g for 10 minutes at 2 to 8°C. The RNA pellet was thoroughly washed once with 1 ml of 75% ethanol and centrifuged at 7,500 x g for 5 minutes at 4°C. The ethanol was removed and the RNA pellet was dissolved in RNase-free water .

Adherent cells were treated with RNeasy Mini Kit®: cells in monolayer or on three-dimensional scaffolds were directly lysed in the cell-culture vessel after removal of culture medium following 2 washes with ice-cold Ca-Mg free PBS. 350 µl of buffer RLT were added and cells were collected by scraping and transferred to an RNAse free tube. After vortexing to homogenize, the solution was transferred to a QIAshredder spin column placed in a 2 ml collection tube and centrifuged for 2' at 13,000 x g. A volume of 70% ethanol was added to the homogenized lysate and mixed well, then the solution was transferred to a RNeasy spin column placed in a 2 ml collection tube and centrifuged at 8,000 x g for 15''. The flow-through was discarded and 500 µl of buffer RPE were added to the column that was then centrifuged at 8,000 x g for 15''. The wash with buffer RPE was repeated by spinning the column at 8,000 x g for 2'. An additional centrifugation was performed in order to eliminate any possible carryover of buffer RPE or ethanol. The column was finally transferred to a collection tube for RNA elution: for such purpose a volume of 30 – 50 µl of RNase-free water was added and the column centrifuged at 8,000 x g for 1'.

RNA concentration was estimated by using a Genova spectrophotometer (Jenway), according to Lambert-Beer law for nucleic acids. Measurements were performed at 260 nm wavelength and the OD readings were multiplied by 40, for single strand nucleic acids. The purity of isolated RNA was assessed by checking the 260/280 nm ratio, which was considered good if greater than 1.8.

Reverse transcription and qPolymerase chain /Real Time PCR

1 µg of total RNA was pre-treated with RNase-free DNase, heated at 70°C for 10 min, placed on ice for 1 min and reverse transcribed with Improm II (Promega) kit. The RNA was mixed with 250 ng of random primers and brought to a volume of 10.6 µl with RNAse-free water: the mixture was pre-heated at 65°C for 5' then cooled at 4°C.To the RNA solution a reaction mix was added containing 1U/µl of Improm II enzyme, 1U/µl of RNase inhibitor, 1.25 µl of a 10mM mix of dNTP, 2.4 µl of $MgCl_2$ solution and 4 µl of 5X reaction buffer. The RT solution mix was than heated at 25°C for 5' to allow primer annealing, and the cDNA synthesis

was performed at 42°C for 1h. The enzyme was inactivated by heat at 70°C for 15 min. The duplex of RNA-DNA was treated with 5 units of RNase H (US Biochemicals, Cleveland, OH) at 37°C for 20 minutes. The amount of single-strand cDNA was evaluated by fluorometry (Victor2 1420 Multilabel Counter, Wallac) with the fluorescent probe Oligreen (Invitrogen s.r.l, Milan, Italy) by using phage M13+ as single-strand DNA standard and brought to a concentration of 5 ng/µl with RNAse free water for RT-PCR.

RT-PCR

For RT-PCR 25 ng of cDNA were amplified with 2X Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen s.r.l., Milano, Italy), along with forward and reverse primers (5 pmol each) outlined in table 1 in a total volume of 25 µl. Primer set was designed according to gene sequences reported in GenBank with the help of Beacon Designer 7 software (Premier Biosoft). Quantitative PCR was performed in a 36 well Rotor Gene™ 3000 (Corbett Life Science, Mortlake, Australia). Each cycle consisted of a denaturation step at 95 °C for 15 s, followed by annealing (30 s) and extension (30 s, 72 °C) steps. Fluorescence was monitored at the end of each extension step. A no-template, no-reverse transcriptase control was included in each experiment. Samples were run in duplicate and the average crossing point (CP) value was used for calculations. The analysis of data was made according to the Relative Standard Curve Method. Data were expressed as the ratio between proband cDNA and GAPDH or RPL15 cDNA.

Product specificity was determined by analysis of a melting curve at the end of the reaction, while the correct size of the amplicon was visualized on 2% agarose gels by ethidium bromide staining and estimated by co-migration with a 100-bp ladder marker (Invitrogen srl, Italy). Pictures of electrophoresed cDNAs were recorded with a digital DC 120 Kodak camera.

Gene	GenBank AC	Forward primer	Sequence	Reverse primer	Sequence	T ampl	Product size (bp)	Genomic product (bp)
Stemness								
CD34	NM_001773	CD34le	ACCTTGAAGCCTAGCCTGTC	CD34ri	GATTTCTGCCTTGATGTCACTTAG	53,00	129	no
CD117	NM_000222	CD117for	GTTCTGCTCCTACTGCTTCGC	CD117rev	TAACAGCCTAATCTCGTCGCC	58,14	135	37543
CD133	NM_006017	CD133_for	CCAAGGACAAGGCGTTCAC	CD133_rev	CTGGTCAGACTGCTGCTAAG	55,3	214	5878
HGF	NM_000601.4	HGF_le	TTGCCCTATTTCTCGTTGTG	HGF_ri	TCCATCCTATGTTTGTTCGTGT	52,89	134	7083
IGF1	NM_000618.2	IGF1_fo	TGTCCTCCTCGCATCTCTTC	IGF1_re	ATACCCTGTGGGCTTGTTGA	59,23	158	56109
SDF-1	NM_000609	SDF-1for	ATGCCCATGCCGATTCTTCG	SDF-1rev	GCCGGGCTACAATCTGAAGG	56,19	101	no
MET	NM_000245	METfo	CCGCTGACTTCTCCACTG	METre	TACTTCATATTCACATTCATCTCG	54,90	172	no
Adipogenic differentiation								
FABP4	NM_001442	FABP4_fo	GCAGAAATGGGATGGAAAA	FABP4_re	TGCTTGCTAAATCAGGGAAAA	53,39	219	no
LPL	NM_000237	LPLfor	ACAAGAGAGAACCAGACTCCAA	LPLrev	AGGGTAGTTAAACTCCTCCTCC	58,36	149	1510
GLUT4	NM_001042.2	GLUT4for	TCGGGCTTCCAACAGATAGG	GLUT4rev	AGCCACGTCTCATTGTAGCTC	57,30	176	no
LEP	NM_000230	LEP_le	GAACCCTGTGCGGATTCTTGT	LEP_ri	GGAGGAGACTGACTGCGTGT	56,75	149	no
Chondrogenic differentiation								
AGC	NM_001135	acan_LE	CCATCAACAGAGACCTAC	acan_RI	CGTAGCATTGTGAGATTC	58,6	129	1528
BGN	NM_001711.3	BGN_le	ACCTCCCTGAGACCCTGAAT	BGN_ri	GGACAGAAGTCGTTGACACC	60,34	289	1474
COL2A1	NM_001844	COL2a1-for	CCTGGTGAACCTGGTGAAC	COL2a1-rev	GCCTGGATAACCTCTGTGAC	58,3	204	no
Decorin	NM_001920	Decofor	TTAGTCCTGGAGCATTTACACCT	Decorev	GTGCCCAGTTCTATGACAATCA	55,80	190	no
HES1	NM_005524	HES1_le	AGCACAGAAAGTCATCAAAGC	HES1_ri	TTCCAGAATGTCCGCCTTC	52,6	149	no
SOX9	NM_000346	sox9_LE	GCTCTGGAGACTTCTGAA	sox9_RI	CGGCTGGTACTTGTAATC	61,9	106	no

Table 1 Characteristics of primers used for Real-Time PCR analysis

Osteogenic differentiation								
ALP	NM_000478	ALP-fo	TGATGTGGAGTATGAGAGTGAC	ALP-re	TGAAGTGGGAGTGCTTGTATC	55	110	no
BMP2	NM_001200	BMP2for	ACTGCGGTCTCCTAAAGGTC	BMP2rev	CGTCAGAGGGCTGGGATG	61,20	166	1410
COL1A1	NM_000088	COL1A1for	GTCGAGGGCCAAGACGAAG	COL1A1rev	CAGATCACGTCATCGCACAAC	60,04	143	1606
OC	NM_199173	OC_LE	TCACACTCCTCGCCCTAT	OC_RI	GTCAGCCAACTCGTCACA	60,4	245	873
ON	NM_003118	ON_LE	GCATCAAGCAGAAGGATA	ON_RI	AATAGTTAAGTTACAGCTAAGAAT	57,1	237	no
OPG	NM_002546	OPG_le	TTGGTCTCCTGCTAACTC	OPG_RI	GAAGAATGCCTCCTCACA	56,5	116	no
SP7	NM_152860	SP7_fo	CCCATTCTCCCTCCCTCTC	SP7_re	CTTCTTTGTGCCTGCTTTGC	57	241	no
RUNX2	NM_004348	Runx2es5F	GGAATGCCTCTGCTGTTATG	Runx2es5R	TTCTGTCTGTGCCTTCTGG	54,3	193	no
SOST	NM_025237	SOST_le	CAGGCGTTCAAGAATGAT	SOST_ri	CACGTCTTTGGTCTCAAA	62,6	147	no
SPP1	NM_000582	SPP1-le	CAGCCTTCTCAGCCAAAC	SPP1-ri	CCTCAGAACTTCCAGAATCAG	59,4	127	no
VDR	NM_000376.2	vdrFOR	CGCATCACCAAGGACAACC	vdrREV	CTGGCAGAAGTCGGAGTAGG	58,80	258	no

Table 1 (continued) Characteristics of primers used for Real-Time PCR analysis

Immunostaining

Immunostaining was performed on cell monolayers grown on four-well chamber slides (Falcon Becton Dickinson Labware, Milano, Italy). Cells were washed twice with PBS and fixed with 4% paraformaldehyde (pH 7.4) for 10 min. After additional washing, non-specific absorption of antibodies was blocked by incubation for 1 h in PBS containing 3% bovine serum albumin and 5% of serum of the animal in which secondary antibody was produced; after blocking reaction, cells were incubated overnight at 4 °C with the selected antibody at the proper dilution (table 2). After three washings, cells were incubated for 1 h at room temperature with FITC or TRITC conjugated secondary antibodies in blocking solution. Nuclei were counterstained with DAPI (4',6-diamidine-2-phenyndole) or propidium iodide and cover slips mounted with Vectashield (VECTOR; USA). Cells were finally washed, and slides were mounted with Pro-Long® antifade kit (Invitrogen, Milano, Italy) and examined with Nikon eclipse TE 300 inverted microscope.

Antibody	Dilution
Anti-CK18 mouse mAb (DAKO)	1:100
Anti-C-Kit rabbit polyclonal Ab (Santa Cruz)	1:20
Anti-Oct-4A (C3013) rabbit mAb (Cell signalling)	1:200
Anti-SSEA4 (MC813) mouse mAb (Cell signalling)	1:200
Anti-TRA-1-60 (S) mouse mAb (Cell signalling)	1:200
Anti-TRA-1-81 mouse mAb (Cell signalling)	1:200
Rabbit anti-goat antibody (Sigma) –FITC conjugated	1:70
Goat anti-mouse antibody (Sigma) - FITC conjugated	1:70
Goat anti-rabbit antibody (Sigma) - FITC conjugated	1:70
Rabbit anti-rat antibody (Sigma) - TRITC conjugated	1:70

Table 2: List of antibodies used for immunohistochemistry

Fluorescence-activated cell sorting analysis

To detect surface antigens, cells were detached by short incubation with 0.25% trypsin-1 mM EDTA. Cells were resuspended in Ca-Mg free PBS containing 1% FBS and incubated for 20 minutes at 4°C with fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated control antibodies or antibodies specific for CD13, CD14, CD31, CD34, CD43, CD44, CD45, CD49d, CD52, CD71, CD80, CD90, CD105/SH2, CD117, CD133, CD146, or HLA-DR (human leukocyte antigen-DR). After staining, cells were washed extensively and analysed. Flow cytometry analysis was performed using a fluorescence-activated flow cytometer (FACScan, Becton Dickinson Bioscence). In each case $2x10^4$ events were acquired and analysed with CellQuest software (Becton Dickinson Bioscence).

Negative controls of IgG1 or IgG2a isotype of irrelevant specificity were purchased from Becton Dickinson. They were used to set the limits of non-specific immunoglobulin cell-binding by cells stained with monoclonal antibodies conjugated with the homologous fluorochrome.

Cell culture and differentiation

Cells were grown in incubator at a constant temperature of 37°C and 5% CO_2.

Freshly isolated placental cells were plated in 75 cm^2 flasks and cultivated in a recovering medium composed of IMDM (Sigma-Aldrich) plus L-glutamine (2 mM, Sigma-Aldrich), 20% foetal bovine serum (FBS, EuroClone), penicillin and streptomycin (EuroClone).

Then cell recovering medium was changed to a complete medium (CM) whose formulation was conceived with the target of keeping cell stemness in culture. For this reason, a formulation with β-FGF was chosen, as it was demonstrated that this growth factor supports cell viability and proliferation as well as an undifferentiated state[18,19,20]. Final composition was as follows (table 3):

Component	Concentration
IMDM	
Glutamine	2 mM
FBS	10%
Penicillin	100 U/l
Streptomycin	100 mg/l
Insulin	5 μg/ml
Transferrin	5 μg/ml
Selenite	5 ng/ml
β-FGF	10 ng/ml
Heparin	15 U/ml

Table 3: Composition of complete medium (CM) for placental cells maintenance

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[18] Tsutsumi S, Shimazu A, Miyazaki K, Pan H, Koike C, Yoshida E, Takagishi K, Kato Y. Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. *Biochemical and Biophyisical Research Communications* (2001) **288**: 413-9

[19] Bianchi G, Banfi A, Mastrogiacomo M, Notaro R, Luzzatto L, Cancedda R, Quarto R. Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. *Experimental cell research* (2003) **287** (1): 98-105

[20] Sotiropoulou PA, Perez SA, Salagianni M, Baxevanix CN, Papamichail M. Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. *Stem cells* (2006) **24**: 462-471

Such culture medium was changed every 3 days and maintained for the whole growth phase. The same medium was also the control medium during differentiation experiments.

In vitro differentiation experiments

In vitro differentiation experiments were performed in order to verify placental cell plasticity. Media formulation were taken from literature and adapted to the cell model in use[21].

Adipogenesis

To promote adipogenesis placental cells were plated at a density of 50.000 cell/well in a 6 well plate and cultured in the following medium (table 4):

Component	Concentration
DMEM:Ham's F-12	1:1
FBS	5%
Dexamethasone	1 μM
Insulin	10 μg/ml
Indomethacin	100 μM
Isobutylmethylxanthine	500 μM
Pantotenic acid	17μM
Biotin	33 μM
L-glutamine	2 mM
Penicillin	100 U/l
Streptomycin	100 mg/l

Table 4: Composition of adipogenic medium

[21] Vater C, Kasten P, Stiehler M. Culture media for the differentiation of mesenchymal stromal cells. *Acta Biomaterialia* (2011) **7**: 463-77

After a 4 week induction, differentiation was assessed by gene expression analysis with a specific primer set and by staining with 0.3% Oil-red-O in 60% isopropanol for 10 min at room temperature to visualize lipid droplets.

Chondrogenesis

For the differentiation of placental multipotent cells into chondrocytes we had to face a lot of difficulties in the search for the ideal medium. After a first approach in which cells were grown on plate, a pellet culture system was adopted accordingly to the protocol of MacKay et al[22]. Briefly, 2.5×10^5 cells were placed in a 15-ml polypropylene tube and centrifuged into a pellet. Each pellet obtained was cultured at 37 °C with 5% CO_2 in 500 µl of chondrogenic medium. The composition of medium was changed progressively, as will be discussed later, in order to reach the optimal one, which was formulated as follows (table 5):

Component	Concentration
DMEM high glucose	
Dexamethasone	100 nM
L-ascorbic acid 2-phosphate	200 m
L-proline	40 µg/ml
Sodium pyruvate	100 µg/ml
ITS + 1	1 X
TGF-β3	10 ng/ml
IGF-1	10 ng/ml
FBS	0,1%
Penicillin	100 U/l
Streptomycin	100 mg/l

Table 5: Final composition of chondrogenic medium

Medium was replaced every 3–4 days while control cells were maintained in CM. After a 4-week induction, pellets were embedded in paraffin and cut into 5-

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[22] Mackay AM, Beck SC, Murphy JM, Barry FP, Chichester Co, Pittenger MF. Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng.* (1998) **4** (4): 415-28

μm sections. Differentiation was assessed by gene expression analysis with specific primers and by staining with 1% w/v alcian blue solution (pH 2.5) or by Masson's trichromic stain to detect acidic mucopolysaccharides synthesis and matrix deposition.

Osteoblastic differentiation

For differentiation experiments, cells that were designed for mRNA analysis were seeded in 6 well plates at a density of $3,5 \times 10^4$ cell/well; for histological analysis cells were seeded in 24-well plates at a density of $1,5 \times 10^4$ cells/well or in 4-well chamber culture slides (BD Falcon).

The first formulation of osteogenic medium was composed as follows (table 6):

Component	Concentration
Penicillin	100 U/l
Streptomycin	100 mg/l
L-ascorbic acid 2-phosphate	100 μM
FBS	10%
Dexamethasone	100 nM
β−glycerophosphate	10 mM
1-25 vitamin D$_3$	1 nM
L-proline	40 g/ml
L-Glutamine	2 mM
Medium αMEM	

Table 6: Composition of osteogenic medium (OM)

The choice of medium components was based on literature data that will be discussed with results.

Differentiation was assessed by gene expression analysis with specific primers, as previously described, and by immunostaining for collagen Type I (goat clone C18, Santa–Cruz, 1:400 dilution), osteocalcin (goat clone V19, Santa –Cruz, 1:400 dilution) and osteopontin (mouse, clone 1B20- Assay Design, 1:50 dilution). Mineralization was pointed out by calcein staining of living cells adding

sterile calcein orange solution (final concentration 1µg/ml) to culture medium during the last week of differentiation protocol to document neo-deposition of calcium salts.

A quantitative evaluation of mineralization was obtained accordingly to Alizarin red assay as described by Gregory et al[23]. Briefly, 400 µl of 10% acetic acid was added to each well containing Alizarin Red S stained samples, and the 24 well plates were incubated at room temperature for 30 min in continuous agitation. For each well, slurry of detached cells was transferred to a microcentrifuge tube and, after vortexing for 1 min, it was heated to 85°C for 10 min, transferred to ice for 5 min, spun at 20,000 g for 15 min. Finally, 300 µl of the supernatant were transferred to a new microcentrifuge tube, and 120 µl of 10% ammonium hydroxide were added. The resulting solution was read at 405 nm in an opaque-walled Terasaki plate (Fisher Scientific) using a Wallac 142 multilabel counter (Wallac). Data were expressed as Alizarin red concentration (mM).

Effect of plate surface and microenvironment: in vitro anorganic bovine bone micro scaffolds experiments

Cells grown on untreated plastic dishes only showed a scanty differentiation: for this reason we tried to improve the microenvironment in order to give cells further inducing signals. At first. cells were plated on plastic dishes covered with a bovine type I collagen solution (Sigma-Aldrich): a 0,001% solution of collagen in 0,1 M acetic acid was distributed into the dishes and left at 50°C overnight for drying. The same solution was used to help adhesion of an anorganic bovine scaffold named Bio-Oss®.

Among biomaterial used as bone substitutes in dental and oral surgery, Geistlich Bio-Oss® is one of the most widespread commercially available product for filling large bone defects. It is made of bovine bone that is treated in order to get a spongious matrix by means of a proprietary extraction process (treatment

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[23] Gregory CA, Grady Gunn W, Peister A, Prockop DJ. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. *Analytical Biochemistry* (2004) **329**: 77-84

with strong alkalis and organic solvents, up to 300°C) that renders it antigen- and protein-free. Geistlich Bio-Oss® supports the body's natural healing processes because of its close resemblance to human tissue. The coarsed, meshed interconnecting pores have a size between 300 μm and 1500 μm which allows bone cell migration and enhances blood vessel formation. Moreover the large surface area allows intimate contact with new bone tissue and the fine crystalline structure (10-60 nm) allows integration into the natural bone remodelling process. Despite the good results in clinical treatment, the mechanisms by which Bio-Oss® supports bone healing from a molecular point of view is not completely clear. For this reason, Bio-Oss® was tested on different cell models to clarify its role from a molecular point of view. The product was tested as small granules (0,25-1mm size) or as a block of approximately 0,3 cm^2. This last product was particularly suitable for modelling, in order to fit it into induced bone defects for the in vivo experiments.

For in vitro experiments, 50.000/well multipotent placental stem cells were seeded onto 6-wells dishes with or without granules of Bio-Oss spongiosa. Dishes were previously prepared by allowing Bio-oss® adhesion through a 0,001% bovine collagen solution in acetic acid. Granules were resuspended at a concentration of about 1mg/ml. Each well was covered with 0,2 ml of solution to achieve a coating of 1 μg/cm2 of collagen type I and roughly 100 μg/cm2 of granules and let dry overnight.

Cell culture lasted 4 weeks, after which cells were harvested for RNA expression analysis, fixed in paraformaldehyde for immunocitochemistry or stained to detect mineralization. For this purpose, in one well for each experimental condition, 1μg/ml of calcein was added 7 days before the interruption of the experiment to visualize neo-deposition of calcium salts.

In vivo experiments

Scaffold preparation and cell pre-culturing

A 250 mg Bio-Oss® Collagen block was cut into cylinders with a diameter of 6 mm and 1,5 mm thick (approximate volume 43 mm^2) which were loaded with GFP-positive stem cells.

Placental multipotent cells were infected with a green fluorescent protein carrying-lentivirus that allowed their recognition in host organism. Briefly, cells were cultured in Petri dishes (Falcon) in complete medium then treated with Polybrene (Hexadimethrine Bromide, Sigma) at a concentration of 8 μg/ml and incubated with lentiviral particles that carried, in addition to GFP sequence, a puromycin resistance cassette. After 48h the medium was replaced with fresh medium containing 5 μg/ml puromycin to select uninfected cells. After three days of selection cells were grown to confluence and harvested by trypsinization and resuspended in osteogenic medium. Bio-oss cylinders were plunged in osteogenic medium then GPF+-cells were dropped on them to allow their penetration and adhesion to the scaffold.

Surgery

Surgery was executed by Prof. Macaluso and Dr. Lumetti from the Unit of Periodontology of the University of Parma. For in vivo experiments three Wistar Han rats of about 470gr were anesthetized with medetomidine hydrochloride (Domitor, Pfizer Animal Health, USA), 0.015 mg/100g (0.07 ml) and ketaminum hydrochloride 1000, 0.04/100mg (0.2 ml) (Imalgene 1000) by intraperitoneal injection. The skull was shaved in the parietal zone and cleansed with povidon-iodine (Betadine). A single incision was made along the sagittal line of the skull from the line above eyes to occipital process. Skin was peeled off from calvaria, and tissue layers were dissected until reaching the periosteum where a critical size cranial defect was created. This consisted of two craniotomies on the two sides of sagittal suture which were executed with a trephine (Biomet 3i, USA) of 6 mm diameter mounted on a 20:1 scaler (Sirona) and a surgical micromotor (ATR 3000 Plus, Simit), taking care not to damage meninges. Surgery was performed at 1400 rpm under abundant irrigation with pre-cold saline solution. Bone chips were

detached from dura mater and were substituted with Bio-Oss® Collagen cylinders: the right side was filled with an empty one, while the left side was filled with a cylinder carried with placental cells. Cylinders were fitted to calvaria thickness in order to avoid brain compression after suture.

Rat 0 died during surgery, while rats 1 and 2 after successful surgery were sacrificed after 30 and 60 days respectively by a 75 µl dose of Domitor plus 200 µl of Imalgene 1000, followed by euthanasia by chloroform and CO2. Rat 1 weight at sacrifice was 501gr, while rat 2 weight was 475 gr. About 40 days post-interventum rat 2 was injected subcutaneously with calcein orange (5mg/kg, pH 7.5) in order to visualize neo-bone deposition.

Sample fixation and histological analysis

After sacrifice, implants were recovered and fixed by neutral buffered formalin 10% solution. Fixation was followed by dehydration, clarification, infiltration and paraffin inclusion. Embedded sample were then cut into 3 µm slices for staining. A routine hematoxylin-eosin stain was performed in order to identify cell structure.

New calcium deposition was highlighted by Alizarin red stain and, for rats 2 and 3, calcein as previously described. Moreover an immunohistochemical analysis was performed with a CY5-conjugated antibody against GFP (Santa Cruz Biotechnology, CA). Nuclear stain was performed with DAPI.

Materials

Endotoxin-free FBS and Media (DMEM, IMDM, αMEM) were purchased from Celbio. Becton Dickinson was the source of antibodies used in FACS analysis. Sigma-Aldrich Italy was the source of all other chemicals not otherwise specifically indicated.

Data analysis

Statistical analyses of data were performed using unpaired t test or one-way analysis of variance followed by Dunnett's Multiple Comparison Test. P<0.05 was considered significant.

Results and discussion

Part 1- Placenta as a source of multipotent cells

Introduction and aim

Placenta is a quite recent object of research in the field of regenerative medicine, so its cellular composition and gene expression profile are still greatly unknown. At present, however, a standardization concerning isolation and cultivation of placental cells is still lacking[24]: for this reason is quite hard to identify the specific region of sampling and culture conditions that would allow the comparison of work by different groups. So, a standard protocol for isolation of placental cells was drafted, including sampling, cultivation and in vitro differentiation of placenta-derived cells. The first target of the work was organ sampling in order to detect the presence of stemness markers and typical growth factors that could address subsequent work with the aim of figuring out the area which could give more consistent results.

Placenta sampling and cell isolation

Samples were taken from the peripheral or the central area of placenta and from amniotic membrane for histological analysis and for RNA extraction.

Samples for histological analysis were fixed and processed in the Section of Pathological Anatomy of the Hospital of Parma. Following images show hematoxylin-eosin stain of placental villi at different magnifications (fig.1).

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[24] Parolini O, Caruso M. Review: Preclinical studies on placenta-derived cells and amniotic membrane: an update. *Placenta* (2011) **32** (S2): S186-S195
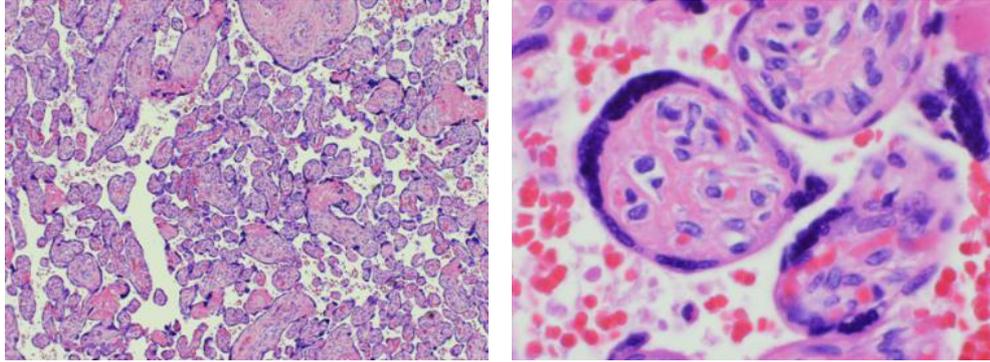
**Figure 1: Placental villi, hematoxylin-eosin stain, light microscopy,
4X (left) and 400X O.M. (right)**

Villi were further analysed with fluorescent antibody in order to visualize stemness markers (fig. 2-4).
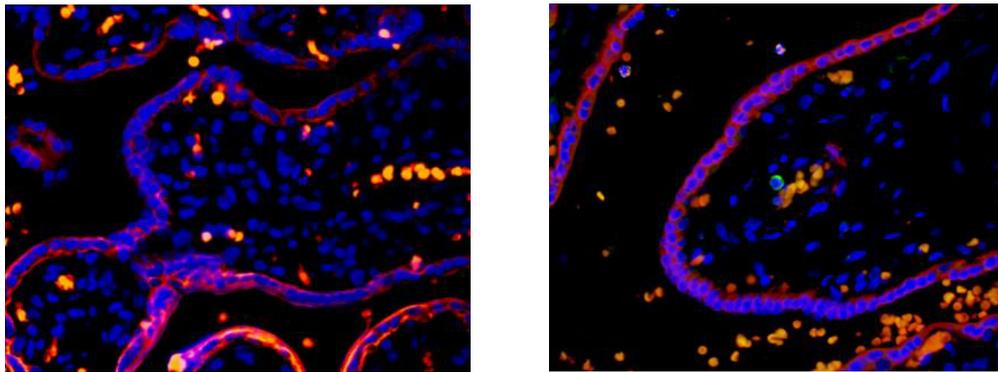
Figure 2: Cytotrophoblastic cells: CK18 (red) and nuclear (blue) stain, fluorescent microscopy, 40x O.M.
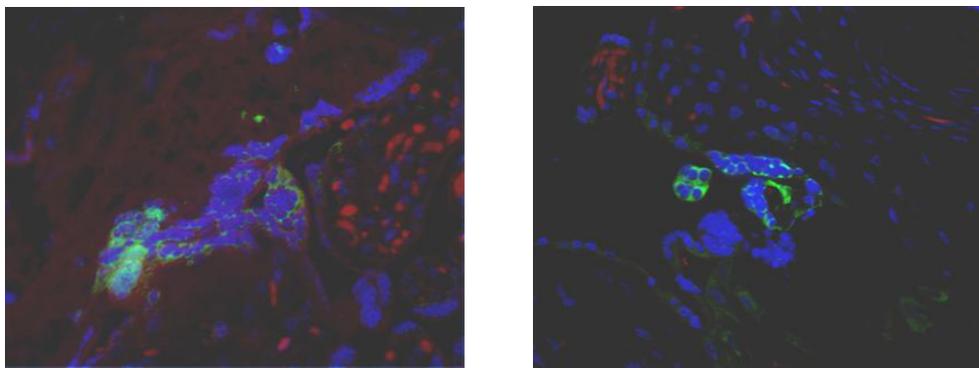
Figure 3: Transverse section of villi, C-Kit (green) and nuclear (blue) stain, fluorescent microscopy, 40x O.M.
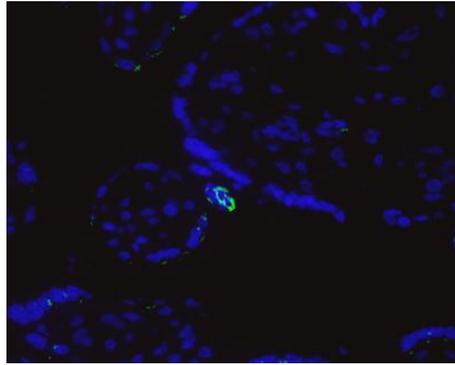
Figure 4: Transverse section of villi, C-Kit (green) and nuclear (blue) stain, fluorescent microscopy, 40x O.M.

These images reveal the presence of markers of an undifferentiated state and the presence of niches identified by C-Kit positive cells: their location is not only into blood filling villous microvessels, but, more interestingly, among parenchyma in strongly positive clusters. This gives a first proof of the presence of cells which hold stemness characteristics in placenta.

The presence of multipotent cells was further verified by qualitative PCR analysis of the extracted RNA. The choice of analyzed markers was based on the search for multipotency markers which are common to many differentiation lineages, including the hematopoietic and mesenchymal ones. We detected the presence of CD34, CD133, and CD117 in whole placental tissue, but with a different expression level among amnios, umbilical cord surrounding area and peripheral portion of placenta. In particular this latter area was the richest one in stemness markers as well as in growth factors involved in stem cells commitment (i.e. IGF-1 and HGF) and chemoattraction (i.e. SDF1). Results are summarized in the following table (table 7):

Group	Gene	Placental area		
		Peripheral villi	*Central villi*	*Amnios*
Hematopoietic progenitors	*CD34*	+ + +	+ +	+
Hematopoietic progenitors-vessels	*CD133*	+	+	+
Multipotent progenitors	*CD117/C-Kit*	+ + +	+ +	+/-
Growth factors for multipotent cells	*Igf-1*	+ + +	+ + +	-
	Hgf	+	+	+/-
	Sdf-1	+	+	-

Table 7: Summary of markers detected in placenta by PCR

These data support the hypothesis that stem cell niches actually reside in placenta, in particular in the peripheral area that, for this reason, was chosen as source of cells for further analysis.

Viable cells isolated after enzymatic digestion of tissue were analysed by FACS: this evidenced that CD117^{pos} cells were less than 1%, CD34^{pos} cells were roughly 1%, while CD90^{pos} cells and CD133^{pos} cells accounted for 3% of the analysed cells. Single cells stained positive for C-Kit and OCT3/4, the latter being in the typical nuclear localization pattern of the isoform detected in embryonic or pluripotent cells (fig. 5-6). Moreover cells stained positive for other typical mesenchymal and stemness markers (fig. 7-12).
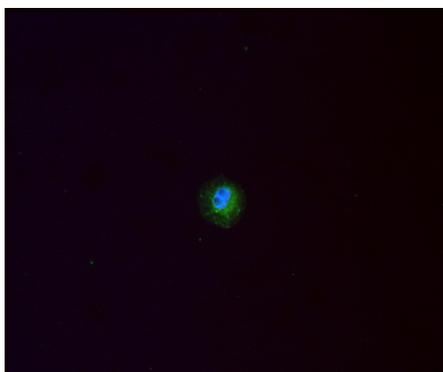
Figure 5: Isolated placental cell, , C-Kit (green) and nuclear (blue) stain, fluorescent microscopy, 40X O.M.
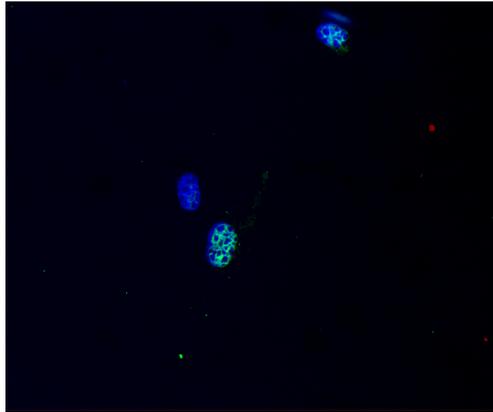
Figure 6: Isolated placental cell, OCT3/4 (green) and nuclear (blue) stain, fluorescent microscopy, 40X O.M.
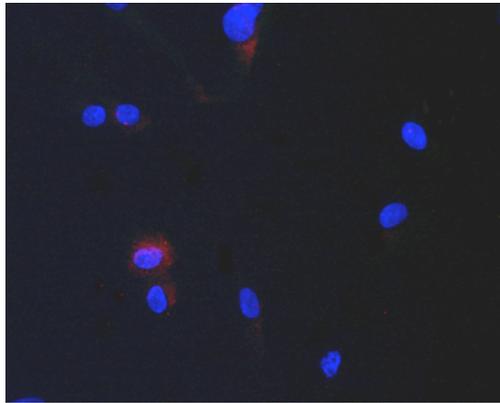
Figure 7: Isolated placental cell, CD34 (red) and nuclear (blue) stain, fluorescent microscopy, 40X O.M.
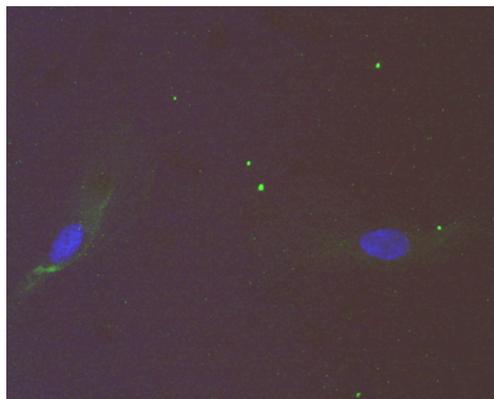
Figure 8: Isolated placental cells, ABCG2 (green) and nuclear (blue) stain, fluorescent microscopy, 40X O.M.
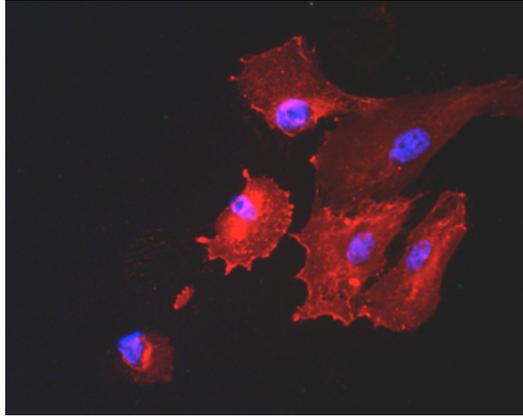
Figure 9: Isolated placental cells, CD44 (red) and nuclear (blue) stain, fluorescent microscopy, 40X O.M.
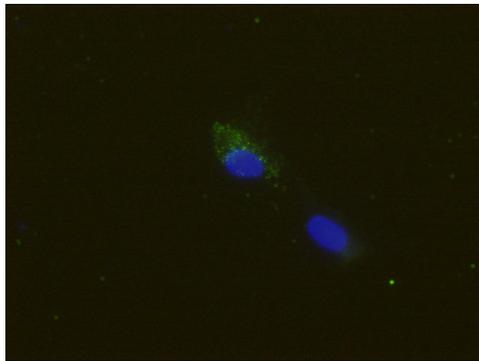
Figure 10: Isolated placental cell, TRA-1-81 (green) and nuclear (blue) stain, fluorescent microscopy, 40X O.M.
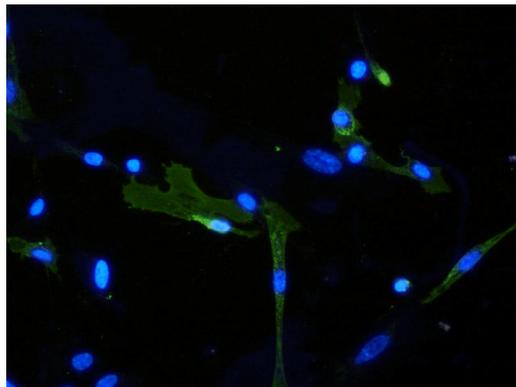
Figure 11: Placental cell, SSEA4 (green) and nuclear (blue) stain, fluorescent microscopy, 20X O.M.
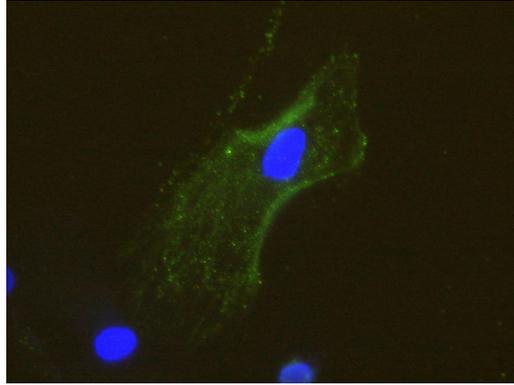
Figure 12: Isolated placental cell, TRA-1-60 (green) and nuclear (blue) stain, fluorescent microscopy, 40X O.M.

Cells further cultured onto plastic dishes gave origin to a mixed population, characterized by fibroblastoid cells together with more polygonal cells having an epithelial-like morphology. As subsequent isolation attempts consistently produced a heterogeneous cell population, a further step was added to enzymatic digestion, consisting of immunoselection of CD34 negative cells. This choice was based on the fact that CD34 is not only a marker of hematopoietic cells but it is also strongly expressed by endothelial cells of small vessels and capillaries, including villi microvessels (fig. 13).
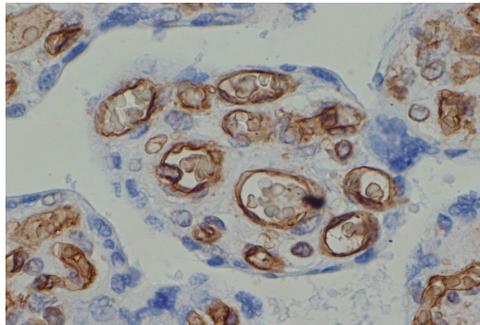
Figure 13:Transversal section of villi showing the location of CD34 by alkaline phosphatase stain, light microscopy, 4x O.M.

Thus, we were able to exclude any contamination deriving from vessels and blood, focusing our work on cells that were actually homed in placental parenchyma.

CD34 negative cells were allowed to adhere to uncoated plastic plates and, after 10-15 days in CM, fibroblast colony-forming units became evident. Those

were detached by trypsin/EDTA and expanded for further characterization and differentiation experiments.

Flow cytometric characterization

When cells were analysed by flow cytometry (fig. 14-16), cell population turned out to be bright positive (3-4 log units) for CD13 (99%), CD44 (95%), CD73 (95%), CD90 (99%), known to bind SH3 and SH4 antibodies; it was low/faint positive (2 log units) for CD49d (88%), CD71 (28%), CD105 (90%), CD146 (60%), known to bind SH2 antibody, HLA ABC (99%). Conversely, cells were negative for CD14, CD31, CD33, CD34, CD43, CD45, CD52, CD80, CD117 and HLA DR. The analysis by FACS showed a good correlation with RT-PCR analysis, which confirmed the lack of expression for CD34, CD117, CD133, CD271 (a mesenchymal cell marker in bone marrow) genes as well as CD14, as previously described by other authors on mesenchymal stem cells[25]. Generally, CD49d is not included in a typical MSC marker panel. In fact, it was detected in only 14% of mesenchymal cells from adipose tissue while it was absent in MSC isolated from bone marrow[26]: however its presence was documented in other MSCs as well as in our cells. On the whole, FACS analysis reasonably allowed us to state that the isolated cell population belongs to the mesenchymal lineage with no contamination by endothelial, hematopoietic or monocytic cells. Moreover it is worth noting that the absence of class II major histocompatibiliy complex molecules suggests a low immunogenicity of these cells, in view of a possible xenogenic graft. Unfortunately, this panel of surface markers by itself is not sufficient for the identification or definition of MSCs, as also skin fibroblasts show almost the same pattern of expression. In order to clearly classify these cells as MSCs we had to carry on with the analysis of their clonogenic potential and plasticity.

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[25] Wagner W, Wein F, Seckinger A, Frankhauser M, Wirkner U, Blake J, Schwager C, Eckstein V, Ansorge W, Ho AD. Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Experimental hematology* (2005) **33** 1402-1416

[26] Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, Alfonso ZC, Fraser JK, Benhaim P, Hedrick MH. Human adipose tissue is a source of multipotent stem cells. *Mol. Biol. Cell.* (2002) **13** (12): 4279-95
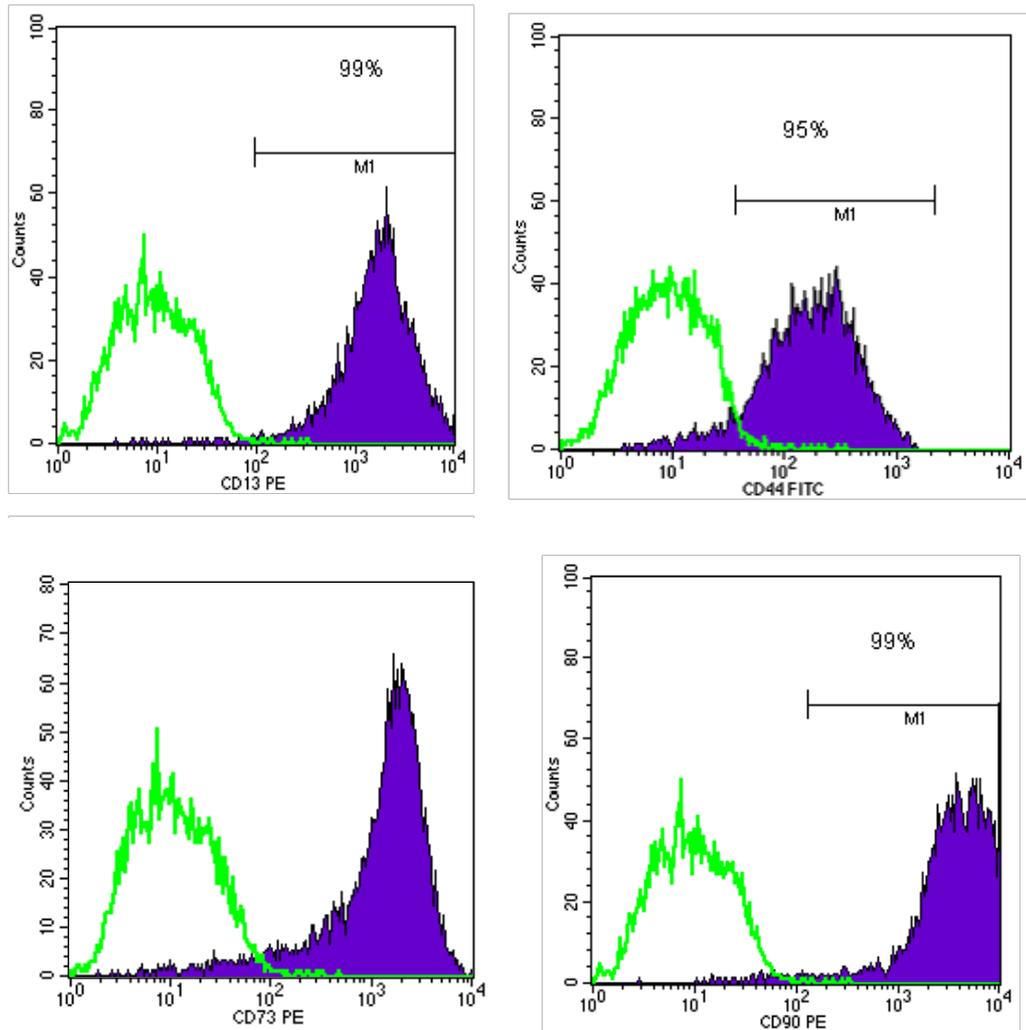
Figure 14: Cytofluorimetric analysis of placental cells, bright positive markers
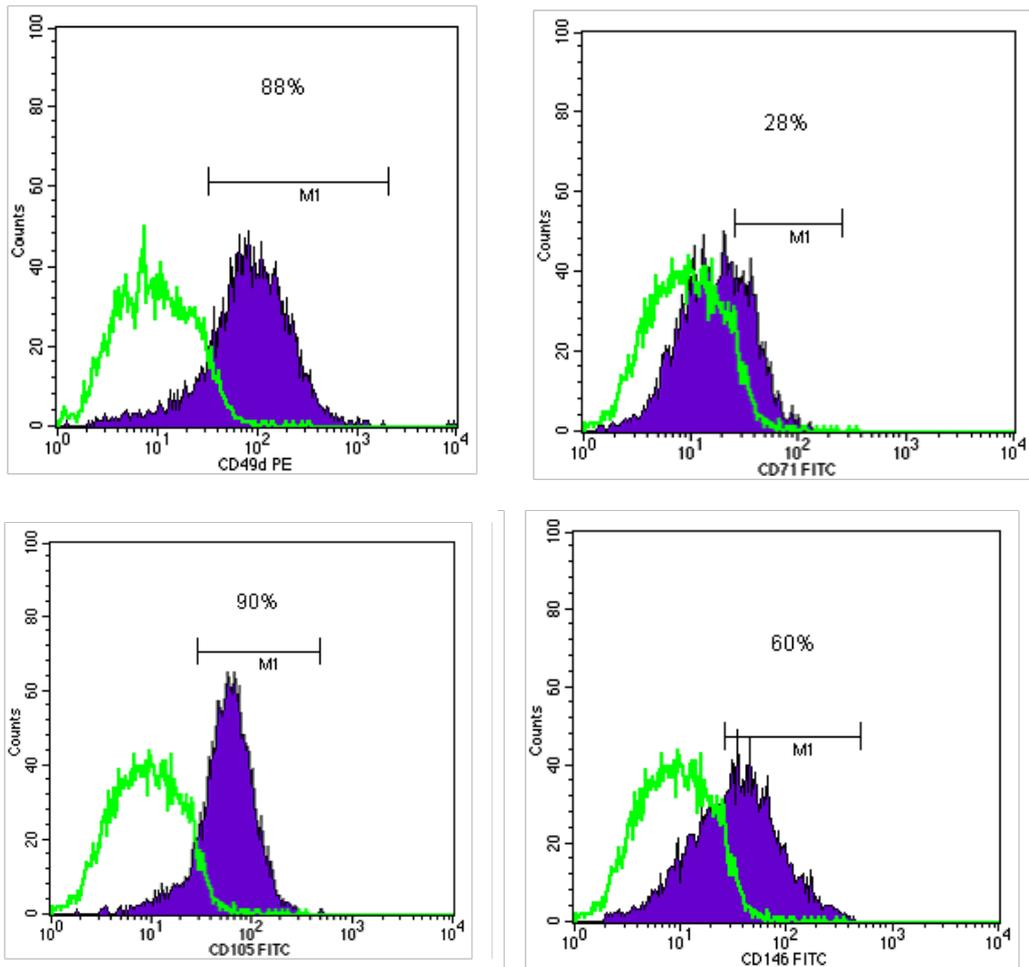
Figure 15: Cytofluorimetric analysis of placental cells, low positive markers
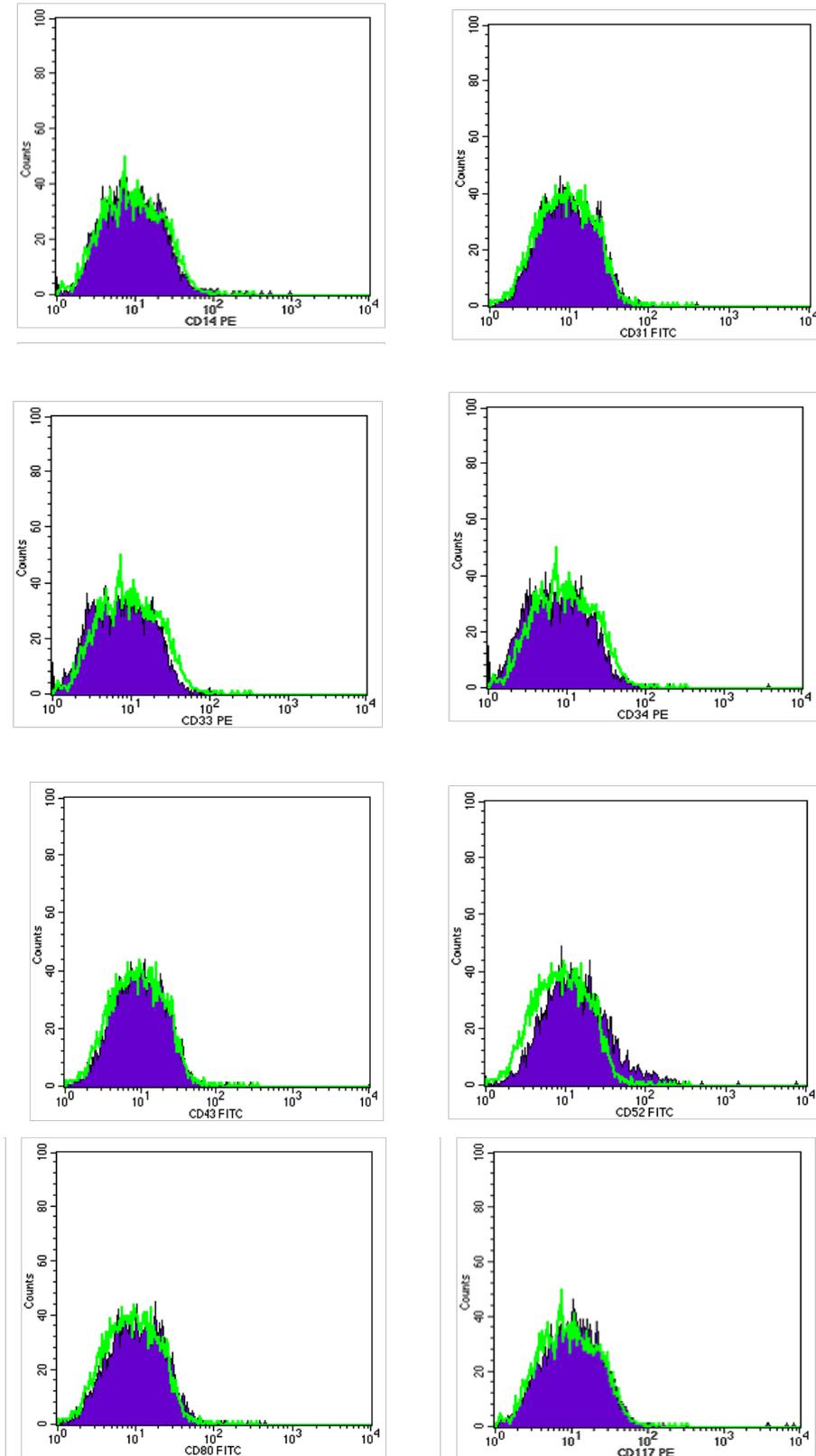
Figure 16: Cytofluorimetric analysis of placental cells, negative markers

Cloning assay and proliferation

The cell population isolated was then seeded at clonal density on 96-wells plates in order to test the presence of colony forming units. After 21 days, colonies were observed in 10% of seeded cells, thus confirming clonal properties of mesenchymal placental cells that seem far above those of bone marrow-derived MSCs.

Moreover the proliferation rate was calculated by regularly counting placental cells grown in complete medium (fig. 17).
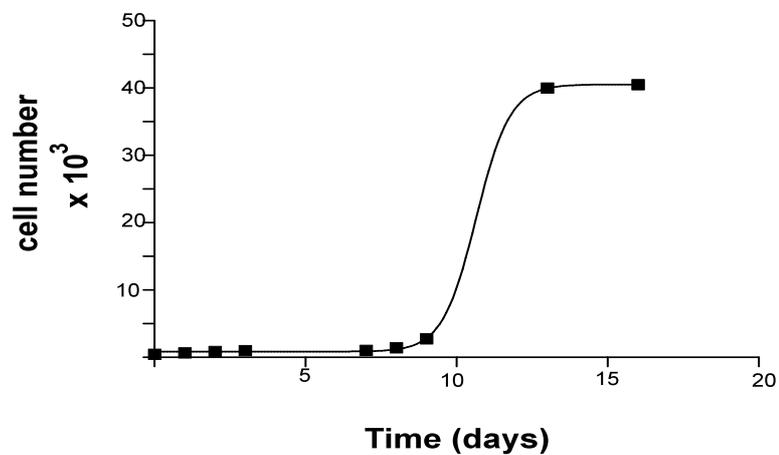
Figure 17: Proliferation rate of placental cells

After a lag phase, cells showed an exponential growth that reached a plateau after 13 days, owing to the reaching of confluence. The analysis of the proliferation rate during exponential growth allowed the assessment of the doubling time which was 23±2,8 hours (fig. 18).
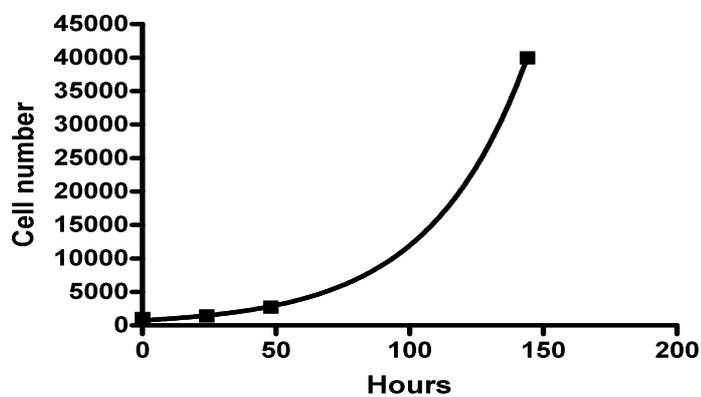
Figure 18: Exponential phase growth rate of placental cells

Isolated cells were also tested for the ability to produce teratomas by injecting them into SCID mice. No signs of teratoma formation were observed at time of sacrifice (data not shown).

Given this starting results, we went further with the analysis of cell plasticity, testing the differentiation potential of placental cells.

Adipogenic differentiation

The formation of adipose tissue by in vitro differentiation of MSCs would constitute an appealing strategy for the repair of soft tissue, as, until now, the use of void-filling material or autologous grafts do not satisfy all clinical needs.

Having this in mind, placental derived cells were subjected to an adipogenic differentiation protocol. As cell density is pivotal for differentiation[27], cells were seeded in 6-well plates at a density of 100.000 cells/well and grown to confluence in CM. After reaching confluence, medium was switched to an adipogenic one, including a combination of 3-isobutyl-1-methyl-xanthine (IBMX), insulin, indomethacin and dexamethasone[28].

IBMX is a cAMP-elevating agent that enhances *PPARγ2* and *LPL* expression and downregulates the expression of osteogenic marker genes. It has a positive role in terminal differentiation of adipocytes.

Insulin is one of the most potent adipogenesis-inducing drugs. It increases and accelerates triglyceride accumulation and its role can be enhanced by the presence of antidiabetic drugs such as thiazolidinediones which stimulate *PPARγ*.

Indomethacin is a non-steroidal anti-inflammatory drug which promotes MSCs commitment to the adipogenic lineage by directly binding to PPARγ receptors, thus acting as a PPARγ agonist.

Serum concentration can have deleterious effects on adipogenic differentiation, by markedly and irreversibly reducing the capability for an adipogenic conversion to mature adipocytes. This is probably due the presence of

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[27] McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Developmental Cell* (2004) **6**: 493-5

[28] Janderova L, McNeil M, Murrell AN, Mynatt RL, Smith SR. Human mesenchymal stem cells as an in vitro model for human adipogenesis. *Obesity research* (2003) **11** 65-74

TNF-α or TGF-β in it. For this reason a 5% serum concentration was adopted in order to reduce this side-effect.

Adipogenic induction protocol lasted 4 weeks, during which lipid droplets appeared an kept widening, as a clear sign of differentiation. This was pointed out by Oil-red-O stain which helps the visualization of lipid droplets deposition (fig. 19)
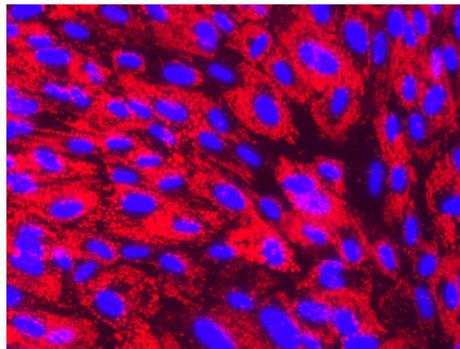
Figure 19: Lipid droplet deposition in placental mesenchymal cells, Oil-red-O stain and DAPI nuclear stain, fluorescent microscopy, 20x O.M.

After nucleic acid extraction, mRNA was analysed by semi-quantitative PCR, in order to support the morphological datum concerning differentiation. Actually adipogenic medium (AM) induced a consistent increase in the expression of typical adipocyte markers, such as *FABP4* (CM: 1±0,01; AM: 23,58±0,86), *GLUT4* (CM: 1±0,02; AM: 7,99±0,04), *LPL* (CM: 1±0,02; AM: 12,77±0,06) and *LEP* (CM: 1±0,02; AM: 9,26±0,04) (fig. 20)
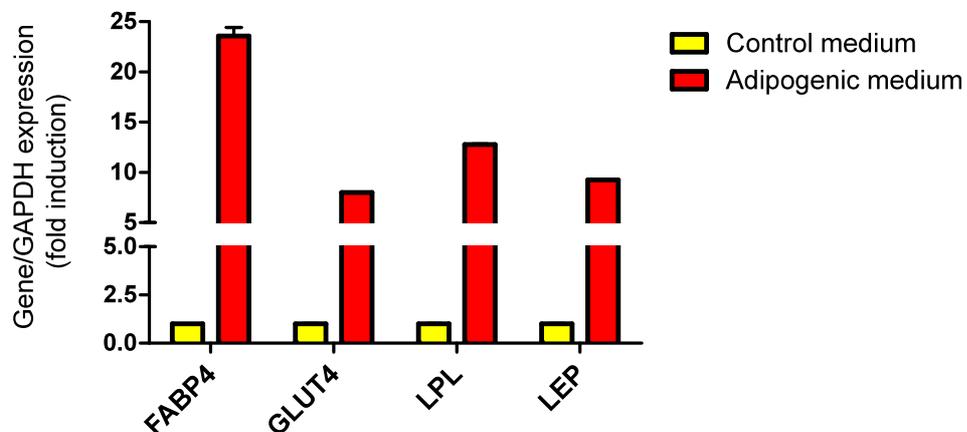
Figure 20: Real Time-PCR analysis of human placental cells- adipogenic differentiation

These results constitute a further milestone in the definition of placental cells plasticity, which will be definitely confirmed by experiments described below.

Summary

The first part of this study reports a protocol for the isolation of a homogeneous MSC population from full term human placenta. Such result is achieved by sorting CD34-negative cells that are successively selected on their ability to adhere and divide in presence of serum on uncoated plastic dishes. When we isolated cells only by plastic adherence we observed a population far to be homogeneous and residual CD34^{pos} cells were still present after 15 population doublings.

Placental mesenchymal cells express almost the same surface markers found on adult MSCs such as bone marrow, adipose or pancreas derived cells. When cells were seeded at clonal density on 96 wells plate the 10% of them gave rise to clones, so MSCs from placenta exhibit clonal proprieties. This is very interesting as this value highly exceeds that referred to bone marrow-derived MSCs which account for about 0.01%.

As far as their differentiation potential, placental stromal cells were able to differentiate into fat tissue, as shown by Real Time PCR results and lipid droplet stain. Moreover these cells possess the ability to differentiate into the osteochondrogenic lineage as will be discussed right below. For these reasons from here on the denomination of human chorionic multipotent stromal cells (hCMSCs) will be adopted referring to this selected population.

Part 2-Cartilage differentiation

Introduction and aim

Cartilage is a tissue that can be found on the articular surface of long bones, in trachea, bronchi, nose, ears, larynx and intervertebral disks. It is an avascular tissue which is constituted by two main components: the collagen- and proteoglycan-rich extracellular matrix and a population of isolated chondrocytes lying within this matrix. Three types of cartilage can be distinguished: hyaline cartilage, elastic cartilage and fibrocartilage[29,30].

Elastic cartilage is present in ears, epiglottis and parts of the larynx. Its main function is ensuring the patency of the lumina of tubes it surrounds.

Fibrocartilage can be found in intervertebral discs, symphysis pubis and junction between large tendons and articular cartilage in large joints.

Hyaline cartilage has a glass-like appearance and is the most abundant type of cartilage, mainly lining the ends of articulating bones. In its articular location, it serves the critical function of permitting movement of one bone against another, thanks to its low friction and capacity to bear load. It is composed of specialized proteins and macromolecules that determine its unique mechanical properties. Though articular cartilage is a metabolically active tissue, the rate and efficiency of turnover is quite peculiar, as not all molecular components are reconstituted at the same rate, and variations exist based on the spatial location within the tissue. This is a quite serious problem considering that articular cartilage undergoes consistent wear throughout lifetime and its complete functionality cannot be restored, resulting in significant pain and reduction and loss of mobility, finally leading to a serious decrease in quality of life and high medical costs[31].

The cellular part of cartilage is constituted by chondrocytes, a population of mesodermal origin which undergoes a series of phenotypic changes throughout its maturation process that converts these cells from articular to hypertrophic

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[29] Meyer U, Wiesmann HP. *Bone and cartilage engineering.* (2006) Springer

[30] Athanasiou KA, Darling EM, Hu JC. *Articular cartilage tissue engineering.* (2010) Morgan and Claypool publishers

[31] Sabatini M, Pastoreau P, De Ceuninck F. *Cartilage and osteoarthritis vol. 1.* (2004) Humana Press

chondrocytes. Their role changes with their maturation as well: chondrocyte precursors cells have a general fibroblastic appearance and synthesize type I and III collagen, fibronectin and noncartilage-type proteoglycans; articular chondrocytes produce a matrix which is endowed with tensile strength and flexibility in which they become buried, thus conferring them a spherical shape with scalloped edges; as they become hypertrophic chondrocytes they increase their size and synthesize a matrix that can undergo mineralization and constitutes the basis for endochondral ossification.

By a molecular point of view chondrocyte maturation can be identified by a switch in gene transcription: actually articular chondrocytes mainly express collagen types II, IX and XI, as well as aggrecan. Hypertrophic chondrocytes have collagen type X as an exclusive marker along with alkaline phosphatase, collagen types II and IX and aggrecan.

Mature articular cartilage is composed primarily of water, approximately 70-80% by weight. The solid fraction of the tissue is primarily collagens (50-75%) and proteoglycans (15-30%), with the remaining balance including minor protein molecules and chondrocytes. This mix of collagens and proteoglycans form an integrated network that provides the basis for the mechanical properties observed in articular cartilage.

Collagens serve a primary role in the structure of connective tissues throughout the body. Collagen type II is the predominant collagen type in articular cartilage, comprising over half the dry weight of the tissue. Collagen fibre orientation varies through the depth of articular cartilage. Hyaline cartilage also contains other fibrillar and globular collagen types, such as types V, VI, IX, and XI but the definitive roles of these other collagen types are not fully known: they are believed to play a role in intermolecular interactions as well as modulating the structure of collagen type II.

Collagen type X, found primarily in the zone of calcified cartilage, appears to play a role in cartilage mineralization at the interface between cartilage and the underlying bone.

Proteoglycans are large macromolecules comprised of a protein core with attached polysaccharide chains (glycosaminoglycans). The primary proteoglycan

in articular cartilage is aggrecan, which consists of a hyaluronan core with numerous glycosaminoglycan side chains. In mature articular cartilage, the dominant polysaccharides in this macromolecule are chondroitin and keratan sulfates. The protein core contains several distinct globular and extended domains where glycosaminoglycans attach. The conglomeration of many proteoglycans into large macromolecules is critical for proper functionality of cartilage tissue. Proteoglycan networks in articular cartilage can be thought of as a mesh that is interlaced throughout, within the more organized collagen structure. Aggrecan molecules are bound to a single, long chain of hyaluronan to form large proteoglycan aggregates, which result in an overall molecular weight of 50-100 $\times$ 10^6 Da. The large size of this polymer mesh acts to immobilize and restrain it within the collagen network. The presence of carboxyl and sulphate groups gives proteoglycans a negative charge, which in turn gives cartilage extracellular matrix a net negative charge known as a "fixed charge density". Because of this charge, the matrix imbibes fluid, swelling the tissue to maintain equilibrium. The swelling is balanced against the elastic restraint of the collagen network.

Other proteoglycans within articular cartilage include biglycan, decorin, and fibromodulin, which also are comprised of core proteins with various glycosaminoglycan species attached as side chains.

In addition to the proteoglycans and collagens, articular cartilage also contains a small fraction of non-collagenous proteins. These include fibronectin, cartilage oligomeric protein, thrombospondin, tenascin, matrix-GLA (glycine-leucine-alanine) protein, chondrocalcin, and superficial zone protein.

Matrix structure and composition varies with respect to distance from chondrocytes resulting in a microscale territorial structure in which a pericellular and an interterritorial matrix are defined as far as the distance from cells grows. The pericellular matrix is characterized by having fine collagen fibres, high concentrations of proteoglycans, and the presence of fibronectin and collagen type VI. Its role is probably to protect the physical integrity of articular chondrocytes during compressive loading. The interterritorial matrix contains large collagen type IV fibres and varying concentrations of proteoglycans, comprises the bulk of articular cartilage, providing the tissue with its mechanical properties. Loading of

articular cartilage involves force transmission through the interterritorial, territorial, and pericellular matrices before reaching the chondrocytes.

At an upper level, hyaline cartilage becomes organized according to chondrocyte differentiation state, thus resulting in a zonal structure that varies from the surface of the tissue to bone. Different regions (superficial/tangential, middle/transitional, deep/radial, calcified) can be identified by extracellular matrix structure and composition, as well as cell shape and arrangement within the tissue. The superficial, or tangential, zone of articular cartilage is characterized by having small diameter, densely packed collagen fibres that are oriented parallel to the cartilage surface. The matrix has a relatively low proteoglycan content as well as low permeability. Cells in this layer are densely packed and exhibit flattened, discoidal shapes and secrete specialized proteins that are hypothesized to facilitate the wear and frictional properties of the tissue. In the middle, or transitional, zone the collagen fibres exhibit an arcade-like structure interspersed with randomly oriented fibres. Proteoglycan content reaches its maximum in the middle zone. The cell density is much lower in this region than the superficial zone, and the cells themselves are more spherical in shape. The deep zone is the last region of purely-hyaline tissue before reaching bone. Its collagen structure is characterized by large fibres that form bundles oriented perpendicular to the articular surface and are anchored in the underlying subchondral bone. Proteoglycan content is much lower than in the middle zone, and the cell density is also the lowest of the three cartilaginous zones. Cells in the deep zone often group together in a columnar organization. They are slightly elongated and oriented in the direction of collagen fibres, perpendicular to the articular surface. A thin line termed the "tidemark" is present between the deep zone and calcified zone of articular cartilage. The calcified zone is a region of the tissue that transitions into the subchondral bone, minimizing the stiffness gradient between the rigid bone and the more pliable cartilage. Underlying this region of the cartilage is the subchondral bone, which is the ultimate anchorage point for cartilage tissue as a whole.

Chondrocyte cultures remain one of the most powerful tools for investigating intracellular and molecular events associated with chondrocyte differentiation and activation. However, chondrocytes are known to lose their phenotypic markers in vitro and dedifferentiate into fibroblasts. Factors associated with an increased tendency toward dedifferentiation include low-density plating, monolayer culturing, treatment with proinflammatory cytokines, such as interleukin-1 (IL-1), or extraction from human adult cartilage. Conversion from the chondrocyte to the fibroblast phenotype can be detected based on a switch from collagen type II or type X to collagen types I and III, and from high-molecular-weight (aggrecan) to low-molecular-weight proteoglycans (biglycan and decorin). Many efforts were made in order to minimize dedifferentiation, in particular by creating a 3D environment for chondrocyte growth such as alginate culture and use of scaffolds.

Despite many attempts, in vitro production of functional hyaline cartilage still has to deal with quantitative and qualitative challenges which makes this quite far from becoming reality: in this contest the search of an alternative source of mesenchymal cells, as placenta can be, still retains its charm.

Results and discussion

Preliminary experiments on placental stromal cells were designed for classical monolayer culture, giving as a result a small increase in the expression of chondrogenic genes (data not shown). From a morphological point of view cell organization didn't go beyond the formation of zones with high cellular concentration that edged empty areas (fig. 21).
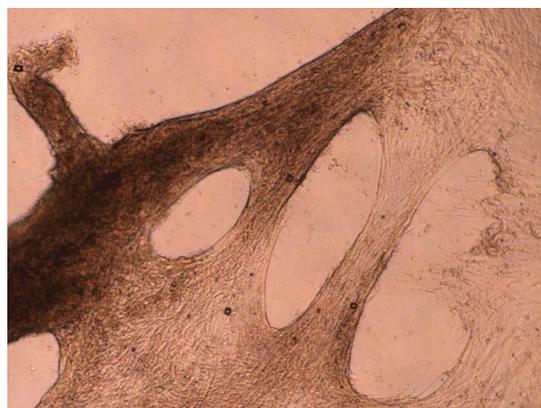
Figure 21: hCMSCs, 28 days chondrogenic differentiation 4x O.M

The use of chondrogenic bioactive factors is just one of the two principles involved in enhancing chondrogenic differentiation, being the second high-density growth which guarantees close cell-to-cell contact. For this reason the protocol by Mackay et al. was adopted as described in experimental procedures.

The choice of tridimensional culture versus traditional culture in monolayer produced a significant increased in the expression of typical cartilage genes, as reported also from Bosnakovski et al[32].

As for medium composition, its formulation was revisited through a series of experiments, which are resumed below, in order to detect the most suitable medium for the differentiation of placental stromal cells.

Sodium pyruvate was added as a further energy source on a basis of high glucose DMEM: this last medium was preferred to DMEM normal glucose on the basis of a preliminary experiment in which differentiation medium, containing the same inducing factors, was prepared with normal or high glucose DMEM. Cells grown in pellets couldn't survive culture in normal glucose DMEM, while cells in high glucose formed rounded aggregates and preserved a good viability. This choice was further supported by literature data[33] which report that, as a major energy substrate and precursor for the synthesis of glycosaminoglycans and extracellular matrix, glucose is an inducer of chondrogenesis. The transport of glucose through the articular cartilage and into chondrocytes is essential for the growth, development, maintenance and structural integrity of articulating joints. Actually, in a model of mesenchymal chick cells, a proportional increase in size of micromass nodules was observed in correspondence with increasing levels of glucose; glucose induced the stimulation of sulphated proteoglycan accumulation and cartilage nodule formation for chondrogenic differentiation. Such mechanism is probably mediated by PKC signalling pathway and leads to the down-regulation

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[32] Bosnakovski D, Mizuno M, Kim G, Takagi S, Okumura M, Fujinaga T. Chondrogenic differentiation of bovine bone marrow stem cells (MSCs) in different hydrogels: influence of collagen type II extracellulare matrix on MSC chondrogenesis. *Biotechnology and Bioengineering* (2006) **93** (6): 1152-1163

[33] Han YS, Bang OS, Jin EJ, Park JH, Sonn JK, Kang SS. High dose of glucose promotes chondrogenesis via PKCa and MAPK signalling pathways in chick mesenchymal cells. *Cell Tissue Res* (2004) **318**: 571-578

of ERK and to the phosphorilation of p38 which affects the expression of cell adhesion molecules thus favouring pre-cartilage condensation. Moreover this phosphorilation leads to *SOX9* mRNA stabilization and post-transcriptionally regulation, thus favouring chondrogenic differentiation[34,35].

Further components were always kept in inductive medium, as they were reported to be indispensable for differentiation. Among these were dexamethasone, ascorbic acid, L-proline and TGF-β.

Dexamethasone was added to medium considering its role in the induction of *COL2A1* and *SOX9*[36,37]. Moreover it promotes the expression of cartilage extracellular matrix genes and enhances TGF-β-mediated up-regulation of their expression[38]. Dexamethasone is indispensable until a typical cartilage matrix is acquired[39]. A 100 nM dose was considered suitable to induce chondrogenic differentiation.

L-ascorbic acid 2-phosphate causes collagen hydroxylation via modification of proline and lysine residues: for this reason it was added to the medium, together with L-proline, in order to give suitable substrates for type II collagen synthesis.

TGF-β plays a role in all phases of chondrogenesis, meaning mesenchymal condensation, chondrocyte proliferation, extracellular matrix deposition and

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[34] Tew SR, Hardingham TE. Regulation of SOX9 mRNA in human articular chondrocytes involving p38 MAPK activation and mRNA stabilization. *The journal of biological chemistry* (2006) **281**, 39471-39479

[35] Tew SR, Peffers MJ, McKay TR, Lowe E, Khan WS, Hardingham TE, Clegg PD. Hyperosmolarity regulates SOX9 mRNA post transcriptionally in human articular chondrocytes. *Am J Physiol Cell Physiol* (2009) **297** (4) C898-C906

[36] Oshina H, Sotome S, Yoshii T, Torigoe I, Sugata Y, Maehara H, Marukawa E, Omura K, Shinomiya K. *Effects of continuous dexamethasone treatment on differentiation capabilities od bone marrow-derived mesenchymal cells.* Bone (2007) **41** 575-583

[37] Sekiya I, Koopman P, Tsuji K, Mertin S, Harley V, Yamada Y, Shinomiya K, Nifuji A, Noda M. Dexamethasone enhances SOX9 expression in chondrocytes. *Journal of Endocrinology* (2001) **169**, 573-579

[38] Derfoul A, Perkins GL, Hall DJ, Tuan RS. Glucocorticoids promote chondrogenic differentiation of adult human mesenchymal stem cells by enhancing expression of cartilage extracellular matrix genes. *Stem cells* (2006) **24** 1487-1495

[39] Locker M, Kellermann O, Boucquey M, Khun H, Huerre M, Poliard A. Paracrine and autocrine signals promoting full chondrogenic differentiation of a mesoblastic cell line. *Journal of bone and mineral research* (2004) **19** (1): 100-110

finally terminal differentiation. TGF-β is the key initiator of chondrogenesis by mesenchymal precursor cells and has a positive role in cartilage growth and maturation[40]. At the same time it inhibits chondrocyte terminal differentiation, thus preventing cartilage ossification. Moreover TGF-β directly controls *Sox9* expression[41]. Different TGF-β isoforms show slightly different effects during development, in a time-dependent manner[42, 43]. The choice of isoform to be used in our experiments was based on some literature data in which TGF-β3 has been shown to be more pro-chondrogenic compared to TGF-β1, having the ability to promote a greater accumulation of glycosaminoglycans and earlier and more extensive deposition of type I collagen[44,45]. As a 2 ng/ml dose is considered the lowest active dose for differentiation, a 10 ng/ml concentration was adopted for more consistent results.

IGF-1 stimulates MSCs proliferation, induces the expression of chondrocyte markers, increases the synthesis of matrix proteins, including collagen type II and proteoglycans and promotes the survival, development and maturation of chondrocytes in vitro. Contrasting data exist in literature concerning the effective role of IGF-1 in chondrogenic differentiation, but, for sure, it plays an important

[40] Van Der Kraan PM, Blaney Davidson EN, Blom A, Van Der Berg WB. TGF--beta signalling in chondrocyte terminal differentiation and osteoarthritis. *Osteoarthritis and cartilage* (2009) **17** (12): 1539-45

[41] Kawakami Y, Rodriguez-Leon J, Izpisua Belmonte JC. The role of TGF-βs and Sox9 during limb chondorgenesis. *Current opinion in cell biology* (2006) **18**: 723-729

[42] Chimal-Monroy J, Diaz de Leon L. Differential effects of transforming growth factors β1, β2, β3, β5 on chondrogenesis in mouse limb bud mesenchymal cells. *Int. J. Dev. Biol.* (1997) **41**: 91-102

[43] Cals FLJ, Hellingman C A, Koevoet W, Baatenburg de Jong RJ, van Osch GJV. Effects of transforming growth factor-β subtypes on *in vitro* cartilage production and mineralization of human bone marrow stromal-derived mesenchymal stem cells. *Journal of Tissue Engineering and Regenerative Medicine* (2011)

[44] Barry F, Boynton RE, Liu B, Murphy JM. Chondrogenic differentiation of mesenchymal stem cells from bone marrow: differentiation dependent gene expression of matrix component. *Experimental cell research* (2001) **268**: 189-200

[45] Mohan N, Nair PD, Tabata Y. A 3D biodegradable protein based matrix for cartilage tissue engineering and stem cell differentiation to cartilage. *J. Mater. Sci: Mater Med* (2009) **20**: S49-S60

role in chondrocyte hypertrophy. According to Indrawattana et al[46], IGF-1 is capable of inducing chondrogenic differentiation on a level comparable to TGF-β and Matsuda et al.[47] reported the combination of TGF-β, dexamethasone and IGF-1 as the most effective cocktail to stimulate differentiation of MSCs to chondrocytes. For the differentiation of hCMSCs, a first formulation without IGF-1 was afterwards substituted with one containing IGF-1, leading to a significant increase in the dimensions of cell pellets.

Usually, chondrogenic differentiation of MSCs is performed in serum-free media in order to reduce serum-induced apoptosis. In experiments with hCMSCs serum couldn't be eliminated, as this lead to a sudden decrease in cell viability in 3D cultures that was testified by the failure to generate strongly adhering cell masses and severe cell suffering that couldn't be fixed even by taking cells back to monolayer culture in maintenance medium containing serum. Moreover, the use of serum substitutes such as Knockout® serum replacement (Invitrogen) wasn't suitable for this kind of culture, as it couldn't sustain cell viability as well. This was testified by the fact that cells which were collected on the bottom of a polypropylene tube for pellet culture failed to form a compact rounded structure that usually appears 24-48 hours after seeding in appropriate conditions. For this reason a low quantity of serum was kept in order to sustain cell viability: a concentration of 1% serum was adopted that shouldn't compete with dexamethasone action and shouldn't induce apoptosis.

The final formulation of medium was evaluated in a time course experiment, in order to follow the variation of gene expression with time. Cells were grown in pellet culture and RNA was extracted after 7, 14 or 28 days of induction. The following image groups Real Time PCR results concerning the expression of

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[46] Indrawattana N, Chen G, Tadokoro M, Shann LH, Ohgushi H, Tateishi T, et al. Growth factor combination for chondrogenic induction from human mesenchymal stem cells. *Bioche Biophys Res Commun* (2004) **320** 914-9

[47] Matsuda C, Takagi M, Hattori T, Wakitani S, Yoshida T. Differentiation of human bone marrow mesenchymal stem cells to chondrocytes for construction of three-dimensional cartilage tissue. *Cytotechnology* (2005) **47**: 11-7

some of the typical chondrogenic genes[48]: for each gene, samples analysed are referred to the respective sample grown in control medium (fig. 22).
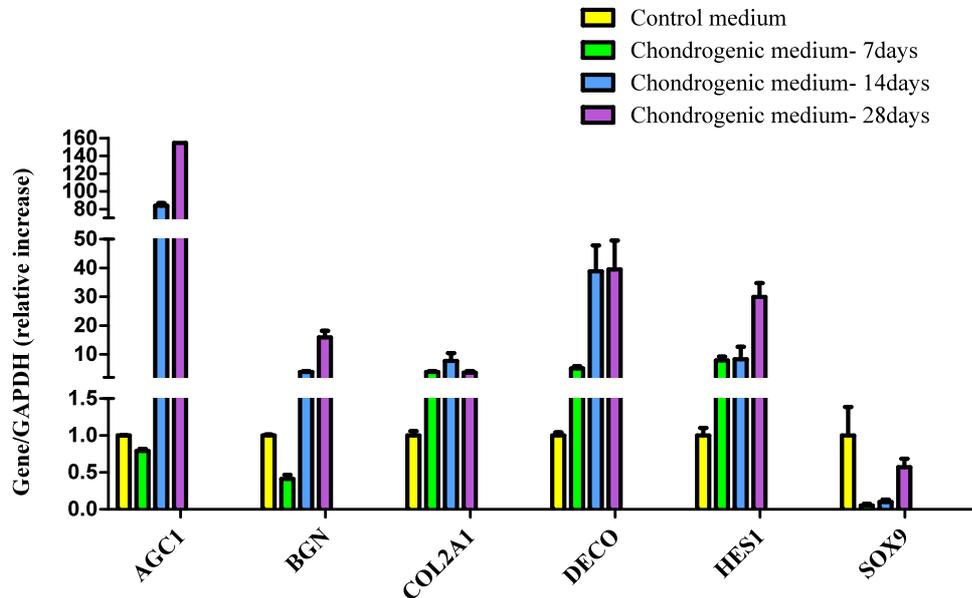
Figure 22: Real-Time PCR analysis of hCMSCs- chondrogenic differentiation, time course

In general, chondrogenic medium was able to induce the expression of all cartilage markers analysed. As far as biglycan (*BGN*), its expression was strongly induced by differentiating conditions, reaching a 15-fold of increase if compared to control medium culture condition: this suggests that the differentiation pathway had actually begun, as this is considered an early gene in chondrocyte maturation. *Decorin* too is an early gene in cartilage differentiation and its expression was induced as well during the 28-days differentiation protocol, reaching a 40-fold induction compared to control. Decorin shares with biglycan a close structural relationship that, however, doesn't seem to correspond to an overlapping function[49]. Decorin was shown to bind to collagen fibrils in vitro thus delaying their association: this is further confirmed by a mouse model in which decorin gene was deleted that showed fragile skin and collagen fibres of irregular and

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[48] Lefebvre V, Smits P. Transcriptional control of chondrocyte fate and differentiation. *Birth defects research. Part C* (2005) **75**: 200-212

[49] Siebel J, Robins Sp, Bilezikian JP. Dynamics of bone and cartilage metabolism. (2006) Academic Press

abnormal diameter. In contrast, biglycan appears not to affect collagen fibril formation, lacking a strong affinity for it. Mice lacking biglycan result in a phenotype with shorter long bones and lower peak bone mass, leading to an osteoporotic-like condition. Anyway both genes take part to cartilage metabolism, in particular they seem to be involved in an effort to compensate proteoglycan loss in osteoarthritis[50]. The high expression rate of these molecules at the end of our differentiation protocol suggests an active role in matrix deposition and organization, supporting the hypothesis of an active chondrogenesis occurring. These small proteoglycans are substituted, as differentiation progresses, by great proteoglycans, such as aggrecan (*AGC*) whose expression was induced by culture condition. This suggests that the maturation stage achieved by placental multipotent cells could be at least that of early chondroblasts[51]. This is further supported by the fact that even type II collagen gene expression was strongly induced after 28 days of differentiation.

As far as transcription factors are concerned, *HES1* expression was induced about 30-fold with respect to control condition. This is a Notch effector that, together with *HES5*, has different functions during chondrogenesis. It has been shown that *NOTCH1* is expressed in the initial stage of chondrogenic development in mice. HES1 decreases the expression of hyaline cartilage markers like collagen type II and aggrecan. Karlsson et al[52] suggested that "the significant increase in *HES1* expression during differentiation of articular chondrocytes in the pellet mass system might be important to balance the action of positive transcription factors promoting collagen type II and aggrecan expression. This thus allows differentiation to be properly controlled." This could be the same even in our case, also considering that appropriate Notch pathway signalling is

[50] Bock HC, Michaeli P, Bode C, Schultz W, Kresse H, Herken R, Miosgue N. The small proteoglycans decorin andbiglycan in human articular cartilage of late-stage osteoarthritis. *Osteoarthritis and cartilage* (2001) **9** (7): 654-663

[51] Lefebvre V, Smits P. Transcriptional control of chondrocyte fate and differentiation. *Birth Defects Res C Embryo Today* (2005) **75** (3): 200-212

[52] Karlsson C, Brantsing C, Svensson T, Brisby H, Asp J, Tallheden T, Lindhal A. Differentiation of human mesenchymal stem cells and articular chondrocytes: analysis of chondrogenic potential and expression pattern of differentiation-related transcription factors. *Journal of orthopaedic research* (2006) **25** (2): 152-163

essential for proper chondrocyte progenitor proliferation and for the normal progression of hypertrophic chondrocyte differentiation into bone.

SOX9 is a key transcription factor that is expressed very early in the chondrogenic mesenchymal lineage. It is essential for mesenchymal condensation and subsequent commitment of a chondrogenic lineage[53,54] and directly regulates cartilage extracellular matrix genes expression: the elimination of *SOX9* before the mesenchymal condensation leads to the absence of cartilage and bones so that *SOX9* presence is considered a marker of osteochondroprogenitor cells. It has an active role in repressing *RUNX2* expression[55] which is its counterpart in osteogenesis: actually, it's the balance between these two transcription factors that drives cell differentiation towards the chondrogenic or osteogenic lineage. *SOX9* actively inhibits chondrocyte hypertrophy and is expressed in all chondrogenic differentiation steps before pre-hypertrophic chondrocytes. Its expression largely overlaps with that of *COL2A1*, suggesting a direct control of *SOX9* on this gene. *SOX9* also induces the expression of other key genes in chondrogenesis belonging to *SRY* family, such as *SOX5* and *SOX6*.

SOX9 expression is directly modulated by TGF-β as well as by FGF: the latter in particular markedly enhances expression of *SOX9* both in chondrocytes and in mesenchymal cells, probably by the MEK-MAPK pathway[56]. So FGF2 acts on mesenchymal stem cells by priming them towards a chondrogenic lineage[57]. This is very interesting considering the fact that, before differentiation, placental mesenchymal cells were grown in a complete medium including 10 ng/ml of FGF2. The same culture condition was used as control medium for samples

[53] Akiyama H. Control of chondrogenesis by the transcription factor Sox9. *Mod Rheumatol* (2008) **18**: 213-219

[54] Quintana L, Zur Nieden NI, Semino CE. Morphogenetic and regulatory mechanisms during developmental chondrogenesis: new paradigms for cartilage tissue engineering. *Tissue Engineering: part B* (2009) **15** (1): 29-41

[55] Yamashita S, Andoh M, Ueno-Kudoh H, Sato T, Miyaki S, Asahara H. Sox9 directly promotes Bapx1 gene expression to repress Runx2 in chondrocytes. *Experimental cell research* (2009) **315** (13): 2231-40

[56] Murakami S, Kan M, McKeehan WL, De Crombrugghe B. Up-regulation of the chondrogenic Sox9 gene by the fibroblast growth factors is mediated by the mitogen-activated protein kinase pathway. *PNAS* (2000) **97** (3): 1113-1118

[57] Handorf AM, Li WJ. Fibroblast growth factor-2 primes human mesenchymal stem cells for enhanced chondrogenesis. *PLOSOne* (2011) **6** (7): e22887

analysed, thus probably explaining the high level of expression of *SOX9* in control cells compared to cells grown in chondrogenic medium in which FGF2 was withdrawn: this choice was based on the fact that FGF family has a main role in controlling chondrogenesis only in certain areas of the body, like the posterior facial suture, suggesting a less ubiquitous effect compared to that of TGF-β superfamily genes. For this reason, FGF2 was kept in maintenance medium and withdrawn in differentiating one as its role in keeping stemness was considered overwhelming compared to its role in chondrogenesis. Moreover the progressive increase of *SOX9* expression that was pointed out during the 28-day differentiation program, suggests that the priming effect of FGF2 on cells was later substituted or carried out by TGF-β, which is more commonly responsible for the activation of *SOX9*.

On the whole Real time PCR analysis suggests that a good chondrogenic differentiation was achieved, as cells show an expression pattern that can be ascribed to that of proliferating chondroblasts.

Pellet samples at 28 days of differentiation were also fixed for histological analysis. Following images show Masson's trichromic stain and alcian blue stain of pellets. Alcian blue shows a massive deposition of mucopolysaccharides and glycosaminoglycans, which correlates with the increase in the relative expression of genes such as decorin and biglycan. Figure 23 in Masson's trichromic stain gives an overall idea of pellet dimensions and structure. As can be seen, a thin layer of cells with few cytoplasm encloses the pellet, forming a capsule around it. Below it, cells are randomly distributed in a matrix with variable density and composition. In fact the peripheral zone shows a stronger green stain, suggesting an abundant presence of collagen fibres which become looser towards the centre. The faint green stain in the central region suggests that collagen is not the main matrix component and that non-collagenous fibres prevail.

On the whole, however, cell/matrix ratio and overall cell distribution doesn't seem to correspond to a hyaline cartilage, but rather to fibrocartilage. Actually cells are not organised in typical isogenous groups nor even distributed in a columnar orientation which would be both typical characteristics of hyaline

cartilage. The presence of serum in the formulation of differentiation medium could be the cause of such result, as this is associated with the emergency of a fibrocartilaginous phenotype. Moreover, the lack of a clear fibre orientation can also be attributed to the fact that those cells weren't subjected to mechanical loading thus missing part of the classical stimuli leading to cartilage formation.
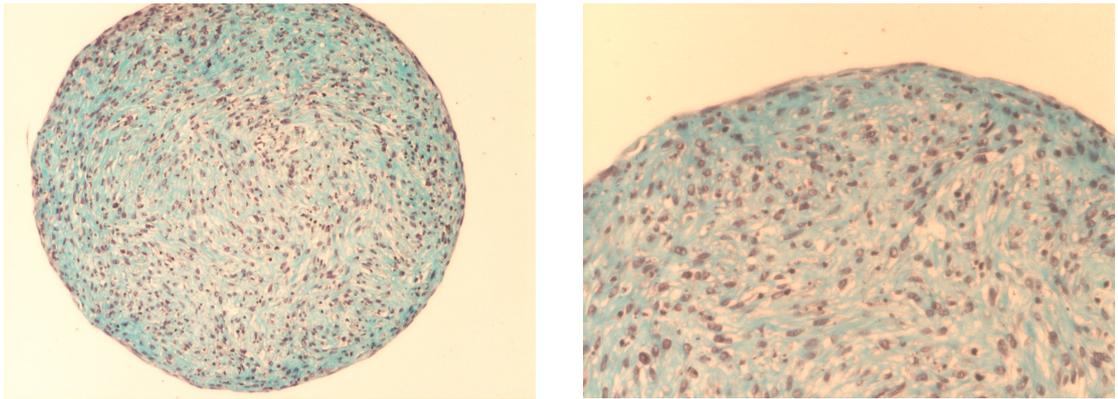
Figure 23:hCMSC pellet, 28-days differentiation, transverse section; Masson's trichromic stain 10X O.M. (left) and 100X O. M. (right)
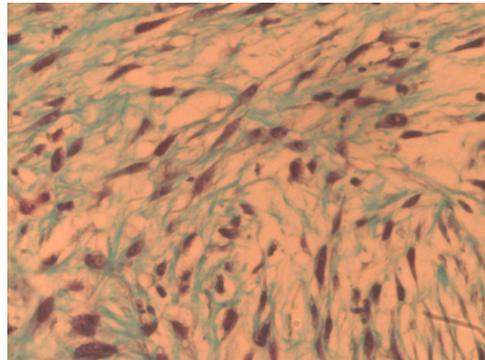
Figure 24:hCMSC pellet, 28-days differentiation, transverse section; Masson's trichromic stain 40X O.M. detail
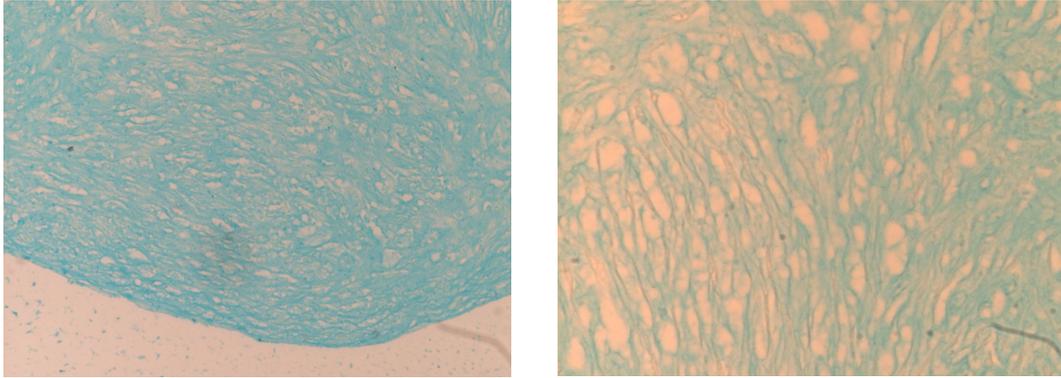
**Figure 25: hCMSC pellet, 28-days differentiation, transverse section;
Alcian blue stain 10X O.M. (left) and 40X O.M. (right)**

Summary

Despite great progresses in orthopaedics, cartilage defects still constitute a major medical issue leading to serious decrease in quality of life and high medical costs. For this reason many strategies to improve cartilage regeneration are under investigation in order to get satisfying results, including the use of cell transplantation.

Results shown so far suggest that, even if adopted culture conditions are actually useful to get the induction of the expression of chondrogenic genes on hCMSCs, some work still has to be done in order to obtain a clear hyaline cartilage to satisfy the target of articular cartilage regeneration. Such aim could be obtained by further modifying culture conditions, starting first of all by changing the timing of serum addition. Given the fact that, as previously mentioned, serum is indispensable for hCMSCs pellet formation, tests should be made to evaluate if a subsequent depletion is compatible with cell survival and differentiation to a hyaline-like cartilage. Serum depletion would also be desirable to increase the translatability of such technique to in vivo cartilage repair. Moreover, other growth factors can be taken into consideration in order to improve cell commitment toward the chondroblastogenic lineage: among others, BMP family members are some of the most promising growth factors that could efficiently help cell differentiation.

However, the type of cartilage which was so far obtained could constitute a good basis for musculoskeletal fibrocartilages regeneration (e.g. knee meniscus, temporomandibolar joint disc and intervertebral discs). Moreover fibrocartilage, which expresses type I collagen, could be a starting point for differentiation protocols towards the osteoblastic lineage that plan to recapitulate endochondral ossification, as this seems to be a new promising route for bone regeneration[58]. As this last topic is also a very burgeoning field in the context of regenerative medicine, the third part of this thesis work was focused on strategies for hCMSCs differentiation into bone which will be elucidated below.

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[58] Gawlitta D, Farrell E, Malda J, Creemers LB, Alblas J, Dhert WJA. Modulating endochondral ossification of multipotent stromal cells for bone regeneration. *Tissue Engineering: Part B* (2010) **16** (4): 385-395

Part 3-Bone differentiation

Introduction and aim

Bone, as well as cartilage[59], derives from mesenchyme, the germ layer that generates all connective tissues. Bone peculiarity is the presence of a mineralized matrix: in fact, calcium phosphate crystals are embedded into an organic intercellular matrix.

Bone tissue is constituted by four types of cells, which are osteoprogenitor cells or pre-osteoblasts, osteoblasts, osteocytes and osteoclasts.

Pre-osteoblasts, osteoblasts and osteocytes are functional phases of the same cell type, thus sharing the same mesenchymal origin.

Osteoprogenitor cells have a fibroblastoid shape, with a scanty cytoplasm rich in polyrybosomes. They line on the free surface of bone, just below periosteum, or on endostium, near to blood vessels.

The osteoblast is an immature cell of bone and can go through one of three fates: it can embed in its own osteoid and differentiate into an osteocyte, quiesce into a lining cell or undergo apoptosis. Osteoblasts are big round cells, about 20 μm wide, with a cytoplasm rich in dense granules that testifies an intense protein synthesis for the production and secretion of the organic matrix.

Osteocytes are the most abundant cell type of bone, exceeding in number osteoblasts of about 10 times. They actively make and calcify matrix and can be identified by their stellate shaped morphology and their localization deep in mineralized matrix within the lacunocanalicular network of bone. In fact, as those cells mature and deposit new bone matrix, they become entrapped into it but keep in touch with other cells by extending their connections deeper and deeper through the small canals of bone matrix. Some of this processes pass through the cell layer establishing a direct contact between the osteocyte syncytium and the extraosseous space, suggesting the existence of a signalling system between the osteocyte and the bone marrow. Moreover the typical cytoskeletal organization of osteocytes is important for their ability to respond to loading and mechanical stress. This network guarantees a tremendous cell-bone surface contact area, and

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[59] Bilezikian JP, Lawrence GR, Martin TJ *Principles of bone biology* (2008) Academic press

an extensive intracellular and extracellular communication system between sites within the bone and the bone surface. Osteocytes typically express osteocalcin, osteonectin, osteopontin and casein kinase II, and show little alkaline phosphatase activity; mature, but not early osteocytes, express sclerostin too. All this markers are also expressed by osteoblasts, and, at present, no osteocyte-specific markers are known. Such markers suggest a role for osteocytes in the mineralization of matrix, in particular by regulating phosphate metabolism and blood-calcium homeostasis. Osteocytes must inhibit mineralization of the matrix directly surrounding them to ensure the diffusion of oxygen, nutrients and waste products through the lacuno-canalicular system. This role is probably in charge to osteocalcin and osteonectin proteins. The regulation of osteocytes activity is strongly submitted to mechanical and hormonal signals, as testified by the presence, between others, of receptors for parathyroid hormone, vitamin D and estrogens.

Osteoclasts are multinucleated giant cells (100-200 μm) that, unlike other bone cells, develop from hematopoietic cells of the monocyte-macrophage lineage and not from the mesenchymal lineage. Those cells are responsible for bone resorption, thus allowing the continuous remodelling of bone in a delicate equilibrium with the activity of osteocytes. Their action targets both the mineral and the proteic component of bone: in fact, the mineral portion is resorbed by a local acidification mechanism that allows the solubilisation of hydroxyapatite crystals; on the other hand the organic matrix is degraded by the activity of lysosomal cysteine proteinases and matrix metalloproteinases.

Osteoclasts development is tightly linked to complex signalling networks that associate them to osteoblasts. An early model of osteoclasts differentiation suggests that the cooperation of vitamin D, PTH and IL-11 signaling pathways leads to the production of an osteoclast differentiation factor by osteoblasts, later identified as RANKL, which would be recognized by osteoclast progenitors through a specific receptor (RANK) that drives the commitment of osteoclast precursors to mononuclear preosteoclasts and then to osteoclasts. The binding of RANK is in competition with that of a soluble decoy receptor, osteoprotegerin (OPG), that can occupy RANKL binding sites thus preventing osteoclast

progenitor maturation. The regulation of RANKL and OPG expression is at the basis of bone homeostasis, by ensuring an equilibrium between bone synthesis and resorption.

From a macroscopically point of view, bone can be distinguished into trabecular and compact bone.

Trabecular or spongy bone is a porous bone, whose internal structure is characterized by the presence of trabeculae that arrange along the lines of stress. This characteristics renders it very light and flexible. It is found in areas which are not subject to mechanical stress, such as epiphyses of long bones, ribs, shoulder blades and in general flat bones.

Compact or cortical bone is the denser kind of bone that forms a shell around trabecular bone. It is the primary component of long bones, where strength and rigidity are needed. Mature compact bone gains a lamellar structure due to the progressive apposition by osteocytes. The structural unit of this organization is the osteon, which is centred around a Havers' channel and composed of many tight bone layers.

From an embryological point of view, bone formation can be distinguished into intramembranous and endochondral.

Intramembranous ossification is the result of the direct transformation of mesenchymal cells into osteoblasts. This type of ossification involves flat bones, such as the cranial vault, some facial bones, mandible and clavicles. On the other hand, endochondral ossification requires an intermediate step of differentiation in which mesenchymal cells form a dense area called growth plate with cartilaginous characteristics that is then invaded by blood vessels and mineralized leading to new bone formation by apposition. This kind of ossification is typical of long bones.

Whatever the type of ossification, bone is composed of a complex matrix in which many structural proteins are embedded.

Collagen type I is the most abundant extracellular protein of the organism. It has a triple helix structure composed of three polypeptide chains (two $\alpha 1$ chains and one $\alpha 2$ chain) whose amino acid sequence consists of Gly-X-Y repeats. This gives to each chain a left-handed coil thus favouring their assembly in a right-

handed triple helix, where Gly residues are in the centre of the triple helix while X and Y (typically proline and hydroxyproline) are on the surface. The presence of hydroxyproline is unique to collagen molecules and is essential for their stabilization.

Collagen type I belongs to the class of fibrillar collagens which share a very high-tensile strength and play a key role in providing a structural framework for body structures.

Its gene expression is regulated by many factors including TGF-β, FGF, IGF-1, TNFα, IFN γ and some interleukins.

Even if collagen is the major organic constituent of bone matrix, it is not the direct nucleator of hydroxyapatite deposition. This role is in charge to non-collagenous bone matrix proteins such as proteoglycans and glycoproteins.

Proteoglycans are characterized by the covalent attachment of long chain polysaccharides (glycosaminoglycans, GAGs) to core protein molecules. GAGs are composed of repeating disaccharide units variously sulphated, such as chondroitin sulphate, keratan sulphate, hyaluronic acid, etc.

Aggrecan and versican belong to the family of chondroitin sulphates, characterized by having core proteins with globular domains at the amino and carboxy termini and by binding to hyaluronan to form bigger aggregates. As previously described, aggrecan is a cartilage specific marker, but its mRNA was detected during bone development too. Versican is a proteoglycan that is mainly found in interstitial mesenchyme but is destroyed as osteogenesis progresses and is replaced by decorin and biglycan.

Decorin and biglycan are both small proteoglycans sharing a core protein that contains a leucin-reach repeat. Both markers can be found in cartilage or bone but they show a slightly different localization. While decorin in cartilage is in the interterritorial matrix away form chondrocytes, biglycan is preferentially located near them. Moreover, during endochondral bone formation decorin pattern of expression parallels that of collagen type I, as it is present in osteoblasts, increases during their maturation and is downregulated in osteocytes. On the other hand, biglycan shows a pericellular location and is found in areas undergoing morphological delineation: it is upregulated in osteoblasts and its expression is

maintained high even by mature osteocytes. Finally, decorin has a low affinity for calcium so that it has not effect on hydrohyapatite precipitation while biglycan shows a concentration-dependent effect in two phases: when calcium is at low concentration hydroxyapatite crystals precipitate, but this is inhibited by higher concentrations. However both decorin and biglycan are not the initiators of matrix mineralization, as this role is assigned to glycoproteins.

Osteonectin was the first glycoprotein isolated by bone in intact form and is known to bind Ca2+, hydroxyapatite and collagen. It is expressed in many connective tissue but it is highly enriched in bone as it is a nucleator of HA deposition.

Some of the major glycoproteins in bone matrix also contain the RGD sequence, thus helping cell adhesion. Osteopontin belongs to this group of proteins: it is a very polyvalent protein which was found independently in many organs, including those without a matrix and in plasma. This suggests for this protein at least a double role, as a structural molecule or as a humoral factor. Even if the gene can encode for more isoforms, their role and tissue expression are unknown. The negative charge of protein probably promotes its binding to mineral bone and increases its affinity for calcium. This is confirmed by structural analyses that show that calcium binding modifies its conformation. The protein is mainly found in a secreted form but an intracellular form exists that is often colocalized with CD44, probably helping osteoblasts movement. The secreted form is recognized by many integrins (in particular by $\alpha_v\beta_3$) and is mainly produced by osteoblasts. Its expression is induced by vitamin D_3 and high phosphate levels as well as by mechanical stimuli. It is mainly deposited along the cement line and lamina limitans of bone, where it could constitute a signal for osteoblasts for new matrix deposition. Osteopontin is also secreted by osteoclasts during bone resorption and a role in their activation seems possible.

Placenta is among tissues expressing osteopontin[60]. In this organ its role consists mainly in helping cell adhesion, which can determine the success of implantation in early phases of pregnancy[61].

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[60] Johnson GA, Burghardt RC, Bazer FW, Spencer TE .Osteopontin: roles in implantation and placentation. *Biol Reprod* (2003) **69,** 1458–1471

Bone sialoprotein is another RGD-containing glycoprotein, mainly associated to mineralization phenomena, as demonstrated by the fact that it is expressed at late stages of osteoblastic differentiation, as soon as mineralization begins. It supports mineralization thanks to its Ca^{2+} binding properties and mediates cell attachment. Outside the skeleton BSP is also found in trophoblasts in placental membranes.

Bone replacement is a very interesting opportunity, especially in those cases in which a critical size defect is created thus preventing common healing processes. In surgical dentistry, for example, deficiency of bone volume is a primary pretreatment complication precluding a correct insertion of dental implants or even an absolute contraindication to implant-prosthetic therapy. At present, as suggested by experimental research and clinical outcome, autologous bone graft seems to provide the best results. However, this procedure implies high morbidity and invasiveness as well as a limited availability of collectable material, boosting the search for biomaterials to substitute autologous bone. Biomaterials give the chance, rather than to repair or to replace lost bone, to recreate bone architecture in vitro and can serve as scaffolds to support growth and osteoblastic differentiation of stem cells that could be implanted afterwards. The following part of this thesis work is focused on the analysis of osteogenic potential of hCMSCs, combining the effect of an inductive medium and of a commercially available biomaterial named Bio-oss spongiosa®.

Results and discussion

In vitro experiments

In vitro experiments were first of all conceived with the purpose of identifying a suitable medium to induce the osteogenic differentiation of hCMSCs. Starting from data taken from literature a first medium was prepared as described in

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[61] Briese J, Oberndorfer M, Patschenik C, Schulte HM, Makrigiannakis A, Loning T, Bamberger A. Osteopontin is colocalized with the adhesion molecule CEACAM1 in the extravillous trophoblast of the human placenta and enhances invasion of CEACAM1-expressing placental cells. *J. Clin. Endocrinol. Metab.* (2005) **90**, 5407-5413

experimental procedures: the choice of each component is detailed below[62,63,] [64,65,66,67,68,69,70].

L-proline, as previously mentioned, is a fundamental amino acid for collagen synthesis as it constitutes the main component of alpha chains. For this reason this amino acid was added to culture medium which, by itself, would be otherwise quite poor of this amino acid.

Also ascorbic acid is involved in collagen synthesis, as it is a cofactor necessary to keep in an active state (by intervening on iron oxidation) enzymes such as proline hydroxylase, procollagen-proline-2-oxyglutarate, 3-dioxygenase and lysine hydroxylase. Those enzymes take part in proline and lysine hydroxylation in tropocollagen synthesis. Cell treatment with ascorbic acid induces cell differentiation toward the osteoblastic lineage by increasing matrix

[62] Liu P, Oyajobi BO, Russell RGG, Scutt A. Regulation of osteogenic differentiation of human bone marrow stromal cells: interaction between transforming growth factor-β and 1,25(OH)$_2$ vitamin D$_3$ in vitro. *Calcified Tissue International* (1999) **65**: 173-180

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[64] Chen TL, Cone CM, Feldman D. Effects of 1,25-dihydroxyvitamin D3 and glucocorticoids on the growth of rat and mouse osteoblast-like cells. *Calcified Tissue International* (1983) **35**: 806-811

[65] Chung CH, Golub EE, Forbes E, Tokuoka T, Shapiro IM. Mechanism of action of beta-glycerophosphate on bone cell mineralization. *Calcified Tissue International* (1992) **51**: 305-311

[66] Jaiswal N, Haynesworth SE, Caplan AI, Bruder SP. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells in vitro. *Journal of Cellular Biochemistry* (1997) **64**: 295-312

[67] Jorgensen NR, Henriksen Z, Sorensen OH, Civitelli R. Dexamethasone, BMP2 and 1,25-dihydroxyvitamin D enhance a more differentiated osteoblast phenotype: validation of an in vitro model for human bone-marrow-derived primary osteoblasts. *Steroids* (2004) **69**: 219-226

[68] Cheng SL, Yang JW, Rifas L, Zhang SF, Avioli LV. Differentiation of human bone marrow osteogenic stromal cells in vitro: induction of the osteoblast phenotype by dexamethasone. *Endocrinology* (1994) **134**: 277-286

[69] Subramaniam M, Colvard D, Keeting PE, Rasmussen K, Riggs BL, Spelsberg TC. Glucocorticoid regulation of alkaline phosphatase, osteoclacin, and proto-oncogenes in normal human osteoblast-like cells. *Journal of Cell Biochemistry* (1992) **50**: 411-424

[70] Chen TL, Hauschka PV, Feldman D. Dexamethasone increases 1,25- dihydroxyvitamin D3 receptor levels and augments bioresponses in rat osteoblast-like cells. *Endocrinology* (1986) **118**: 1119-1126

deposition [71] and by inducing the expression of genes like osteocalcin, osteopontin[72] and alkaline phosphatase[73]. This molecule was added to culture medium in the derivative form of ascorbic acid-2- phosphate which is stable under conventional culture conditions, thus avoiding the need of daily supplementation[74].

The activity of alkaline phosphatase brings to the release of inorganic phosphates from β−glycerophosphate. This is essential for bone mineralization as inorganic phosphates, in particular calcium phosphates, constitute the basic unit of hydroxyapatite crystals. This is why β−glycerophosphate was added to differentiation medium[75,76].

1-25 vitamin D_3 is the active form of cholecalciferol and is involved in the homeostasis of calcium and phosphorus[77]. It acts through a nuclear receptor named VDR and, at a transcriptional level, it stimulates the production of *OC*[78]

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[71] Choi KM, Seo YK, Yoon HH, Song KY, Kwon SY, Lee HS, Park JK. Effect of ascorbic acid on bone marrow-derived mesenchymal stem cell proliferation and differentiation. *Journal of Bioscience and Bioengineering* (2008) **105** (6): 586-594

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and *OPN* mRNAs and regulates collagen quality[79]. Moreover it acts directly by controlling the transmembrane calcium influx[80].

Dexamethasone is a synthetic glucocorticoid that acts via nuclear receptors. It has an active role in osteoblasts proliferation and differentiation. It induces the expression of some typical bone markers such as alkaline phosphatase, ostepontin, osteocalcin and BSP. Under specific experimental conditions, glucocorticoids are anabolic[81] on bone organ and cell cultures; they are able to stimulate osteoblasts differentiation, function and bone formation. Glucocorticoids increase the number of mineralized, bonelike nodules and the expression of osteoblast marker genes in rat osteoblast cultures grown in the presence of osteoinductive medium. This anabolic effect is due to the proliferation and differentiation of glucocorticoid-dependent osteoprogenitors. All these elements would support the necessity to add dexamethasone to culture medium.

The formulation described was adopted and hCMSCs were grown for 28 days in inductive conditions to get a differentiation into osteoblastic cells. At the end of the protocol RNA was extracted to assess changes in its expression which could be ascribed to cell differentiation. As shown by the graph below (fig. 26), osteogenic medium can induce a slight increase in the expression of typical bone genes such as *Alkaline Phosphatase* (*ALP*, CM: 1±0.242, OM: 1.331±0.605), *Collagen α1 chain* (*COL1A1*, CM: 1±0.104, OM: 2.337±0.379), *Osteocalcin* (*OC*, CM: 1±0.034, OM: 7.898±1.139), *Osteonectin* (*ON*, CM: 1±0.06, OM: 1.897±0.305), *RUNX2* (CM: 1±0.136, OM: 2.276±0.545), *Osteopontin* (*SPP1*, CM: 1±0.382, OM: 3.415±0.565) and *Vitamin D receptor* (*VDR*, CM: 1±0.729, OM: 2.282±0.284).

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[79] Nagaoka H, Mochida Y, Atsawasuwan P, Kaku M, Kondoh T, Yamauchi M 1,25(OH)$_2$D$_3$ regulates collagen quality in an osteoblastic cell culture system. *Biochemical and Biophysical Research Communications* (2008) **377** 674-678

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[81] Sher LB, Woitge HW, Adams DJ, Gronowicz A, Krozowski Z, Harrison JR, Kream BE. Transgenic expression of 1b-Hydroxystoeroid dehydrogenase type 2 in osteoblasts reveals an anabolic role for endogenous glucocorticoids in bone. *Endocrinology* (2004) **145** (2): 922-929
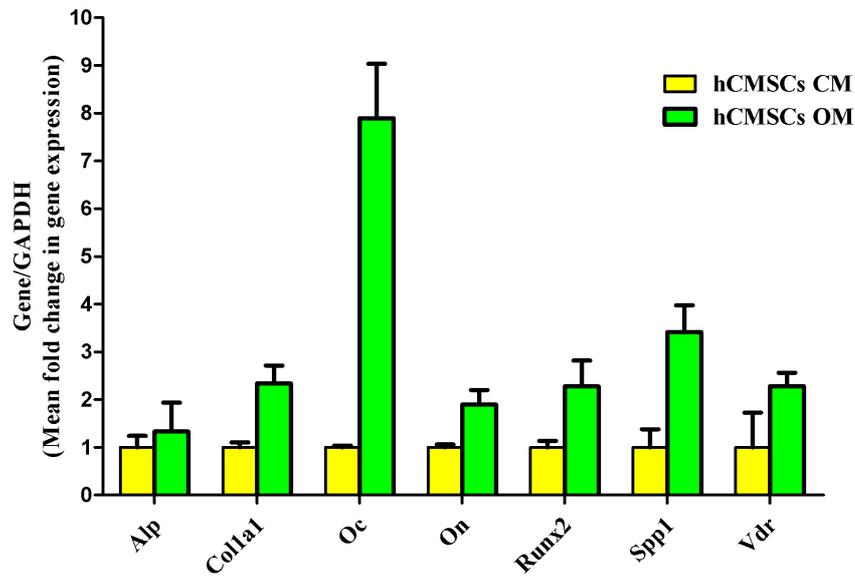
Figure 26: Real Time PCR analysis of gene expression in hCMSCs after osteogenic induction

This suggests that culture conditions actually prompted multipotent placental cells to differentiate towards the osteoblastic lineage. This is in agreement with Alizarin red S stain of calcium deposits that were observed on culture surface. Image below (fig. 27) shows the deposition of some calcium crystals: it is clearly visible that the deposition is not uniform and just covers a small area on cell surface, suggesting that overall differentiation was scanty.
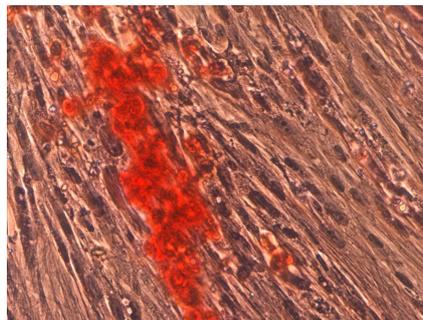
Figure 27: Alizarin red S stain, light microscopy, 4X O.M.

This lead us to improve culture stimuli by taking into consideration the natural conditions occurring in bone in order to get a massive calcium deposition which would be a sign of further maturation towards the osteoblastic lineage.

In order to have a robust term of comparison, we isolated human osteoblasts from mandibular fragments derived from oral surgery. Those cells were grown in the same conditions as hCMSCs and directly compared to them as a reference of a complete differentiation.

We started by considering that type I collagen is the main component of bone, so we prepared culture dishes by covering them with type I bovine collagen as described in experimental procedures. Cells were seeded on plastic or collagen-coated dishes and grown in complete medium in order to point out collagen influence on gene expression. After 28 days RNA was extracted and analysed by RT-PCR. Results are summarized in the graphs below (fig.28 and 29): collagen itself does not affect *RUNX2* expression both in hCMSCs and hOBs (hCMSCs CM: 1.0±0.16; hCMSCs/col CM: 1.48±0.32, ns; hOBs CM: 1.0±0.05; hOBs/col CM: 1.26±0.13, ns) as well as the expression of its target genes such as *VDR* (hCMSCs CM: 1.0±0.09; hCMSCs/col CM: 1.23±0.38, ns; hOBs CM: 1.0±0.1; hOBs/col CM: 1.49±0.37, ns), *COL1A1* (hCMSCs CM: 1.0±0.09; hCMSCs/col CM: 1.15±0.21, ns; hOBs CM: 1.0±0.06; hOBs/col CM: 0.99±0.05, ns). Only on hCMSCs, collagen had a minor effect on the expression of *BMP2* (hCMSCs CM: 1.0±0.11 hCMSCs/col CM: 2.37±0.36, $P<0.05^*$; hOBs CM: 1.0±0.26; hOBs/col CM: 1.07±0.16, ns), which was not significant for *Osteonectin* (*ON*, hCMSCs CM: 1.0±0.14; hCMSCs/col CM: 2.14±0.36, ns; hOBs CM: 1.0±0.05; hOBs/col CM: 1.29±0.05) and *Osteoprotegerin* (*OPG*, hCMSCs CM: 1.0±0.10; hCMSCs/col CM: 1.65±0.25, ns; hOBs CM: 1.0± 0.08; hOBs/col CM: 0.76±0.03). On the other hand, collagen induced only in hOBs a significant increase in the expression of *ALP* (hCMSCs CM: 1.0±0.09; hCMSCs/col CM: 2.26±0.43, ns; hOBs CM: 1.0±0.05; hOBs/col CM: 1.26±0.05, $P<0.05^*$) as well as of *Osteopontin* (*SPP1*, hCMSCs CM: 1.0±0.09; hCMSCs/col CM: 0.71±0.11, ns; hOBs CM: 1.0±0.19; hOBs/col CM: 7.95±0.45, $P<0.005^{**}$). Moreover collagen did not induce *SP7* (hOBs CM = 1.0±0.43; hOBs/col CM = 1.39±0.05) and strongly inhibited *Osteocalcin* expression in both cell types (hCMSCs CM: 1.0±0.12; hCMSCs/col CM: 0.05±0.02, $P<0.005^{**}$; hOBs CM: 1.0±0.06; hOBs/col CM: 0.05±0.01, $P<0.005^{**}$). This last is considered a marker of

osteoblast late differentiation process: given this results, collagen by itself shows a very limited osteogenic potential.
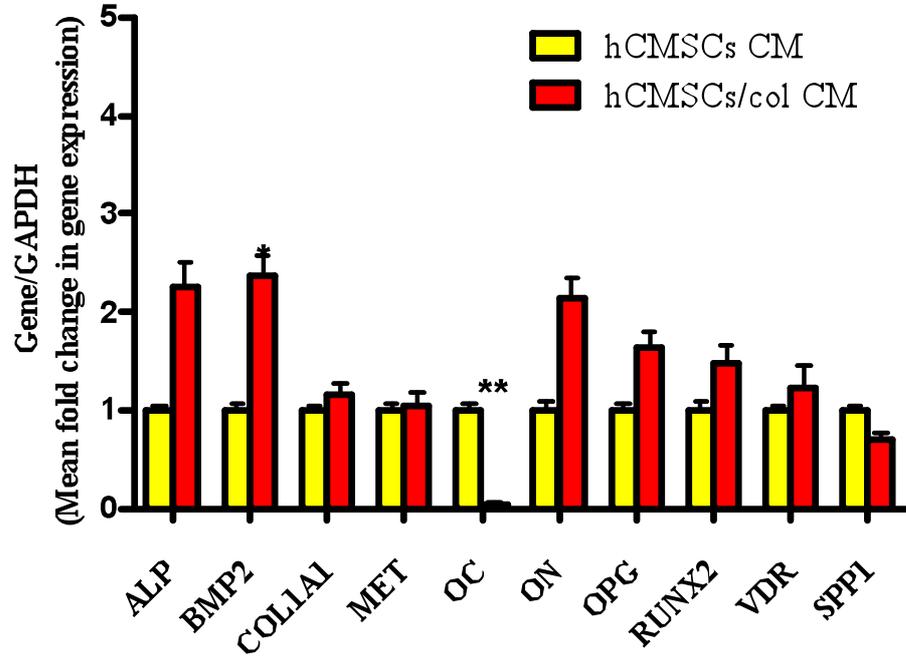
Figure 28: Real Time PCR analysis of gene expression in hCMSCs grown on collagen
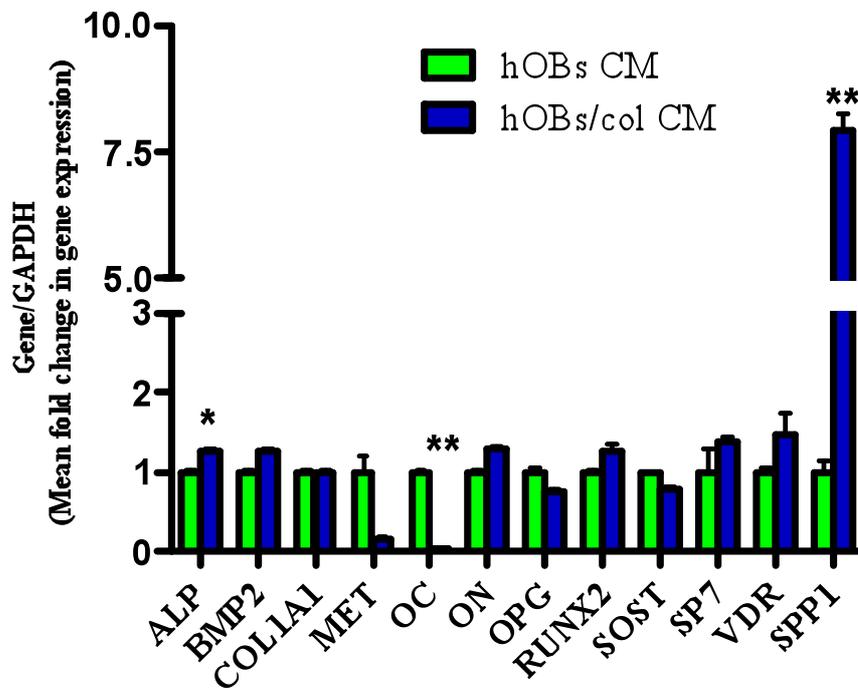
Figure 29: Real Time PCR analysis of gene expression in hOBs grown on collagen

As collagen did not give very satisfactory results, Bio-oss®, a bone substitute currently used in oral surgery, was tested in order to verify any possible supporting role in differentiation. Cells were grown on plates coated with Bio-oss®, as previously described, for 5 weeks.
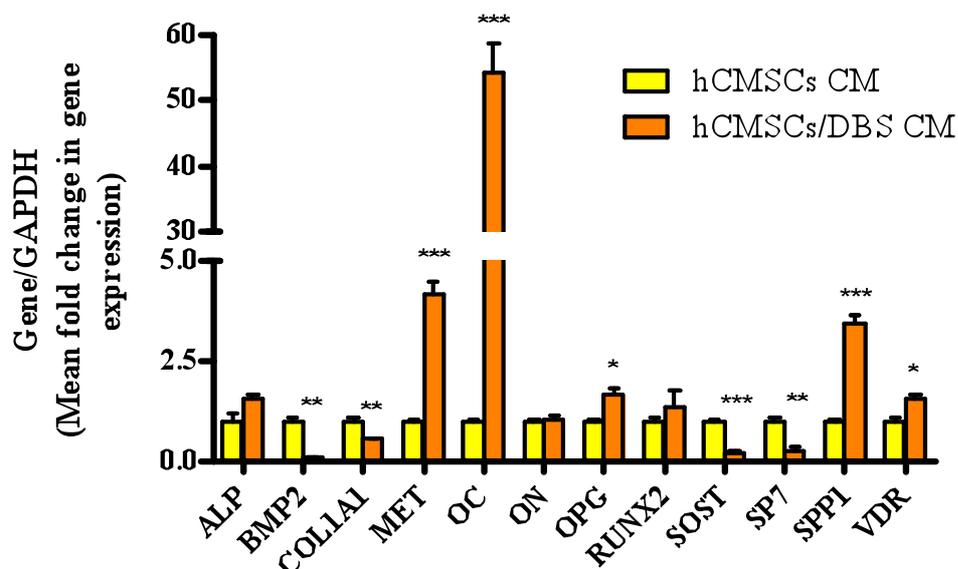
Figure 30: Real Time PCR analysis of gene expression in hCMSCs on Bio-oss®
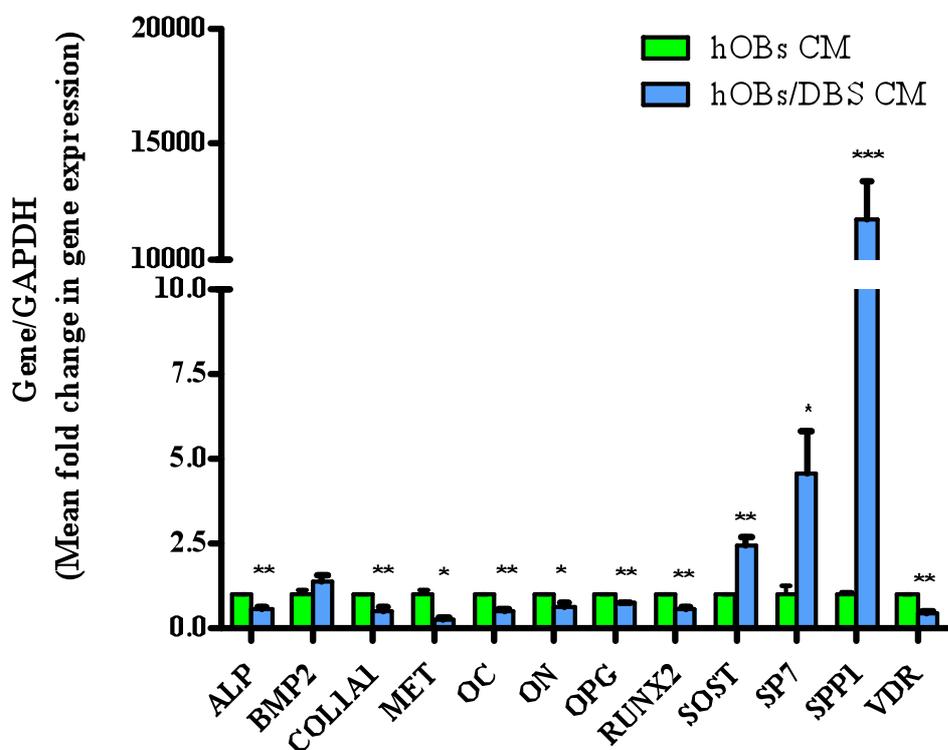
Figure 31: Real Time PCR analysis of gene expression in hOBs on Bio-oss®

The presence of Bio-oss® resulted in contrasting results on hCMSCs or on hOBs, except for a common massive induction of *SPP1* expression (hCMSCs CM: 1.0± 0.09, hCMSCs/DBS CM: 3.46± 0.38 P<0.0001***; hOBs CM: 1.0±0.19; hOB/DBS CM; 36.0±11.6, P<0.0001***) and a reduction of expression of *COL1A1* (hCMSCs CM: 1.0± 0.1; hCMSCs/DBS CM: 0.54±0.05, P<0.005**; hOBs CM: 1.0±0.06; hOBs/DBS CM: 0.55±0.13, P<0.005**).

As far as other typical bone markers are concerned, Bio-oss® presence, only on hCMSCs, resulted in a slight induction of the expression of *ALP* (hCMSCs CM: 1.0± 0.24; hCMSCs/DBS CM: 1.57± 0.17, P<0.05*), *OPG* (hCMSCs/DBS CM: 1.0±0.10; hCMSCs/DBS CM: 1.65±0.25, P<0.05*), and *VDR* (hCMSCs CM: 1.0± 0.1; hCMSCs/DBS CM: 1.57± 0.2, P<0.05*)[82]. On the other hand, the expression of those genes was significantly reduced by the presence of Bio-oss® in hOBs (*ALP*: hOBs CM: 1.0±0.05; hOBs/DBS CM: 0.57±0.14, P<0.005**; *OPG*: hOBs CM: 1.0±0.08; hOBs/DBS CM: 0.76±0.03, P<0.005**; *VDR*: hOBs CM: 1.0±0.1; hOB/DBS CM: 0.45±0.11, P<0.005**).

Similarly, Bio-oss®, only on hCMSCs, lead to a strong induction of the expression of genes such as *MET* (hCMSCs/DBS CM: 1.0±0.04; hCMSCs/DBS CM: 4.19±0.55, P<0.0001***), and *OC* (hCMSCs CM: 1.0± 0.06; hCMSCs/DBS CM: 54.0± 8.19, P<0.0001***), and suppressed the expression of *BMP2* (hCMSCs/DBS CM: 1.0±0.12; hCMSCs/DBS CM: 0.07±0.01, P<0.0001***), but, on the contrary, did not affect the expression of *BMP2* in hOBs (*BMP2*: hOBs CM: 1.0±0.26; hOB/DBS CM: 1.39± 0.34). In hOBs a strong decrease in the expression of *MET* (hOBs CM: 1.0±0.30; hOBs/DBS CM: 0.29±0.07, P<0.05*) and *OC* (hOBs CM: 1.0±0.06; hOB/DBS CM: 0.52±0.16, P<0.005**) was observed.

The presence of Bio-oss® didn't affect the expression of *Osteonectin* (hCMSCs/DBS CM: 1.0±0.06; hCMSCs/DBS CM: 1.05±0.12) and *RUNX2* (hCMSCs CM: 1.0± 0.15; hCMSCs/DBS CM: 1.34.0± 0.729) on hCMSCs, while the expression of the same genes was reduced in hOBs: (*Osteonectin*: hOBs CM:

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[82] Bellows CG, Reimers SM and Heersche JN. Expression of mRNAs for type-I collagen, bone sialoprotein, osteocalcin, and osteopontin at different stages of osteoblastic differentiation and their regulation by 1,25 dihydroxyvitamin D3. *Cell Tissue Res* (1999) **297**: 249-59

1.0±0.05; hOB/DBS CM: 0.67±0.16, P<0.05*; *RUNX2*: hOBs CM: 1.0±0.05; hOB/DBS CM: 0.57±0.14, P<0.005**).

Opposite results were obtained considering the effect of Bio-oss® on markers of late differentiation such as *SOST* (hCMSCs/DBS CM: 1.0±0.06; hCMSCs/DBS CM: 0.21±0.04, P<0.0001***; hOBs CM: 1.0±0.01; hOBs/DBS CM: 2.48±0.36, P<0.05*) and *Sp7* (hCMSCs/DBS CM: 1.0±0.16; hCMSCs/DBS CM: 0.25±0.16, P<0.005**; hOBs CM: 1.0±0.43; hOBs/DBS CM: 4.6±2.1, P<0.05*), whose expression was reduced in hCMSC while was induced in hOBs.

On the whole these results suggest a different effect of Bio-oss® on cell behaviour depending on the starting condition of the population considered. Actually, growing hCMSCs on Bio-oss® leads to an increase in the expression of some typical osteogenic genes which however can't be ascribed to a mature differentiation. The presence of the mineral scaffold hampered the expression of *BMP2* thus blocking de facto the possibility of an autocrine and paracrine stimulus toward hard tissue commitment. The most unexpected datum concerns the massive induction of expression of *Osteocalcin*, which is considered to be unique to bone tissue and predominantly limited to the cells of osteoblastic lineage, in particular in the late phase of osteoblasts maturation. The main gene responsible for the induction of the expression of *OC* is *RUNX2* but this is not the case as Bio-oss® has no effect on the expression of this transcription factor: so, it is not clear by which mechanism Bio-oss® could promote *OC* expression. Actually, Beck et al[83] reported that inorganic phosphates, that could be released from Bio-oss®, are able to induce *SPP1* and reduce *COL1A1* expression, which is in accordance with what we observed on hCMSCs, but no data were found in literature that could link the induction of expression of *OC* to the presence of phosphates. We hypothesize that such increase in *OC* expression could be ascribed to the production process of Bio-oss®, which is subjected to very high temperatures in order to get a protein-free scaffold. Termic treatment of

[83] Beck GR, Moran E, Knecht N. Inorganic phosphate regulates multiple genes during osteoblast differentiation, including Nrf2. *Experimental Cell Research* (2003) **288** (2): 288-300

hydroxyapatite, as previously reported by Wang et al[84], affects crystal particle size and can induce a significant increase in osteocalcin expression. A further, though minor, effect on *OC* expression could be attributed to the slightly acidic treatment to which Bio-oss® was subjected in order to help its adhesion to plastic surface in the presence of collagen in acetic acid solution. In fact Takemura et al[85] showed that acid treatment of hydroxyapatite improves its performance on mature osteoblasts by inducing osteocalcin and alkaline phosphatase. In our experiments the sum of treatments to which Bio-oss® was subjected could explain its effect on *OC* expression in hCMSCs, but such hypothesis needs to be further verified. Anyway, the fact that osteocalcin is expressed even by immature mesenchymal cells challenges its role as an exclusive bone marker[86].

On the whole, it seems rather that the presence of Bio-oss® drives the maintenance of a state of uncommitment, as testified by high values of *MET* expression.

On the other hand, hOBs, which are already committed towards an osteogenic fate, are prompted to go further in their differentiation pathway by expressing late osteogenic genes, which suggest a maturation of those cells towards osteocytes.

So Bio-oss® seems to have a cell-specific behaviour that is dependent on the state of differentiation of the population considered. Mesenchymal cells from placenta, which are in an uncommitted state, do not undergo differentiation, even if Bio-oss® seems to be suitable for their proliferation.

On the whole, the presence of Bio-oss® can stimulate the expression of some typical bone markers but, by itself, it is not enough at all to induce a maturation of cells into osteocytes, even for those cells which are already committed towards the osteogenic lineage like hOBs are.

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[84] Wang C, Duan Y, Markovic B, Barbara J, Howlett CR, Zhang X, Zreiqat H. Proliferation and bone-related gene expression of osteoblasts grown on hydroxyapatite ceramics sntered at different temperature. *Biomaterials* (2004) **25** (15): 2949-2956

[85] Takemura T, Ikoma T, Hanagata N. Effect of acid trated hydroxyapatite on osteoblast maturation. *Key engineering materials* (2008) **361-363**: 1029-1032

[86] Malaval L, Modrowski D, Gupta AK, Aubin JE. Celular expression of bone-related proteins during in vitro osteogenesis in rat bone marrow stromal cell cultures. *Journal of cellular physiology* (1994) **158** (3): 555-572

These results suggest that actually Bio-oss® by itself can induce the expression of some typical osteogenic genes thus offering some advantages for cell differentiation. However genes induced by Bio-oss® don't include *COL1A1* and *Osteonectin*: this prevents the deposition of an organized osteoid matrix and narrows the presumptive osteoinductive role of Bio-oss® to an osteoconductive one, supporting cell attachment and migration but not completely inducing cell differentiation.

Considering previous results, we wanted to evaluate the effect of an improved osteogenic medium (OM) on cells cultured in presence of Bio-oss®. The conventional osteogenic medium reported in literature includes dexamethasone, a glucocorticoid whose presence is essential to allow the differentiation of mesenchymal cells into osteoblasts. However, it has been known for many years that the administration of corticosteroids to patients results in osteoporosis and growth retardation. When added to fibroblasts in culture, corticosteroids usually decrease type I collagen synthesis by acting at a pre-translational level. Moreover glucocorticoids have a two-phase effect on bone loss: an early, rapid phase in which bone mass is lost because of excessive bone resorption and a slower, later phase in which bone is lost because of inadequate bone formation. This is probably the result of an additive effect of a decrease in collagen synthesis and an increase in the activity of osteoclasts. Moreover glucocorticoids decrease bone formation by direct and indirect mechanisms which include the downregulation of IGF-1 expression in osteoblasts, the increase in osteoblasts apoptosis and the induction of osteoclastic activity. Glucocorticoids hamper bone formation also by suppressing osteoblasts differentiation and by prolonging osteoclasts lifespan[87]. Glucocorticoids decrease the number of bone forming cells by decreasing cell replication and by preventing the terminal differentiation of cells into mature functioning osteoblasts [88]. Chronic administration of steroids reduces bone

[87] O'Brien CA, Jia D, Plotkin LI, Bellido T, Powers CC, Stewart SA, Manolagas SC, Weinstein RS. Glucocorticoids act directly on osteoblasts and osteocytes to induce their apoptosis and reduce bone formation and strength. *Endocrinology* (2004) **145** (4): 1835-1841

[88] Canalis E, Delany AM. Mechanisms of glucocorticoids action on bone. *Ann. N.Y. Acad. Sci* (2002) **966**: 73-81

formation rate, resulting in decreased osteoid, wall and trabecular width, bone mineral density and bone strength.

Given such controversial effects of corticosteroids, we tried to reach a compromise in order to take advantage of dexamethasone-derived osteoinductive effects without suffering side-effects. For such reason we introduced a new protocol for cell induction in which osteogenic medium (OM) contained dexamethasone for the 3 weeks, then cultures were switched to an osteogenic medium without it (OM-dex) for 2 more weeks.

Both osteoblasts and multipotent placental cells were grown on dishes covered with Bio-oss® for 5 weeks, RNA was extracted and analysed by RT-PCR.

A panel of markers was analysed, in order to check if a differentiation occurred and to which grade. RT-PCR results are shown below, comparing multipotent stem cells or human osteoblasts grown in CM or in osteogenic medium in which dexamethasone was maintained for 5 or 3 weeks.
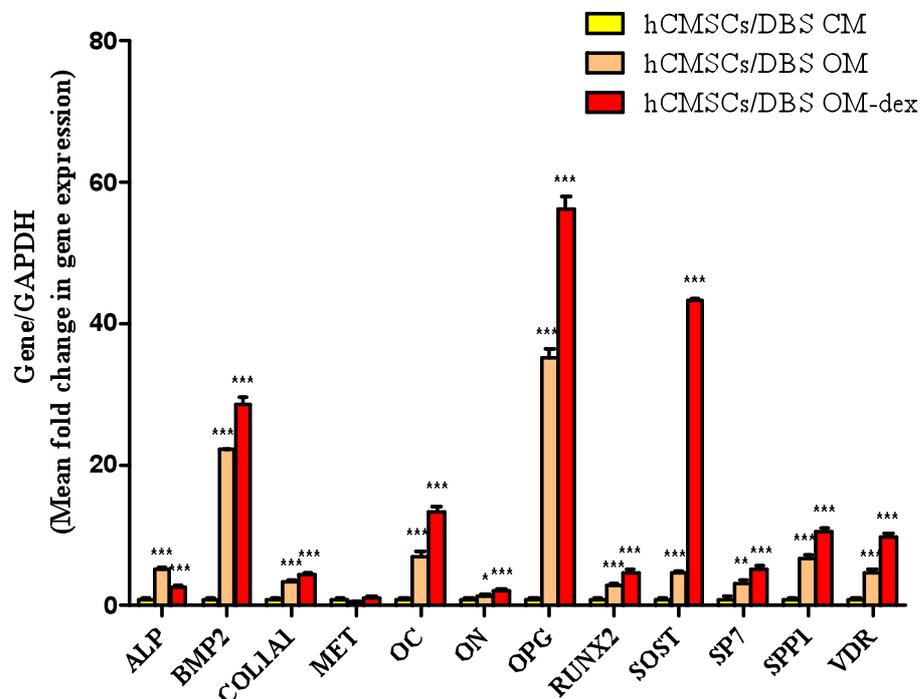
Figure 32: Real Time PCR analysis of gene expression in hCMSCs on Bio-oss®: evaluation of dexamethasone withdrawal
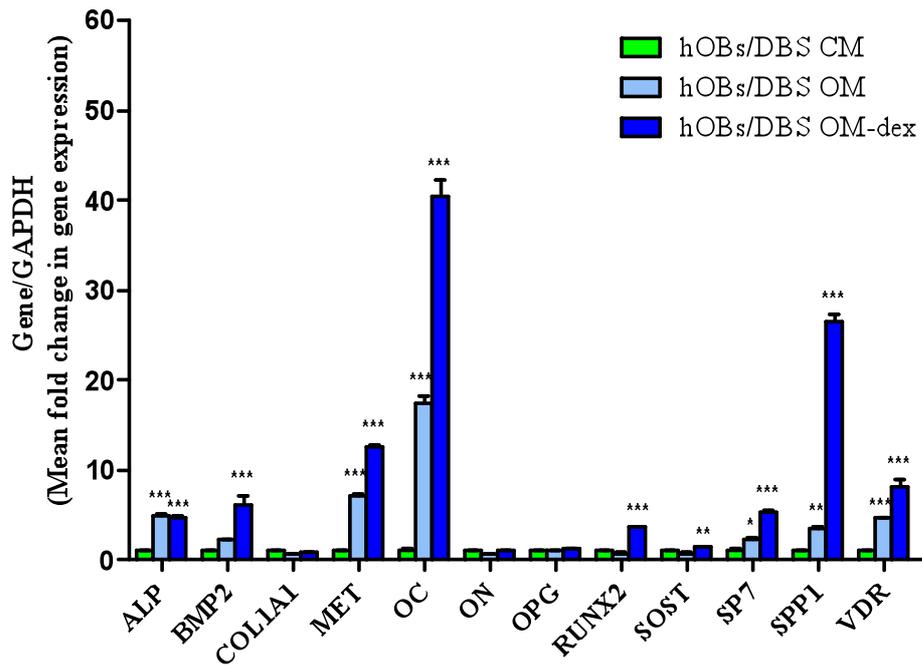
Figure 33: Real Time PCR analysis of gene expression in hOBs on Bio-oss®: evaluation of dexamethasone withdrawal

Data reported in figures 32 and 33 clearly indicate that an advanced differentiation can be achieved both in hCMSCs and in hOBs if a protocol is adopted that considers a maturation time without glucocorticoids. The removal of dexamethasone resulted in an increased expression of BMP2 both in mesenchymal stem cells and osteoblasts (hCMSCs/DBS CM: 1.0±0.21; hCMSCs/DBS OM: 22.281±0.15, P<0.0001***; hCMSCs/DBS OM-dex: 28.64±1.49, P<0.0001***; hOBs/DBS CM: 1.0±0.24; hOBs/DBS OM: 2.23±0.15; hOBs/DBS OM-dex: 6.11±1.55, P<0.0001***). As this is an upstream factor involved in RUNX2 activation, this leads to the increase in the expression of this transcription factor (hCMSCs/DBS CM: 1.0±0.22; hCMSCs/DBS OM: 3.00±0.21, P<0.05*; hCMSCs/DBS OM-dex: 4.74±0.89, ns; hOBs/DBS CM: 1.0±0.24; hOBs/DBS OM: 0.77±0.07, ns; hOBs/DBS OM-dex: 3.69±0.13, P<0.0001***) which was associated with a coherent expression of its target genes: VDR (hCMSCs/DBS CM: 1.0±0.13; hCMSCs/DBS OM: 4.75±0.62, P<0.0001***; hCMSCs/DBS OM-dex: 9.93±0.64, P<0.0001***; hOBs/DBS CM: 1.0±0.23; hOBs/DBS OM: 4.66±0.2, P<0.0001***; hOBs/DBS OM-dex: 8.21±1.12, P<0.0001***), COL1A1 (hCMSCs/DBS CM: 1.0±0.09;

hCMSCs/DBS OM: 3.48±0.36, P<0.0001***; hCMSCs/DBS OM-dex: 4.39±0.4, P<0.0001***; hOBs/DBS CM: 1.0±0.24; hOBs/DBS OM: 0.65±0.04, P<0.05* and hOBs/DBS OM-dex: 0.93±0.03??), Osteonectin (hCMSCs/DBS CM: 1.0±0.12; hCMSCs/DBS OM: 1.52±0.18, P<0.05*; hCMSCs/DBS OM-dex: 2.26±0.26, P<0.0001***; hOBs/DBS CM: 1.0±0.24; hOBs/DBS OM: 0.6±0.05, P<0.05*; hOBs/DBS OM-dex: 1.03±0.03, ns), OPG (hCMSCs/DBS CM: 1.0±0.24; hCMSCs/DBS OM: 35.13±2.43, P<0.0001***; hCMSCs/DBS OM-dex: 56.31±2.87, P<0.0001***; hOBs/DBS CM: 1.0±0.24; hOBs/DBS OM: 1.05±0.11; hOBs/DBS OM-dex: 1.23±0.05), ALP (hCMSCs/DBS CM: 1.0±0.11; hCMSCs/DBS OM: 5.21±0.56, P<0.0001***; hCMSCs/DBS OM-dex: 2.76±0.3, P<0.005**; hOBs/DBS CM: 1.0±0.24; hOBs/DBS OM: 4.92±0.31, P<0.0001***; hOBs/DBS OM-dex: 4.77±0.17, P<0.0001***), OC 89 (hCMSCs/DBS CM: 1.0±0.15; hCMSCs/DBS OM: 7.09±1.07, P<0.0001***; hCMSCs/DBS OM-dex: 13.33±1.47, P<0.0001***; hOBs/DBS CM: 1.0±0.32; hOBs/DBS OM: 17.39±1.32, P<0.0001***; hOBs/DBS OM-dex: 40.44±2.54, P<0.0001***), Osteopontin (hCMSCs/DBS CM: 1.0±0.11; hCMSCs/DBS OM: 6.69±0.75, P<0.0001***; hCMSCs/DBS OM-dex: 10.60±0.77, P<0.0001***; hOBs/DBS CM: 1.0±0.24; hOBs/DBS OM: 3.5±0.23, P<0.005**; hOBs/DBS OM-dex: 26.56±1.0, P<0.0001***); Sp7 (hCMSCs/DBS CM: 1.0±0.21; hCMSCs/DBS OM: 22.281±0.15, P<0.05*; hCMSCs/DBS OM-dex: 28.64±1.49, P<0.0001***;hOB/DBS CM: 1.0±0.46; hOB/DBS OM: 2.83±1.23, P<0.05*; hOB/DBS OM-dex: 5.06±0.58, P<0.0001***), MET (hCMSCs/DBS CM: 1.0±0.13; hCMSCs/DBS OM: 0.58±0.07, P<0.05*; hCMSCs/DBS OM-dex: 1.25±0.17, ns; hOBs/DBS CM: 1.0±0.25; hOBs/DBS OM: 7.2±0.1, P<0.0001***; hOBs/DBS OM-dex: 12.68±0.17, P<0.0001***), SOST (hCMSCs/DBS CM: 1.0±0.2; hCMSCs/DBS OM: 4.821±0.2, P<0.0001***; hCMSCs/DBS OM-dex: 43.27±0.65, P<0.0001***; hOBs/DBS CM: 1.0±0.14; hOBs/DBS OM: 0.77±0.07, P<0.05*; hOBs/DBS OM-dex: 1.39±0.06, P<0.005**).

[89] Paredes R, Arriagada G, Cruzat F, Villagra A, Olate J, Zaidi K, van Wijnen AJ, Lian JB, Stein GS, Stein JL, and Montecino M. Bone-specific transcription factor Runx2 interacts with the 1a,25-dihydroxyvitamin D_3 receptor to upregulate rat osteocalcin gene expression in osteoblastic cells. *Mol. Cell. Biol.* (2004) 24, 8847–8861

On the whole the extent of cell differentiation looks different between the two cell types, as it is plain that while the panel of expression of hCMSCs after differentiation can be compared to that of mature osteoblasts, that of hOBs can be referred to that of osteocytes, as the expression of late stage genes prevails.

Alizarin red stain and quantification

As already demonstrated, the presence of Bio-oss®, in combination with an appropriate osteoinductive medium, was able to induce a good differentiation of hCMSCs towards the osteogenic lineage and a massive maturation of hOBs into osteocytes, which was testified by an increase in the expression of typical osteogenic genes; from the hystochemical point of view, the analysis of cells cultured in differentiation medium in the presence of 1 mg/ml Bio-oss®, after staining with Alizarin red S dye, revealed a clear red pattern, thus evidencing a massive deposition of calcium. On the contrary, when cells were cultured in CM even in the presence of 1 mg/ml of Bio-oss®, no calcium deposition was detected and Bio-oss® was not stained. As shown by figures below, cells grown on Bio-oss® in the presence of osteogenic medium showed a significant increase in calcium deposition, which was even greater if dexamethasone was withdrawn from the medium for the last two weeks of culture. The amount of calcium deposition was proportional to the ability to bind Alizarin red S stain, thus allowing its quantification through the method described in experimental procedures.
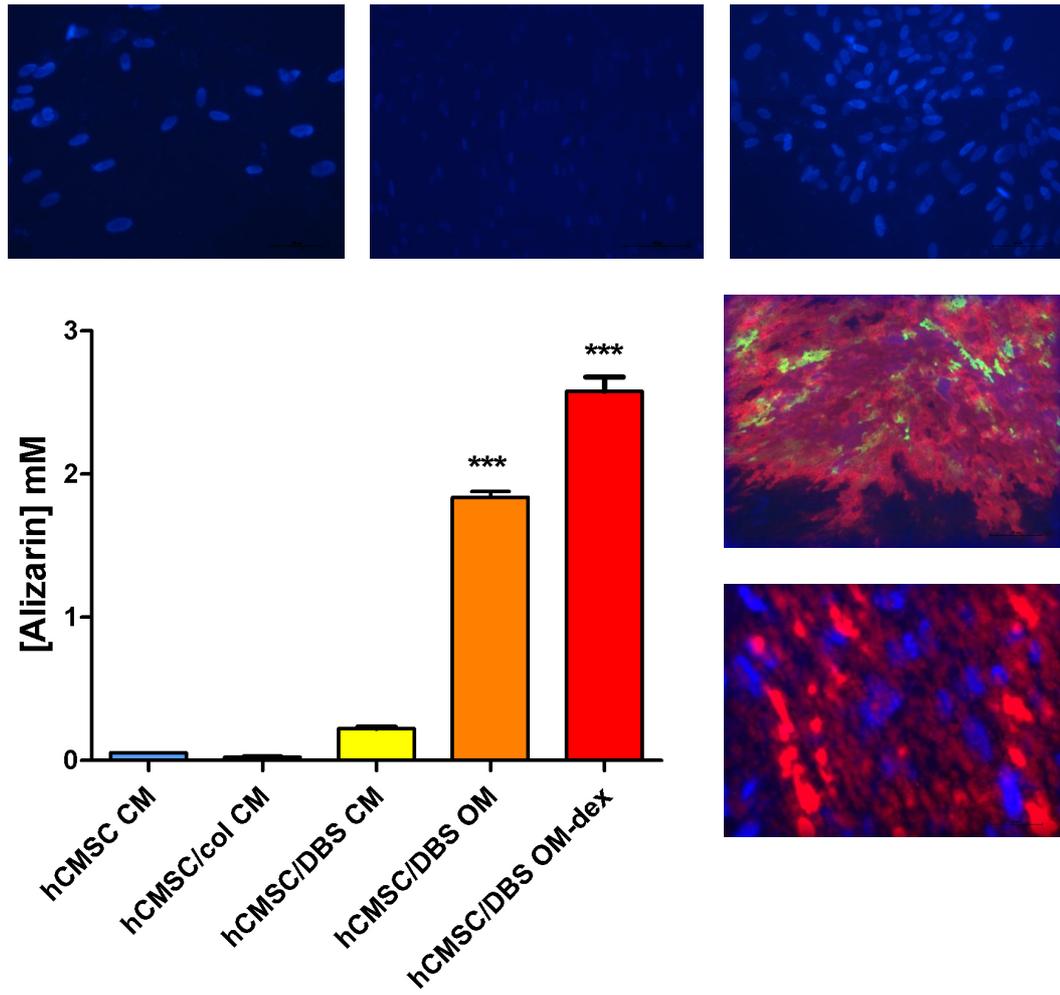
Figure 34: Alizarin red stain and quantification of calcium deposition by hCSMCs on different substrates. From left to right, hCMSCs CM, hCMSCs/col CM, hCMSCs/DBS CM, hCMSC/DBS OM, hCMSC/DBS OM-dex; fluorescent microscopy 20X O.M.
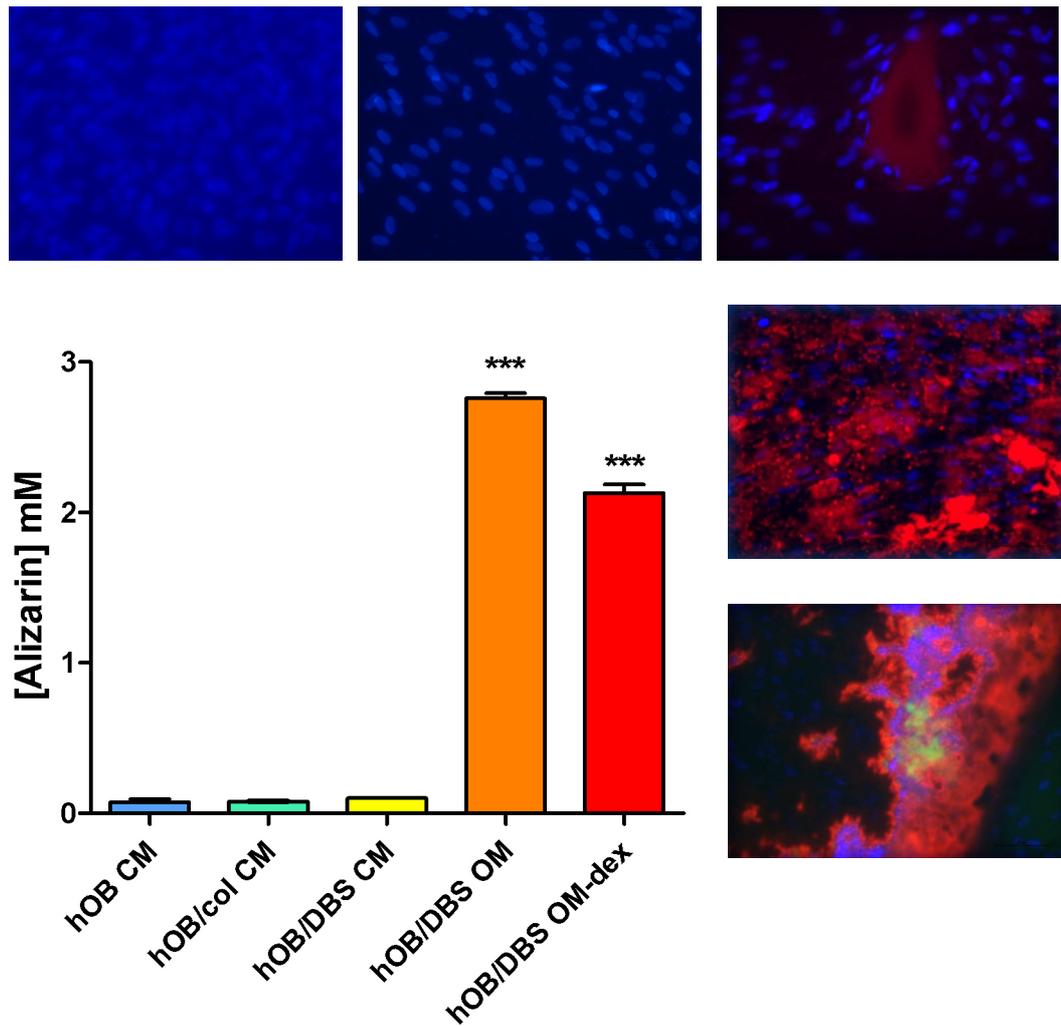
Figure 35: Alizarin red stain and quantification of calcium deposition by hOBs on different substrates. From left to right, hOBs CM, hOBs/col CM, hOBs/DBS CM, hOBs/DBS OM, hOBs/DBS OM-dex; fluorescent microscopy 20X O.M.

An osteoid-like matrix was produced by hCMSCs. This data were in accordance with calcein orange stain, pointing out newly formed calcium phosphate crystals: a bright green colour is well evident in cells cultured in OM; in contrast only a very week signal was observed in the CM cultured cells. To localize the deposition of extracellular matrix protein that occurred in osteogenic differentiation, we evaluated the presence of collagen I with a secondary antibody conjugated with AMCA and the deposition of Osteopontin with a TRITC conjugated secondary antibody and nuclei were stained by DAPI. Only when cells were incubated in OM it was possible to recognize the deposition of adequate extracellular matrix (fig 36). To demonstrate if bone matrix was

synthesized, we verified the co-localization of the different markers tested by superimposing the different pictures with DAPI, AMCA, calcein and TRITC.
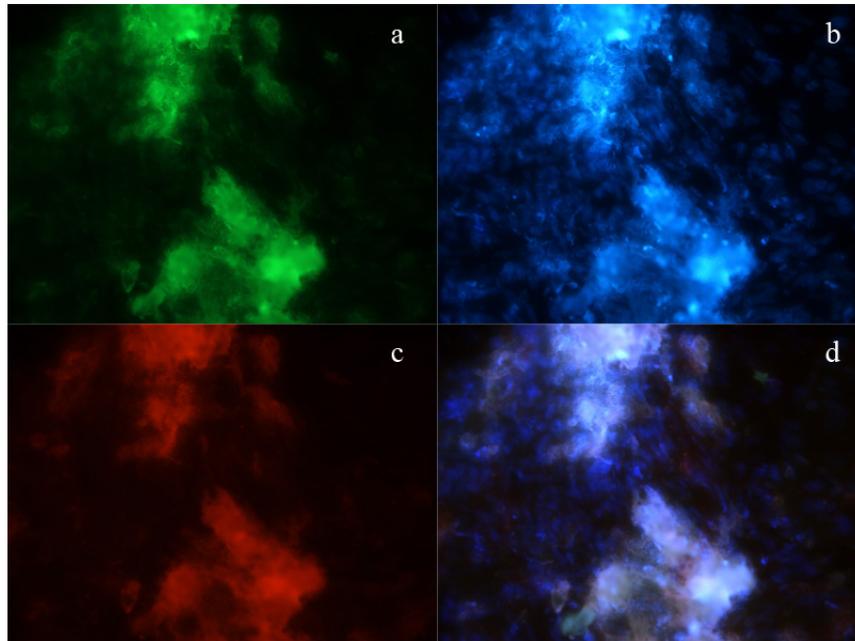
Figure 36: hCMSCs stain (a) calcein orange, (b) AMCA-conjugated anti-COL1A1 antibody+ nuclear stain with DAPI (blue), (c) TRITC-conjugated anti-Osteopontin antibody (red), (d) merge. Fluorescent microscopy 20X O.M.

Data described so far demonstrate that hCMSCs are endowed with an osteogenic potential that can be accomplished at its best by the combination of a suitable osteogenic medium and of an appropriate support such as Bio-oss[®]. Actually a synergistic effect can be observed between Bio-oss[®] and osteogenic medium, which is much greater than the effect of those components taken by themselves.

In vivo experiments

Considering the osteogenic potential of hCMSCs, these cells were further used for an in vivo experiment, in order to evaluate their ability in healing a critical size defect if implanted on Bio-oss® scaffolds.

As explained in experimental procedures, hCMSCs were infected with a GFP-carrying lentivirus then grown in presence of puromycin to select resistant cells. Transfection didn't affect cell viability and proliferation, and after expansion in complete medium cells looked as follows.
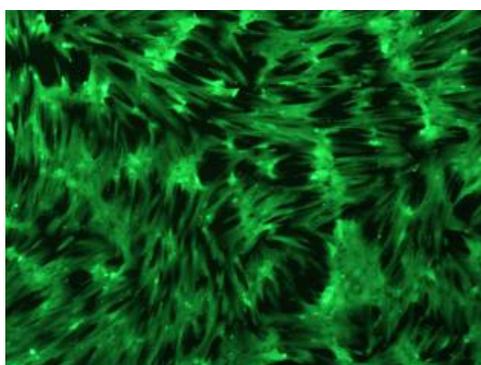
Figure 37: hCMSC GFP+, fluorescent microscopy, 10X O.M.

Meanwhile Bio-oss® spongiosa scaffolds were prepared in order to get 1mm-high dishes with a diameter of about 6 mm.

Figure 38: preparation of Bio-oss spongiosa scaffolds for in vivo implantation

Cells were harvested and seeded on dishes which had been previously wetted with osteogenic medium.

After rats sedation and skull shaving, an incision was made along the sagittal line and calvaria was exposed by peeling off tissue layers.

Figure 39: exposition of calvaria

Critical size defects were created on both sides of calvaria and were filled with Bio-oss dishes: in particular, the one on the right side was empty, while the left one was carried with placental cells. Cylinders were fitted to calvaria thickness in order to avoid brain compression after suture.

Figure 40: left, critical size defects created on both sides of calvaria; right, filling of defects with Bio-oss cylinders

Histological analysis of Bio-oss scaffolds

Time 0 samples

As rat 0 died during surgery, Bio-oss® dishes were anyway recovered and analysed by immunohistochemistry. Unfortunately, sample processing including demineralization generated an autofluorescence that partially hampered a clear analysis of slides. Moreover the treatment altered GFP fluorescence, so, to discern human cells from rat one, an immunostaining with a CY5-conjugated anti-GFP antibody was performed.

The right dish, not carrying hCMSC, showed no evidence of cellular colonization by rat cells, as shown by negative nuclear stain with DAPI. As was expected no positive red fluorescence, corresponding to human cells was evidenced (fig. 41).
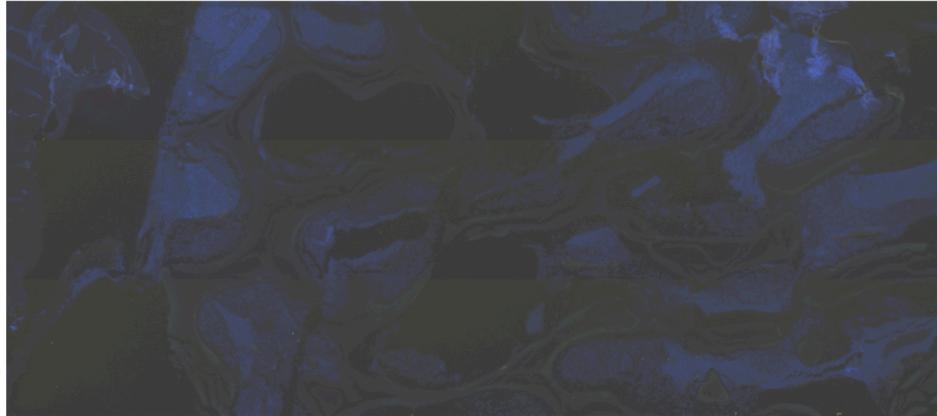
Figure 41: Bio-oss scaffold at time 0 without human cells, CY5 stain +DAPI; fluorescent microscopy 10X O.M.

On the other side, Bio-oss® scaffold carrying GFP+ placental cells was positively stained with CY5-anti-GFP antibody. As stated before, in this case too, nuclear stain was unfortunately useless to identify cells. As shown below, cells had efficiently settled on the scaffold (fig. 42).

Errore. L'origine riferimento non è stata trovata.

Figure 42: Bio-oss scaffold at time 0 carrying hCMSCs, CY5 stain+DAPI; fluorescent microscopy 10X O.M. (left) and light microscopy of hematoxylin-eosin stain 10x (right)

Image below (fig. 43) shows a detail of previous image that points out cell presence and distribution, as in this case cells are clearly visible as single red units on the scaffold.

30-days samples

Samples recovered from rat sacrificed at day 30 were analysed as well. Image below (fig. 44) shows hematoxylin-eosin stain of the dish without human cells. As can be better appreciated from following details (fig. 45), a clear cell infiltrate is present, which looks like a soft connective tissue. Healing was efficient without significant inflammation. This supports the idea that the scaffold actually offers a suitable environment for cell colonization.
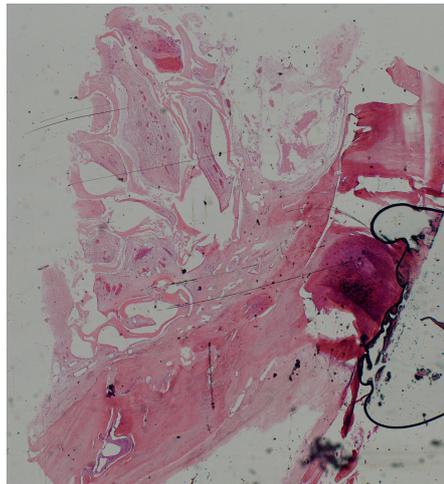
Figure 44: Bio-oss scaffold at 30 days without hCMSCs, hematoxylin-eosin stain; light microscopy 4X O.M.

In the analogous sample bearing hCMSCs, a clear sign of human cell proliferation is detectable along trabecular structure and pores (fig. 46), as shown by GFP stain which has a rosettelike distribution (fig. 47). This confirmed that placental cells actually survived after transplantation and found in the scaffold a suitable environment for proliferation. No placental cells are present in the lining soft connective tissue, which was formed by host cells, and stained positive for DAPI but not for CY5-GFP. Rat cells can be appreciated filling some spaces of the scaffold as well (fig.48).
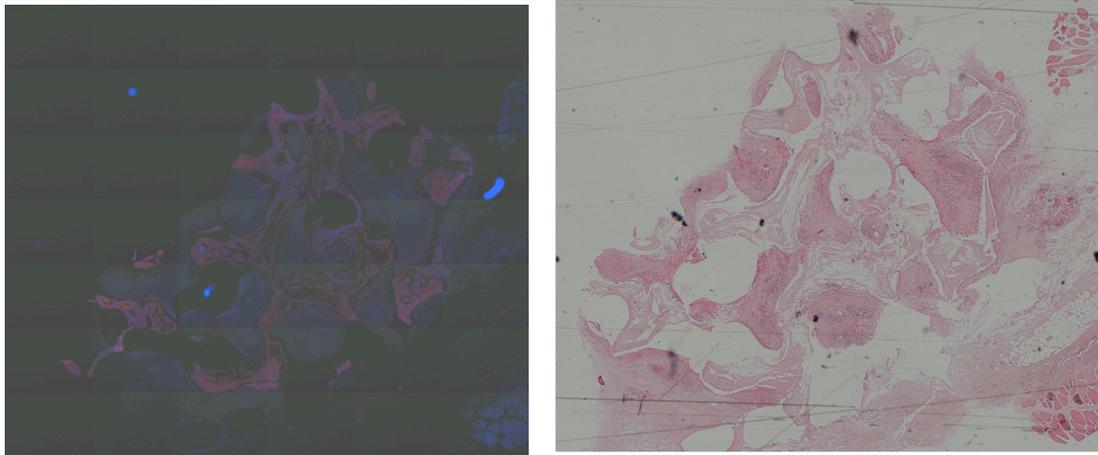
Figure 46: Bio-oss scaffold at 30 days with hCMSC, left, CY5 stain, fluorescent microscopy; right, hematoxylin-eosin stain; light microscopy 10X O.M.
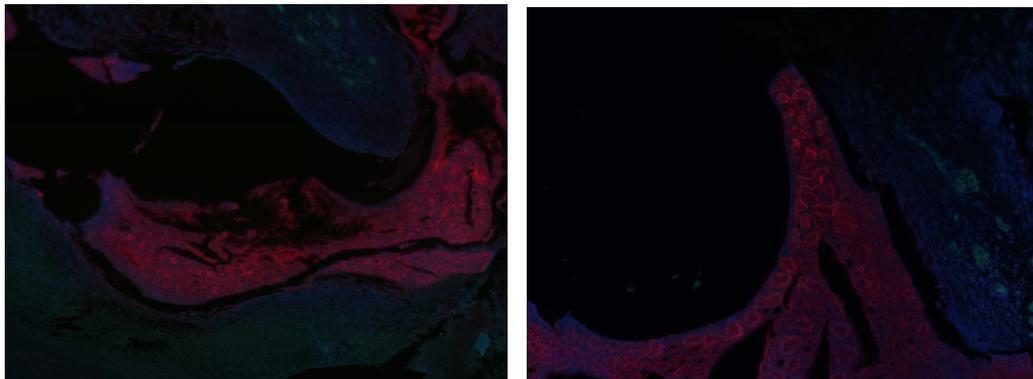
Figure 47: Bio-oss scaffold at 30 days with hCMSCs: detail of human cells colonizing Bio-oss scaffold; fluorescent microscopy 40X O.M.
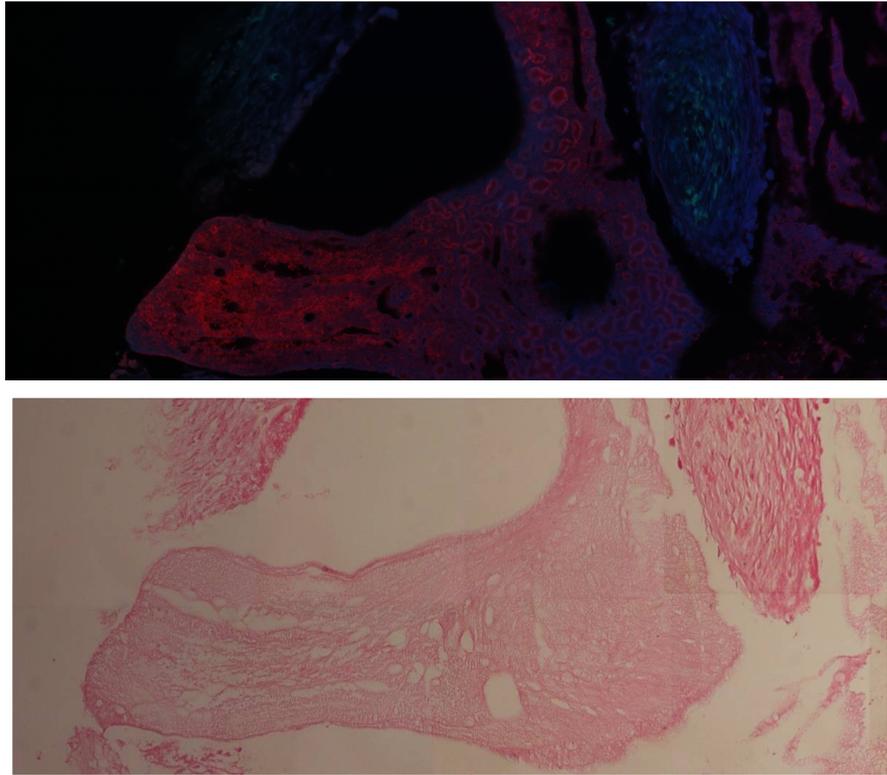
Figure 48: Bio-oss scaffold at 30 days with hCMSC, up, CY5 stain + DAPI, fluorescent microscopy; down, hematoxylin-eosin stain; light microscopy 63X O.M.

60-days samples

The sample not bearing placental cells recovered from rats sacrificed 60 days after surgical intervention showed no significant differences from the analogous sample at 30 days.

On the other hand, the scaffold bearing placental cells shows a strongly positive reaction to anti-GFP antibody, testifying a consistent proliferation of placental cells, much greater than the corresponding 30-days sample. Also at this time point, hCMSCs show a lining distribution on scaffold surface but not a colonization of intratrabecular spaces.
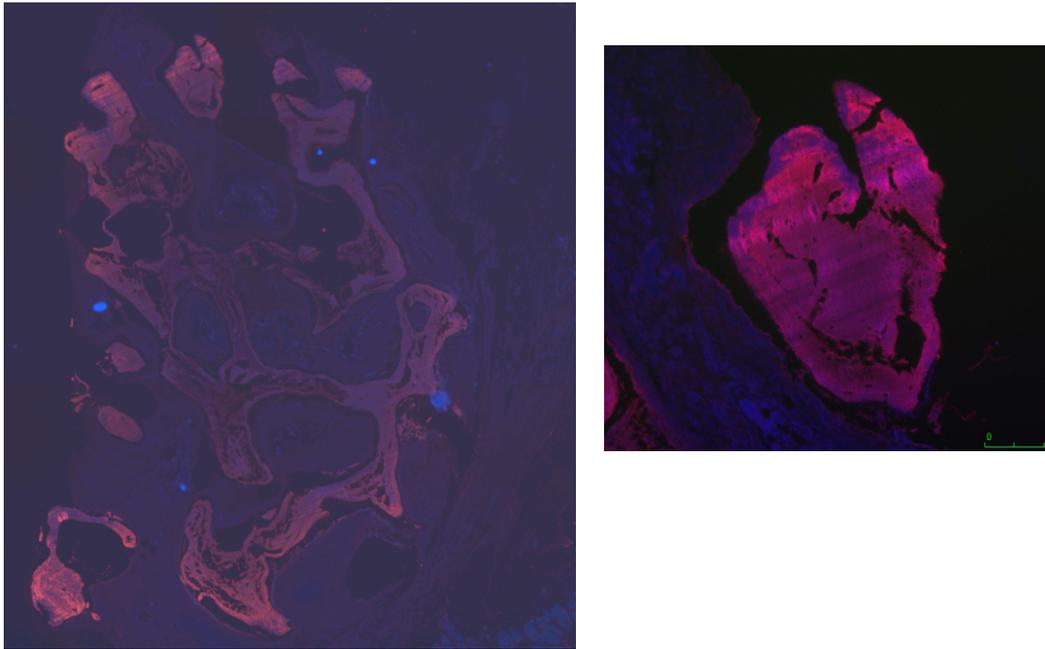
Figure 49: Bio-oss scaffold at 60 days with hCMSCs, CY5 stain + DAPI, fluorescent microscopy 4X O.M. (left) and detail 63X O.M. (right)

The general appearance of tissue filling trabecular space reminds that of a soft spongious tissue, growing as a discrete unit from the central to the peripheric zone of the scaffold without a continuity with native bone. This is in accordance with the observation of Wang et al who reported that in cranial defects "ossification process occurs at different discrete sites simultaneously and […] from those sites, the ossification spreads out to form new ossification sites. Maintaining a high number of these sites with continually stimulated cells that are proliferating and differentiation is an absolutely necessary event to assure the healing of a critical size defect in the cranium."[90] In our case the source of proliferating cells are not the dura and underlying subcutaneous tissue as proposed by Wang but rather hCMSCs which filled the empty spaces and, perhaps, restricted the role of dura in healing to a nutritious one.

On the whole, mesenchymal placental cells seem to have a positive role in healing of critical size defects. It is very worth noting that no signs of rejection or inflammation due to the presence of foreign cells can be observed in rats treated: this can be due to the peculiar immunological characteristics of placenta and of

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[90] Wang J, Glimcher MJ. Characterization of matrix-induced osteogenesis in rat calvarial bone defects: II. Origins of bone-forming cells. *Calcif Tissue Int* (1999) **65**: 486-493

cells deriving from it, which makes them suitable even for heterologous transplantation. As for the ability to replace damaged tissue and to restore tissue functionality, some improvement should be made in order to drive cell commitment towards the tissue of interest. Actually the filling of cranial defects by placental cells is massive and on the whole significantly better if compared to the natural healing potential of the organism, however the stiffness of the newly formed tissue is suggestive of a soft tissue rather than a stiff cranial bone. This could be overcome by using cells which are previously grown on the scaffold, or by adding locally growth factors that can help ossification.

Summary

Results collected in the last section of this PhD thesis, testify that hCMSCs possess an osteogenic potential if cultivated in appropriate condition. Actually if a canonical medium was used, hCMSCs showed only a scanty differentiation towards the osteogenic lineage and only small calcified nodules were visualized. In order to improve this results, cells were grown in presence of collagen or Bio-oss$^®$. The presence of collagen didn't influence gene expression of hCSMCs as well as that of human osteoblasts taken as term of comparison. On the other hand, the presence of Bio-oss$^®$, especially if combined with an osteogenic medium, allowed a better differentiation and maturation of cells into osteoblasts or osteocytes.

In experiments performed in vivo, hCMSCs confirmed their ability to settle and proliferate on Bio-oss$^®$ scaffolds. Moreover those cells were able to fill the created defect by forming a soft compact tissue: by driving their commitment with the help of growth factors, those cells could constitute a good cell source for tissue regeneration.

Conclusions

The improvement of life quality and lifespan lengthening in Western countries leads as a consequence a more and more urgent need for organs and tissues replacement. As organs and tissues derived from heterologous donors are not easily available and, moreover, oblige to a life-spanning use of immunosuppressive drugs, effective alternatives are needed.

The use of adult stem cells for tissue repair, and in particular mesenchymal stem cells, is at present focused on cells deriving from bone marrow that is currently the main source of those cells. However MSCs in bone marrow constitute a rare population, accounting for 0.01-0.001% of the nucleated cells population.

Present work reports a protocol that allows the isolation of a homogeneous population of mesenchymal stem cells from placenta by the combination of plastic adherence and subsequent cell sorting of CD34^{neg} cells. Such homogenous population displays the typical characteristics of mesenchymal stem cells derived from bone marrow, adipose or pancreas derived cells and exhibits clonal properties, as testified by the analysis of surface markers and doubling time. The percentage of cells showing clonal characteristics account for about 0.01%: this is a very outstanding datum, if compared to bone marrow-derived MSCs as both the size of the organ and the high percentage of cloning forming units constitutes, together with their relative ease of isolation, a promising starting point in terms of quantity and efficiency of cells that could be used for transplantation.

Moreover, placental cells display low immunogenicity and immunomodulatory properties and do not give rise to teratomas in SCID mice

As far as their differentiation potential, evidence was provided on the ability of placental cells to differentiate into osteoblastic cells as well as into adipoblasts and chondroblasts when proper culture conditions are adopted.

In particular, concerning chondrogenic differentiation, adopted culture conditions are actually useful to get the induction of the expression of

chondrogenic genes on hCMSCs, but some work still has to be done in order to obtain a clear hyaline cartilage to satisfy the target of articular cartilage regeneration. Anyway, the fibrocartilage which was so far obtained could constitute a good basis for musculoskeletal fibrocartilages regeneration or for osteogenesis recapitulating endochondral ossification.

Results collected in the last section of this PhD thesis, testify that hCMSCs possess an osteogenic potential if cultivated in appropriate conditions. Actually if a canonical medium was used, hCMSCs showed only a scanty differentiation towards the osteogenic lineage and only small calcified nodules were visualized. In order to improve these results, cells were grown in presence of collagen or Bio-oss®. The presence of collagen didn't influence gene expression of hCSMCs as well as human osteoblasts taken as term of comparison. On the other hand, the presence of Bio-oss®, especially if combined with an osteogenic medium, allowed a better differentiation and maturation of cells into osteoblasts or osteocytes.

In experiments performed in vivo, hCMSCs confirmed their ability to settle and proliferate on Bio-oss® scaffolds. Moreover those cells were able to fill the created defect by forming a soft compact tissue: by driving their commitment with the help of growth factors, those cells could constitute a good cell source for tissue regeneration.

Data collected in this PhD thesis suggest that placenta is a promising source of cells for tissue regeneration. The sum of phenotypic characteristics and differentiation potential described so far encourages to perform further in-depth examinations, which could lead to useful applications in the field regenerative medicine.

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