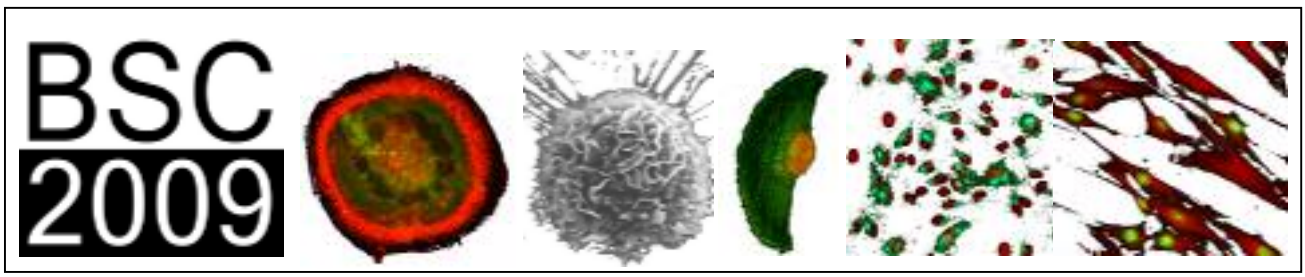

Bone Stem Cells

A European Science Foundation Workshop

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MACROPOROUS BRUSHITE BONE CEMENT

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*Aims:*The aim of the current work was to create a bone cement having a macroporosity able to let bone grows and to study the decrease of mechanical strength due to the presence of macroporous.
Method: Brushite bone cements were produced by manual mixing of a solid phase made of equimolar quantities of β-tricalcium phosphate (β-tcp: $Ca_3(PO_4)_2$ assay ≥96% (Fluka Germany), Monocalcium phosphate monohydrate (MCPM: $Ca(H_2PO_4)_2XH_20$,
assay min. 98%) (Sharlau, Spain) and mannitol single crystals in volume fractions between 0 and 15% vol, with a liquid phase consisting in a sodium citrate solution. The ratio between the amounts of the solid and liquid phase was fixed at a value of 3.
The mannitol crystals, used as a porogen and obtained from a re-crystallization in a saturated solution in de-ionized water of commercial mannitol were of acicular shape and were sieved for a diameter in the range 250-500 μm, with a shape factor between 3 and 6 (Figure 1).
Compressive test of brushite samples having cylindrical shape with 16mm in length and 8mm in diameter, were performed using a servo-hydraulic machine Instron 8501, at a loading rate of 1mm/min starting from a preload of 0.2kN
The osteoblast cell line MG63 was used to test the cellular affinity to the surface of differently produced samples.
Results: Results of the compressive test, made on 6 samples for each formulation are given in Table I as mean values and standard deviation. Citotoxicity and cells adhesion efficiency of different samples was qualitatively assessed through optical microscope by using Toluidine Blue (TB) staining, respectively after 2 hours and 48 hours of cells culture onto scaffolds (Figure 2).
SEM photograph of the surface of macroporous Brushite cement is shown in Figure 3.
Conclusion:

- the maximum compressive strength of all samples tested was found higher than that of cancellus bone (12 MPa)*
- the dissolution of mannitol by cement during adhesion test does not negatively affects cellular adhesion
- cells were able to adhere and spread on the surface scaffolds

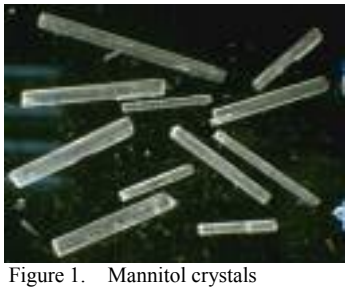

Figure 1. Mannitol crystals

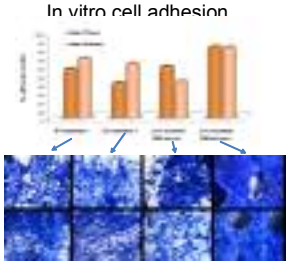

Figure 2. Cells adhesion

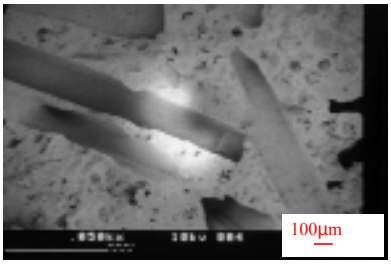

Figure 3 . SEM photograph (magnification 50 x)

% v/v mannitol	<σc> [MPa]	Stand.Dev. σc [MPa]	<E> [GPa]	Stand.Dev. E [GPa]
0	32.6	0.8	4.2	0.3
5	24	1.1	2.8	0.6
10	20.4	1.7	2.6	0.4
15	17.5	1.6	2.5	0.2

Table I . Compressive test.

* Hench LL. Bioceramics. J Am Ceram Soc. 1998; 81: 1705–28

2D AND 3D DIFFERENTIATION OF HUMAN STEM CELLS FOR BONE TISSUE ENGINEERING

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Aims: Mesenchymal stem cells (MSC) are a useful source for bone tissue engineering strategies, however, the self-renewal and proliferative capacity of these multipotent cells is restricted and appears to decrease with age.
Human embryonic stem cells (hESC) have unique dividing capacities and pluripotent characteristics making them a promising source for tissue engineering and regenerative medicine. To make *in vivo* applications with hESC possible, the current culture methods in the field of expansion as well as differentiation should be optimized and/or adapted. More specific for bone tissue engineering, differentiated cells should be available on a 'cell delivery' system that protects them during the encapsulation of cells in an *in situ* hardening material in the bone defect.
The purpose of this study is to compare hESC, bone marrow stromal cells (BMSC) and adipose tissue derived cells (ATMSC) on their: 1) expansion efficiency, and 2) osteogenic differentiation in 2D and 3D on a 'cell delivery' system
Methods: Genetic normal hESC (H1, VUB01) are used in all experiments. hESC are cultured on MEFs in serum-free stem cell medium and passaged by mechanical splitting of the colonies. The cells are screened on the maintenance of their pluripotent characteristics and embryoid body formation. Morphological, immunocytochemical and RT-PCR analyses are performed. Pluripotency is evaluated by alkaline phosphatase (ALP), Oct-4, Nanog, SSEA-3 and SSEA-4, TRA-1-60 and TRA-1-81. To induce differentiation, hESC colonies were collected after 40 minutes of collagenase type IV treatment, washed and centrifuged. The cell clumps were re-suspended in differentiation medium (containing 20 % FCS) and plated on 0.1 % gelatin-coated culture dishes. After 24 days in culture, the cells were trypsinized and passaged at a 1/3 ratio until P5. This induction method was compared with obtaining single cells by trypsinization of the detached colonies. These single cells were then further differentiated until P5.
BMSC (Lonza) and ATMSC were expanded in MesenPRO culture medium until P5.
The osteogenic differentiation was studied in 2D and 3D cultures.
2D: hESC-derived cells, BMSC and ATMSC are seeded at a concentration of 40 000 cells/well in 24-well culture plates in osteogenic medium (alfa MEM + 10 % FBS + 100 µM L-ascorbic acid 2-phosphate + 10 nM dexamethasone + 10 mM β-glycerophosphate). The cells were analyzed after 4, 14 and 21 days.
3D: hESC-derived cells, BMSC and ATMSC are seeded at a concentration of 2-6 million cells on 0.09 g CultiSpher S microcarriers. After 2 days, the cell-seeded microcarriers were transferred to a dynamic culture system and cultured in osteogenic medium The cell-seeded microcarriers were cultured for 40 days and analyzed at specific time-points.
The cultures were evaluated by fluorescence microscopy (calcein AM and propidium jodide staining) and by histology techniques (ALP, H&E, Trichrome Masson, von Kossa, cbfa-1 and osteocalcin staining).
Results: During 24 days of pre-differentiation of hESC, different cellular morphologies appear in the culture dish. After trypsinization and repeated passaging, a morphologically homogeneous population of fibroblast-like cells was obtained after 4 passages. The cell yield varied from 1-12 million differentiated cells derived from 1 culture dish of colonies. For the 2nd pre-differentiation protocol, also a homogeneous population of fibroblast-like cells was obtained after 4 passages. The cell yield varied from 1,3-3,7 million differentiated cells derived from 1 culture dish of colonies. BMSC and ATMSC had a population doubling time of 52 hours respectively 24 hours.
2D osteogenic differentiation revealed strongly positive ALP and von Kossa staining. ALP and von Kossa staining was most intense for hESC and ATMSC in comparison to BMSC.
During the 3D osteogenic differentiation of hESC-derived cells, BMSC and ATMSC on microcarriers, the cells remained viable even after 40 days in culture. The cell-seeded microcarriers formed clusters after 14-21 days. Histology of the microcarriers revealed fully colonization of the microcarriers. The cells formed an extracellular matrix (ECM) with eosinophilic structures resembling bone structure (osteoblasts secreted an ECM with embedded osteocytes with a lacunae around) and was most obvious for ATMSC. With Trichrome Masson staining, a distinction can be made between newly formed osteoid (pink) and mineralized ECM (blue).
Conclusions: Osteogenic differentiation of hESC- and adipose tissue derived cells can be studied in 2D, but the risk of dystrophic calcification due to senescence is high. A better method is to study the osteogenic differentiation in 3D on microcarriers. This gives also the advantage that the differentiated cell-seeded microcarriers can be encapsulated in an *in situ* forming material in the bone defect.
Bone formation of hESC- and adipose tissue derived cells on CultiSpher S microcarriers can be obtained but the bone formation is heterogeneous and limited to specific areas.

PREDICTION PARAMETERS FOR THE FUNCTIONAL SUITABILITY OF HUMAN MESENCHYMAL STEM CELLS FOR ECTOPIC BONE FORMATION

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Aims: Usually, bone defects are successfully treated with autogenous or allogenic transplantation. However, in patients at risk, such as diabetics or smokers, large bone defects or fractures often do not heal sufficiently. New techniques to heal bone defects include bone substitute materials seeded with human mesenchymal stem cells (MSC). Considerable donor variability was reported for human MSC in ectopic bone formation assays, indicating functional deficits in expanded human MSC of some donors compared to others. The aim of this study was to identify functional donor-dependent correlates of ectopic bone formation in order to predict the ability of human MSC to form bone.
Methods: MSC were isolated from fresh bone marrow samples of 16 donors (aged 10-74 years) and expanded until passage 2 or 3. ß-TCP granules (Ceros®82, RMZ Switzerland) were mixed with undifferentiated MSC and fibrin glue and implanted into subcutaneous pouches of SCID mice. All constructs were explanted 8 weeks after transplantation to determine the amount of ectopic bone by histological analysis (H&E staining, Alu in situ hybridisation). At the time of transplantation, the following parameter were determined in vitro: calculation of generation time, determination of growth rate (3H-Thymidine assay), adipogenic differentiation (Oil Red O) and osteogenic differentiation (measurement of calcium content and alkaline phosphatase (ALP) activity). In addition, total RNA of undifferentiated MSC samples were stored for later microarray analysis.
Results:
Ectopic bone formation in vivo
Ectopic bone formation occurred in 8 out of 16 MSC samples. Pores within the ceramics were either filled with loose fibrous connective tissue or with newly synthesized bone matrix. Alu in situ hybridisation confirmed the human origin of bone forming cells. In constructs without ectopic bone, most of the human cells had disappeared.
Proliferation, generation time and multipotency of MSC in vitro
Both, growth rate and generation time of MSC in vitro showed huge donor variability. MSC, which were able to form ectopic bone in vivo showed a significantly higher 3H-Thymidine uptake ($p \leq 0.001$) and a shorter generation time ($p \leq 0.016$) in vitro than MSC without bone formation. MSC from all donors were able to differentiate along the adipogenic and osteogenic pathway according to lipid deposition and accumulation of a calcium-rich matrix. However, donor MSC which formed bone in vivo had significantly higher ALP values during osteogenic in vitro induction ($p \leq 0.005$) than MSC which did not form bone.
Conclusions: Our experiments showed that a low generation time prior to transplantation and high ALP activity during osteogenic differentiation in vitro correlated positively to ectopic bone formation in vivo. In contrast to this, slowly growing MSC had not the ability to survive inside the host and to differentiate into osteoblasts in a calcium-rich environment. The amount of deposited mineralized matrix in vitro did not correlate with the capability to form bone in vivo. Further analysis has to focus on the identification of parameters that stimulate slow growing MSC to proliferate and to regain their capability to reproducible form bone in vivo.

OSTEOBLAST FUNCTION/DIFFERENTIATION MODULATED BY INTRACELLULAR NANOTRANSDUCERS BASED ON PIEZOELECTRIC NANOTUBES

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Aims: Boron nitride nanotubes (BNNTs) have been recently drawn attention from the scientific community further to the multidisciplinary exploration of carbon nanotubes. BNNTs show unique chemio-physical properties, such as an excellent piezoelectricity. To date, because of their novelty, interactions of BNNTs with biological systems are still largely unexplored. Bone tissue and osteoblasts are known to be responsive to both mechanical and electrical stimulation, representing interesting biological models to study the efficacy of BNNTs as intracellular nanotransducers
In this study, BNNTs were innovatively investigated as non-viral nanovectors to convey electrical/mechanical signals to a cellular system exploiting BNNT piezoelectricity [Patent # FI2009A000076, 19/04/2009]. Cell uptake and cytotoxic effects of BNNTs were studied. Afterwards, intracellular electrical stimuli were wirelessly administrated to cell cultures using an outer mechanical stress (i.e., ultrasounds). The *in vitro* effect of ultrasound-activated BNNT-induced stimulation on both primary human osteoblasts (hOBs) and human mesenchymal stem cells (hMSCs) isolated from the dental pulp (DP) (undifferentiated, osteo- and chondro-induced) was explored by patterning the main markers involved in their function and differentiation pathways.
Methods: Primary hOBs were isolated from trabecular bone fragments and expanded in non-differentiating culture medium (CM). BNNTs were suspended in poly-L-lysine phosphate buffer solution as a homogeneous dispersion and used to supplement the CM. As a first step, cytocompatibility of BNNTs was investigated up to 72 h with MTT test. Afterwards, live HOB uptake was evaluated using quantum-dot labeled BNNTs and BNNT intracellular localization was detected via Transmission Electron Microscopy (TEM). Finally, HOBs, either incorporating or not BNNTs, underwent *in vitro* ultrasound (US) stimulation for 1 week. The treatment consisted of multiple 5 s exposure to a US source working at 40 kHz and 20 W. Osteoblast-related gene expression (Runx2, Coll I, ALP, OPN and OCN) was evaluated by reverse transcriptase polymer chain reaction (RT-PCR). This study was also repeated using DP-hMSCs cultured in regular, osteo- and chondro-inducing CM for 21 days.
Results: Not significant cytotoxicity up to 15 μg BNNTs/ml CM was observed, so working concentrations ranging in 5-10 μg/ml were selected. BNNTs resulted homogeneously internalized in about 6 h. Additionally, TEM revealed that BNNT were internalized inside cytoplasm vesicles. HOBs and DP-hMSCs, subject to US daily cycles, were viable and metabolically active till the endpoints. In the hOB experiment, expression of Runx2 resulted downregulated specifically by BNNTs, while OPN expression level was upregulated specifically by US. Coll I expression was unaffected, while ALP and OCN expressions were synergistically affected by both BNNT and US treatments. In particular, ALP was synergistically downregulated, while OCN was synergistically upregulated by the combined stimuli (BNNTs+US). Preliminary results with DP-hMSCs also suggested strong influence of BNNTs/US on their differentiation pathways.
Conclusions: After supplementing BNNTs up to 10 μg/ml in the CM, neither short-term cytotoxic nor significant apoptosis was revealed. We conversely obtained, for hOBs, a panel of findings compatible with osteoblastic maturation. This method was found to act through modulating the expression of several osteoblast-related genes, confirming evidences of osteoblast sensitivity to piezoelectricity mediated by BNNT-based nanotransducers. Investigations on MSC differentiation are in progress to assess a potential role of BNNT nanotransducers as bone-inducing non-viral nanovectors for bone repair and tissue engineering.

ADHESION AND PROLIFERATION OF HUMAN MESENCHYMAL STEM CELLS ARE INFLUENCED BY DIFFERENT BIOMATERIALS

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Introduction: In the orthopaedic perspective, tissue engineering is focused on the development of innovative materials, whose action consists in recruiting bone progenitor cells and in stimulating their proliferation [1]. In this study, we investigated the effects of hydroxylapatite (HA) biomaterials, which differ in composition and form, on human mesenchymal stem cells (hMSCs). Doubling time variability, among hMSC samples and their passages, was investigated in "in vitro" cultures The AlamarBlue test was employed to analyze the cell viability, biocompatibility, adhesion and proliferation on different biomaterials. hMSCs morphology was evaluated during their growth on biomaterials by scanning electron microscopy (SEM) analysis. Cytoskeleton was investigated by immunofluorescence assay to evaluate the effects of biomaterials on cell structure and organization [2]. Quantitative data were statistically analyzed by Student t test.
Results and Conclusions: hMSCs were isolated from bone marrow samples of orthopaedic patients by density gradient. Flow cytometric analysis was performed to characterize cell population towards specific mesenchymal stem cell surface markers. hMSCs were characterized with specific monoclonal antibodies and selected for the antigenic profile showing the markers CD105+, CD29+, CD44+, CD71+, while were CD45- and CD235-. These hMSC populations grown as a confluent monolayer in approximately 3-4 weeks and exhibited a fibroblast-like morphology. Comparative analyses of cell viability, adhesion and proliferation have been carried out on biomaterials. The best result has been obtained with the biomaterial in block, named ENGIpore, which is composed of a high porosity hydroxyl-apatite. Specifically, the cell adhesion increased of the 40% compared to the control represented by the plastic material of the Petri dish. The proliferation assay, at 120h, indicated that the number of cells grown on biomaterials was higher than that of the control. SEM and immunofluorescence analysis showed that hMSCs morphology and cytoskeletal architecture on biomaterials was indistinguishable from that of hMSCs grown on plastic petri dishes, used as a control. Our data indicate that the specific shape and the chemical composition of different biomaterials influence the viability, adhesion and proliferation of hMSCs [3].

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Acknowledgments
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A MODEL OF BIOINSPIRED COMPOSITE MATERIAL WITH IMPROVED BONE FORMING EFFICIENCY

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The combination of synthetic polymers and calcium phosphates represent an improvement in the development of scaffolds for bone-tissue regeneration. Ideally, these composites provide both mechanical and architectural enhanced performances. However, the design and processing of such composites is often lacking biological properties, such as osteoconductivity and cell bioactivation.
In our study we attempted to generate, through a biomimetic approach, a composite bone substitute maximizing the available osteoconductive surface for cell adhesion and activity.
Highly porous scaffolds were prepared through particulate leaching method, combining poly-epsilon-caprolactone (PCL) and hydroxyapatite (HA) particles, previously coated with a sucrose layer, to minimize their embedding by the polymer solution; composite performances were evaluated both in vitro and in vivo.
In PCL-sucrose-coated HA samples, HA particles were almost completely exposed and physically distinct from the polymer mesh, while control samples displayed ceramic granules massively covered by the polymer. In vivo results revealed a significant extent of bone deposition around all sucrose-coated HA granules, while only a part of HA granules of PCL-uncoated-HA scaffolds were surrounded by bone matrix.
These findings highlight the possibility of generating osteoconductive substitutes

SELF-MINERALIZING POLYMER BASED POROUS SCAFFOLDS FOR BONE TISSUE ENGINEERING

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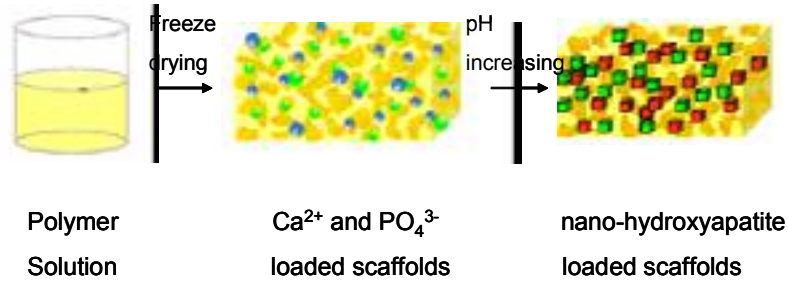
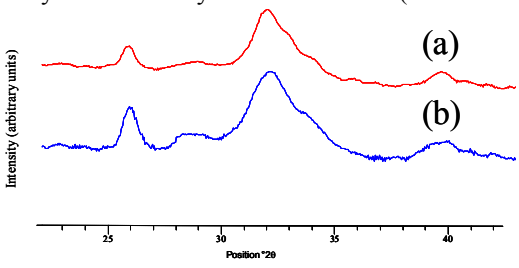
Aims: One goal of tissue engineering is to replace lost or compromised tissue function, and an approach to this is to control the interplay between materials (scaffolds), cells and growth factors to create environments that promote the regeneration of functional tissues and organs. In addition to acting as a bare physical support, scaffolds can be engineered to provide biological functions and actively induce bone tissue regeneration.
Aim of this study is the developing of simple novel fabrication processes tuned to the production of polymer based, self-mineralizing porous scaffolds. As self-mineralizing we intend their ability to *in situ* promote bone-like, nano-crystalline hydroxyapatite (HA) growth.
Methods: Typically a ranging from 1 to 5 % *wt* polymer solution in aqueous medium has been prepared using low and high density chitosan and polyvynil-pyrrolidone (PVP) powders. They have been loaded with calcium and phosphate salts in a 1.67 molar ratio useful to induce the nano-hydroxyapatite crystallization. Chitosan solutions have undergone freeze drying while PVP solutions have been cross linked by γrays to obtain gels prior the lyophilisation. The obtained polymeric porous sponges went through freeze drying at −60 °C under vacuum (3 mbar) for 12 hours.
HA crystallization has been induced by dipping the obtained scaffolds in either sodium hydroxide, either arginine (pH = 9) solution. Contemporarily, unloaded chitosan and PVP scaffolds have been prepared in the same condition for comparison. The *in situ* produced composites have been characterized by Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), X-ray diffraction (XRD), Fourier Transform and Attenuated Total Reflectance Infrared Spectroscopy (FTIR-ATR) and Thermogravimetric analysis.
Studies on cell attachment and proliferation, cell morphology and osteoblasts differentiation are currently in progress.
Results: Presumably osteoinduttive, nanohydroxyapatite loaded polymeric scaffolds have been obtained by a simple novel "in situ" crystal growth method. The scheme below resumes how hydroxyapatite nanocrystals can be formed into the scaffold structure by an external switching corresponding to a pH increasing.



The HAps diffraction pattern was very similar to that recorded for deproteinated bone apatite, and this was quantitatively confirmed by the similarity of the two crystal domain sizes (230 and 213 Å, respectively).



Powder X-ray diffraction patterns, relative to: deproteinated bone HA nanocrystals (a) and HA nanocrystals grown inside the chitosan and PVP scaffolds (b).

The biological properties of these materials and their potential application in bone tissue engineering, examining growth and differentiation of murine osteoblasts are currently under investigation.
Conclusions: A sort of "stimuli responsive material", based on polymeric scaffolds able to store calcium and phosphate ions and to produce bone-like hydroxyapatite has been assessed. The property of this assembly could open nice perspectives in mineralized and particular bone tissue regenerative medicine for the implication derived from the composite ability to *in situ* generate biomimetic HA nanocrystals.

A BIOMIMETIC BONE SCAFFOLD FOR POTENTIAL USE AS A CARRIER FOR STEM CELLS

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Introduction: Tissue engineering aims to create replacement tissues in situations where the body no longer has the potential to do so. In the body, tissues are organized into three-dimensional structures and in order to engineer functional tissues and organs successfully, the scaffolds have to be designed to facilitate cell distribution and guide tissue regeneration in three dimensions. In addition to providing tissues with the appropriate architecture and acting as a template, scaffolds can also act as carriers incorporating biological molecules that are known to promote signaling pathways that influence key cell functions such as migration, proliferation and differentiation.
Calcium phosphates materials have been shown to interact strongly with bone, due to their similarity to bone mineral. In this study, a porous β-metacalcium phosphate scaffold material was developed, and further optimized by the incorporation of bone stimulating factors BMP-7 (a pleitrophic morphogen) and PDGF (pro-osteogenic factor) both of which play critical roles in the regulation of cell migration, proliferation and differentiation
Materials and methods:
1. Scaffold material preparation
A mixture of monocalcium hydrogen phosphate and poly(vinylalcohol) powders in 4:1 weight ratio were pressed under a force of 10MPa to yield discs. These discs were subsequently sintered at 9000C to yield porous scaffolds via the burn out of the PVA and were sterilised by γ-radiation for biological evaluation.
2. Incorporation of growth factors
Growth factors, BMP-7 and PDGF-BB were infiltrated on to the β-calcium metaphosphate scaffolds at a concentration of 400 ng per sample (approximately 0.1g) prior to cell seeding. Loaded and unloaded scaffolds were investigated and a commercial HA was used as a control. Tissue culture plastic was used as a cell culture control. In vitro studies were performed using primary human alveolar osteoblasts (aHOB, 1x105 cell/ml); cells were cultured in Dulbecco's Modified Eagles Medium (DMEM), with 10% FCS (Foetal Calf Serum), 0.02M HEPES, 2mM L-Glutamine, 1% Penicillin/streptomycin (all Life technologies) and 150µg/ml Ascorbate. Cytocompatibility was assessed and proliferatio measured using alamar BlueTM assay. SEM was performed to assess cell morphology and adherence.
Results and Discussion
The micrograph shown in Figure 2 showed mixed macro and microporosity (80µm up to 400µm) (Fig2). The HOB cells were observed to grow on the surface of the scaffold with extended filopodia and also within the macropores (Fig 3). The scaffold was confirmed to biocompatible and proliferation in all cases was comparable to the commercial HA. An increasing trend was however, observed on days 1 to 21 including Thermanox, HA, β-calcium metaphosphate scaffolds (CMP) with loaded and unload growth factors (Fig 4).
Conclusions/Summary: A porous β-calcium metaphosphate biocompatible scaffold has been developed. Incorporation of BMP-7 resulted in an enhanced cellular response. SEM confirmed numerous cells within the macroporous structure. This scaffold has the potential as a tissue engineering scaffold and further work is ongoing to exploit its potential as a carrier for growth factors and for facilitating the formation of functional tissue for engineering bone.

Fig 1 Porous scaffold

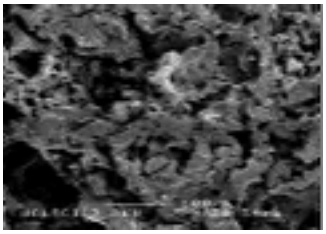

Fig 2 SEM showing open Porous structure

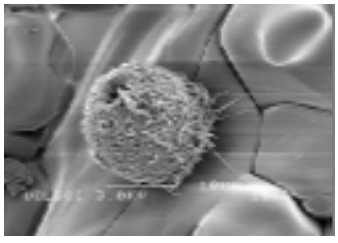

Fig 3

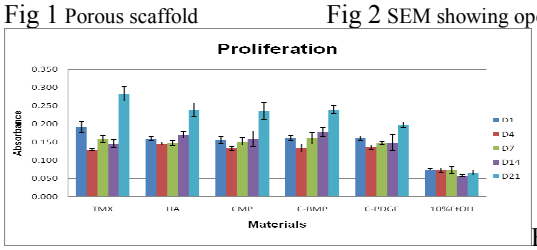

Fig4

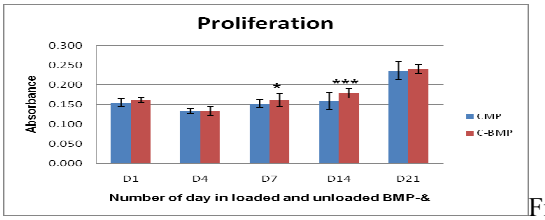

Fig5

Fig 4 Proliferation alamar BlueTM from days1 to 21. A significant increase (*P<0.05,***P<0.001) was observed in the BMP-7 loaded scaffold compared to the unloaded (Fig5). Fig 5 Cell proliferation data loaded and unloaded of BMP-7 on scaffolds

CHARACTERIZATION OF DIFFERENT MESENCHYMAL STROMAL CELLS ISOLATED FROM UMBILICAL CORD BLOOD (UCB-FMSC), AMNIOTIC FLUID (AF-FMSC) AND BONE MARROW (BM-MSC) AND COMPARISON OF THEIR OSTEOGENIC POTENTIAL.

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The presence of tissue injury triggers cellular mechanism favouring tissue-specific engraftment, and multipotent MSCs infusion has been proposed to treat severe genetic disorders or acute tissue injury because of their ability to self-renew and differentiate into specific functional cell types. The capacity of MSC to differentiate down the osteogenic lineage has been broadly documented and with increasing evidence for the presence of MSC in various fetal tissues, the challenge remains to identify an optimal MSC source for bone repair and regeneration.
Aim: Here we defined the differences between MSCs isolated from umbilical cord blood (CB), amniotic fluid (AF) and bone marrow (BM). Our first aim was to characterize the isolated populations by FACS analysis and proliferation capacity. For this purpose, we grew all the differents cells under exactly same conditions. In the second part of our study we better define the UCB-fMSC and AF-fMSC osteogenic potential.
Methods: Of all the generated hMSC lines their growth rate and viability have been tested at each passage. To confirm their identity and purity, hMSCs have been characterized. Following standardized differentiation protocols, we have been also able to demonstrate that MSC differentiate into all mesenchymal cells types. In this study, AF and CB samples were collected in the third trimester of pregnancy. The isolation efficiency for AF and CB was 62% and 15% respectively. Interestingly, in three cases we have been able to isolate fMSC from UCB and AF collected from the same donor at the same gestational age.
Results: When cultured at a low cell density, all the populations formed similar adherent clonogenic cell clusters; for the 3 populations a similar rate of cycling cells could be detected at different time points.
The purity of different MSCs populations was confirmed by the lack of expression of haematopoietic and endothelial-specific surface antigens such as CD45, CD34, CD14 and CD31 and by bright expression of CD73, CD146 and CD29. There was no difference between the immunophenotypes of the different MSC with the exception of CD105 (16% AF vs. 99 % BM $p<0.001$), HLA-ABC (56% CB vs. 80% AF vs. 37% BM) and CD146 (36% AF vs. 80% CB vs. 76% BM). Karyotype of the three populations revealed chromosomal stability.
Furthermore, chondrogenic and adipogenic differentiations were similar among different MSC sources; adipogenic differentiation showed that AF-fMSCs and CB-fMSCs produced few and small lipid vacuoles stained by oil red O.
To better evaluate their differentiation into osteogenic lineage, we cultured the cells in osteogenic medium and evaluated their capacity to form bone nodules at three different time points (at 14, 21 and 28 days) after osteogenic induction. The staining with alizarin red revealed that CB-fMSC and BM-MSC were able to form nodules earlier in the differentiation process. AF-fMSC formed small bone nodules only at the later time points.
Our second aim was to better evaluate the osteogenic differentiation potential of fetal MSC derived from CB and AF. AF-fMSC and UCB-fMSC share similar properties, so it might be useful define and compare their potential.
In vitro osteogenic differentiation of fMSCs expanded in osteogenic medium was assessed via expression of osteogenic markers, such as Runx 2, osteopontin (OP), osteocalcin (OC), alkaline phosphatase (ALPL), collagen type I alpha 2 (Col1A2) and osteonectin (OTN). Using quantitative RT-PCR we analyzed the expression level of these genes under basal and osteogenic permissive conditions during a controlled time-course study. At each time point the expression level of Runx 2, OP and OC was significantly higher in CB-fMSC compared to AF-fMSC. Differently, ALPL, Col1A2 and OTN have been detected at higher levels in AF samples. Ongoing studies will elucidated if similar results will be obtained analyzing the profile of a larger panel of genes involved in osteogenic differentiation.
Conclusion: The presented data have shown that AF-fMSC, UCB-fMSC and BM-MSC are good sources for bone repair and regeneration, even if some peculiar differences between the three populations should be considered and further research is still necessary to delineate their suitable clinical application.

MESENCHYMAL STEM CELLS FROM WHARTON'S JELLY: ENCAPSULATION IN ALGINATE MICROBEADS AND OSTEOGENIC DIFFERENTIATION

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Aims: We are valuating the potential of Mesenchymal Stem Cells from Wharton's Jelly of umbilical cord (WJMSCs) as osteoprogenitors, and identifying new potential osteoblast-specific proteins during osteoblastogenesis.
The advantages of using this source of stem cells are its non-invasive procurement and its vast abundance. This makes the use of WJMSCs particularly attractive for the development of innovative therapeutic strategies aimed to repair and replace damaged bone tissue. Nevertheless, many factors still hurdle the extensive clinical use of cell-based therapy. The protection of implanted cells from the host's immune response is, in fact, of primary importance. To solve this problem, one of the most promising approaches is represented by cell encapsulation within semipermeable capsular systems that are also able to prolong the cell viability and to sustain cell function.
In addition, in order to identify new potential osteoblast-specific proteins, we are also investigating the role of Slug transcription factor as mediator of canonical Wnt signaling, in relation to the expression profile of bone-related genes during osteoblast differentiation of WJMSCs.
Methods: We entrapped WJMSCs, after appropriate FACS analysis, in barium-alginate microbeads. Microencapsulation procedure is based on the generation of monodisperse droplets by a vibrational nozzle. An ionic alginate encapsulation procedure was employed for the microbeads hardening. Determination of WJMSCs secretory pattern was made by Bio-Plex analysis. Quantitative RT-PCR was used to analyze expression of specifi genes. ALP and mineralization tests were used to evaluate the osteoblast phenotype.
Results: Our procedure did not alter cell morphology and viability supporting osteoblast differentiation. In addition, as determined by Bio-Plex analysis, encapsulated WJMSCs maintained a secretive activity, suggesting that alginate doesn't prevent cell functionality, but, in some cases may promote it. The encapsulation induced a secretion increase of specific proteins such as GRO, MCP3, and HGF. At present, we don't know the correlation between the functionality of encapsulated WJMSCs and the over-production of two important chemokines (GRO and MCP3) involved in mesenchymal stem cell chemotaxis and a pleyotropic cytokine of mesenchymal origin (HGF, hepatocyte growth factor) promoting migration and survival of MSCs. Nevertheless, for example, it is interesting to underline that HGF is one of the factors with therapeutic potential in regenerative medicine mostly studied in the last years. Significative induction to osteogenesis was observed in microbeads-encapsulated WJMSCs.
Preliminary molecular data obtained by overexpressing some Wnt effectors (including Lef1 and □eta-catenin) and silencing Slug gene suggest a critical role of Slug in osteoblastogenesis.
Conclusions: Our data on the secretion of proteins determined by Bio-Plex suggest that encapsulated WJMSCs maintain a secretive activity. Therefore, it is possible to conclude that alginate doesn't prevent cell functionality, on the contrary, in some cases may promote it. Further studies are required to design and select the appropriate scaffold for specific application of MSCs to the tissue of interest. Nevertheless, our data support the idea that alginate-encapsulated WJMSCs may have an important role for tissue engineering strategies, and may be useful to improve a regenerative medicine approach for tissue repair based on mesenchymal stem cells. This latter point appear particularly important to the potential application of WJMSCs in clinic, in fact, issues about the safety of free Wharton' jelly derived MSC-based therapy should be considered, including the possibility to produce immunogenic responses or to induce tumor formation.
In addition, results about gene expresseion support the hypothesis that Slug functions as a novel regulator of osteoblastogenesis and may be considered a new factor required for osteoblast maturation.

STRONTIUM-INDUCING ADIPOSE TISSUE MESENCHYMAL STEM CELLS: EFFECTS ON DIFFERENTIATION INTO OSTEOBLASTIC PHENOTYPE

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Strontium (Sr^{2+}) is an alkaline earth trace metal cation that has a high affinity for hydroxyapatite. Strontium ranelate, an anti-osteoporotic agent with demonstrated antifracture efficacy, is composed of two atoms of Sr that are combined with ranelic acid. The latter is a carrier, while Sr^{2+} is the active cation with respect to the drug's skeletal effects. Previous work in this laboratory has demonstrated that adipose tissue mesenchymal stem cells (AMSCs) have the same ability to produce bone matrix as bone marrow derived stem cells, while being a better source of stem cells according to their abundance and accessibility. The aim of the present study was to evaluate a possible effect of Sr^{2+}on cell proliferation and on late-stage osteogenic differentiation of AMSCs.
Long-term cultures of AMSCs were performed. Cell growth and viability were assessed by [^{3}H]-thymidine incorporation assay and by growth curve analysis in the presence of Sr^{2+} from 1 to 150 μg/ml. Expression of osteoblastic markers (*ALP*, *COLIA1*, *OCN*, *OPN*, *RUNX2)* and of important osteoclastogenesis regulators (*OPG* and *RANKL*) was examined in all cell lines treated with different concentrations (1-10-100 μg/ml) of Sr^{2+}using quantitative RT-PCR, after 15 and 30 days from osteogenic induction. The data were normalized for *GADPH* housekeeping genes.
No statistically significant difference was observed in cell proliferation for all primary cell lines cultured in presence of the different concentrations of Sr^{2+}. Conversely, gene expression analysis showed that highest Sr^{2+} concentration strongly increased mRNA levels of *ALP*, *OCN* and *RUNX2* after 15 and 30 days from osteogenic induction. In addition, the presence of Sr^{2+} seems to increase the gene expression of the osteoclastogenesis inhibitor OPG and not to influence the mRNA levels of osteoclast formation mediator *RANKL*.
In conclusion our preliminary results showed that Sr^{2+}can promote osteoblast differentiation of AMSCs and also increase the *OPG/RANKL* ratio throughout the culture period, consistent with an effect to inhibit osteoblast-induced osteoclastogenesis. These findings open the possibility of the use of the Strontium ranelate for the *in vivo* treatment of cell transplantation in bone regenerations programs.

PROLIFERATION AND DIFFERENTIATION POTENTIAL OF MOUSE ADIPOSE DERIVED STROMAL CELLS ON TITANIUM SCAFFOLDS

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Aims: Mesenchymal Stem Cells (MSC) were originally isolated from bone marrow, but this source has some limitations, such as an invasive procedure, painful symptoms, low recovery of bone marrow and thus low yield of stem cells. Recently, pluripotent stromal stem cells have been detected within the adipose tissue. They are the so-called adipose-derived stem cells (ADSC). The simple surgical procedure, the easy and repeatable access to the subcutaneous adipose tissue, as well as the easy enzyme-based isolation procedures and the relative large quantities of available adipose tissue suggest that ADSC may represent an important source of autologous stem cells for regenerative medicine.
The aim of this study was to investigate ADSC adhesion, proliferation and osteogenic differentiation potential on titanium scaffolds (Ti-Scaffolds), which are widely used in orthopedic and dental implantology.
Methods: Stromal cells were obtained by collagenase digestion of mouse inguinal adipose tissue. The adherent fraction of stromal cells was cultured in expansion conditions (DMEM added with FBS 10% and antibiotics) and used for experiments after few passages. The MC3T3-E1 cell-line (immortalized osteoblasts) was used as control. ADSC were characterized by stemness gene expression profile in RT-PCR, by clonogenic ability (colony fibroblast forming units - CFU-F) and by multi-lineage differentiation ability (osteogenic, adipogenic and chondrogenic conditions). After few passages ADSC were seeded on Titanium grade 2 scaffolds and cultured in osteogenic differentiation condition for up 4 weeks. Viability and proliferation rate of cells were evalueted by MTT. At early and late experimental times osteogenic differentiation was assessed by alkaline phosphatase activity, expression of osteogenic markers and matrix mineralization. Cell morphology was analyzed with fluorescence and scanning electron microscopy.
Results: Mouse ADSC showed high proliferation capacity (until P_5) in our in vitro expansion conditions; moreover they are clonogenic and display multi-differentiation ability (adipogenic, osteogenic and chondrogenic) which was similar both on plastic and Ti-Scaffolds.
ADSC in expansion condition expressed the stemness genes Sca-1, c-Kit, nucleostemin, Islet-1 but not the embryonic marker Oct-4.
ADSC in osteogenic differentiation condition on Ti-Scaffolds were able to adhere and proliferate similarly to MC3T3-E1. ADSC, after 2 weeks in osteogenic conditions, expressed significantly high ALP activity (early osteogenic differentiation marker) compared to cells maintained in expansion medium. Osteogenic differentiation of ADSC on Ti-Scaffolds was confirmed by determination of cell mineralization by quantitative alizarin staining (late marker) and expression of osteogenic marker by RT-PCR (ALP, pro-collagen 1 and osteocalcin).
Conclusions: This work demonstrates that murine adipose stromal cells display stem-mesenchimal phenotype and are able to differentiate into osteoblasts on Ti-Scaffolds and can be suitable candidate for the generation of a stable cell-line to be used for further studies.

MESENCHYMAL STEM CELLS 'FACE-TO-FACE' WITH THE BONE MATRIX: THE ROLE OF A11B1 INTEGRINS

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Aims: Human mesenchymal stem cells (hMSCs), valuable candidates for bone tissue engineering, are shown to differentiate into osteoblasts in vitro as well as in vivo [1]. HMSCs are equipped with a variety of integrins that mediate essential cell-matrix interactions [2]. Since collagen I is the major protein component of bone, we questioned in our study whether the collagen-I binding, α11β1 integrin is indispensable for hMSC survival and osteogenic differentiation.
Methods: HMSCs (Lonza, Belguim) were cultured on collagen I-coated surfaces. A lentiviral transfer (Invitrogen, Germany) of α11-specific shRNA was applied for downregulating integrin α11β1. Quantitative PCR and Western Blot were used to validate the knockdown efficiency. Colorimetric adhesion assay was used to estimate the extent of cell attachment. HMSC spreading and migration was observed by time lapse (Zeiss, Germany). JC-1 staining (Invitrogen) was used for investigation of apoptosis. Osteogenic differentiation was performed as described in [3].
Results: In our analysis, α11-shRNA-transduced hMSCs were always compared to hMSCs transduced with control-shRNA (a sequence against non-mammalian mRNA). We achieved 95±4% knockdown of integrin α11 RNA, a finding that was also confirmed on protein level. Subsequently to the transduction, we did not notice pronounced morphological changes. However, a clear decrease of α11-shRNA-hMSC numbers was observed during cell cultivation. Using a quantitative adhesion assay, we estimated that only 30% of α11-shRNA-hMSCs were able to attach to collagen I. In contrast, at the same time point, control-shRNA-hMSCs reached 100% adhesion. Furthermore, a time lapse analysis showed that α11-shRNA-hMSCs took approx. twice the time for spreading on collagen I than control-shRNA-hMSCs and also had a migrational deficit. Since it was observed a progressive loss of α11-shRNA-hMSCs, we next performed JC-1 staining which visualizes mitochondrial depolymerisation, a hallmark of apoptosis. The majority of α11-shRNA-hMSCs exhibited mitochondrial leakage whereas control-shRNA-hMSCs showed intact mitochondrial staining. Finally, we performed osteogenic differentiation experiments in which the control-shRNA-hMSCs deposited calcified matrix. Intriguingly, the osteogenic stimulation of α11-shRNA-hMSCs resulted in further cell loss and not in matrix mineralization.
Conclusions: Our results strongly suggest that α11β1 integrin mediates an important signalling for hMSCs. Once the cells are ablated for this receptor, they reduce adhesion, spreading , migration and survival rates.

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STIMULATION OF VEGF EXPRESSION DUE TO MECHANICAL B1-INTEGRIN STRESS

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Aims: Mesenchymal stem cells possess the potential to differentiate into multiple lineages including osteogenic, chondrogenic or adipogenic cells dependent on environmental stimuli. Concerning the regeneration of bone mechanical forces play an important physiological role. It has been reported that mechanical forces commit the fate of mesenchymal stem cells towards osteogenic differentiation [1]. To study the underlying mechanisms of mechanotransduction in cells we applied mechanical stress to integrins on the apical surface of adherent cells using a technique developed earlier in this laboratory [2]. This study aims at analyzing biological responses to mechanical stimulation on β1-integrin receptors with regard to expression of differentiation markers and the release of cytokines. Furthermore, we hypothesized the involvement of the PI-3K/Akt-signaling pathway in these processes.
Methods: Human mesenchymal stem cells (hMSC) were isolated from bone marrow, which was obtained during median sternotomy. For the experiments the cells were cultured in expansion medium (EM) or in media suitable for osteogenic (ostDIM) or adipogenic (adiDIM) differentiation. Drag forces to the β1-integrin receptor subunits were applied for 15 min with a frequency of 1 Hz by a technique using anti-β1-integrin antibody coated paramagnetic microbeads which were exposed to an inhomogeneous magnetic field [3]. After application of mechanical stress, mRNA expression of alkaline phosphatase (ALP), Sox9 and PPARγ as markers for osteogenic, chondrogenic and adipogenic differentiation, respectively, was analyzed via Real Time RT-PCR as well as the expression of relevant proteins by using the xMAP™ Luminex assay and Western Blot. Furthermore, to test the role of the PI-3K/Akt signaling pathway in mediating mechanical loading, the stress was applied in the presence or absence of the PI-3K inhibitor LY294002.
Results: Application of drag forces on β1-integrin-receptors induced local distortions of the cell membrane in the proximity of bound beads, but no changes of the cell shape. This mechanical stimulation was sufficient to induce an immediate transient activation of the MAP kinase Erk as well as Akt, which could be inhibited by LY294002. Then the effect of integrin mediated mechanical stress on the differentiation of hMSC was investigated within 48 h. Application of a mechanical integrin stress induced an increased expression of the chondrogenic marker Sox9, but no increase in the expression of osteogenic and adipogenic markers were detected during this time. To test the effect of mechanical stimulation on a functional activity of hMSC we analyzed the release of VEGF. Both mRNA-expression and release of VEGF were increased when cells were cultured in EM as well as in ostDIM. Treatment with PI-3K inhibitor decreased both the mRNA expression and the release of VEGF in EM and ostDIM.
Conclusions: Mechanical forces mediated by integrins play a significant role in the control of functional activity of hMSC. By short-time mechanical loading the expression and the release of VEGF wer induced prior to an obvious differentiation of hMSC into an osteogenic phenotype. In addition, results indicate that the PI-3K/Akt signaling pathway plays a role in the release of VEGF due to mechanical β1-integrin stress.

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ADIPOSE TISSUE DERIVED MESENCHYMAL STEM CELLS: EFFECTS OF 2 NANOSTRUCTURED TITIANIUM ALLOYS ON OSTEOGENIC DIFFERENTIATION

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Establishing and maintaining mature bone at the bone-device interface is critical to the long-term success of prosthesis. Several variables influence the biocompatibility and osteogenic potency of hard biomaterials such as chemical composition and surface topography. Nano-scale features have been shown to affect cell adhesion, migration, cytoskeleton, proliferation and differentiation of a large range of cell types. Several studies with human mesenchymal and osteoprogenitor cell populations have shown strong cells responses to nanofeatures with increased osteoblastic activity on certain topographies. In our laboratory smooth Ti6Al4V surfaces have demonstrated to exhibit an osteoinductive action on human adipose tissue derived stromal cells (AMSCs), promoting their differentiation into functional osteoblasts and increasing bone formation. The aim of the present work was to evaluate the ability of nanostructured Ti6Al4V and Ti13Nb13Zr, compared to polystyrene (PS), to promote the differentiation and the maturation into functional osteoblasts on three primary cell lines of AMSCs, using normal human osteoblast cell line (NHOst) as control.
Cells were seeded onto Titanium alloys or PS and cultured for up to 40 days. Morphology, cell adhesion, proliferation, and differentiation were evaluated by Laser Scanning Confocal Microscopy analysis, cell counting, alkaline phosphatase activity evaluation, and mineralization study.
Both PA and NHOst cells showed a good viability and growth activity on the two nanostructured Titanium alloys, but lower to that observed on PS. On the contrary, all cells cultured on nanostructured Ti6Al4V and Ti13Nb13V displayed a statistically higher degree of adhesion than cells grown on PS. Interestingly the highest FCA/TCA values were observed when cells grown on Ti13Nb13Zr.
Undifferentiated PA cells are less committed to osteoblastic phenotype comparing to NHOst cells. However, incubation with OM stimulated better osteogenic differentiation in PA cells than in NHost cells, on all tested surfaces. Indeed in PA cells ALP activity increased during induction, whereas in NHOst cells ALP activity slightly increased until the 20th day of induction and then decreased thereafter.
Osteoblastic activity was confirmed, at the mineral level, by the production of calcium deposits and at the protein level, by COLIA1, OPN and OCN immunostaining. Production of hydroxyapatite crystals and bone extracellular matrix proteins were observed in every cell line, but interestingly only PA cells showed a statistically higher deposition of mineralized nodules on nanostructured Ti6Al4V and TI13Nb13V than on PS.
In conclusion, the results of this study showed that Titanium nanostructured alloys are capable to sustain the adhesion and proliferation of both PA and NHOst cells, but promote the maturation into functional osteoblasts only in PA cells.
The finding that nanostrutured Titanium alloys stimulate cell differentiation of PA cells towards an osteoblastic phenotype may lead to interesting alternatives in the design of efficient prostheses.

ENDOCHONDRAL BONE FORMATION BY HUMAN MESENCHYMAL STEM CELLS

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Aim: The general approach to bone tissue engineering is by combining a porous biodegradable scaffold with osteogenic cells. Thereby human bone marrow-derived mesenchymal stem cells (hMSCs) are differentiated *in vitro* directly into the osteogenic lineage by culturing the cells in the presence of the osteogenic differentiation factors such as dexamethasone and ascorbic acid. *In vivo* these cells then form bone via the process of intramembranous ossification. Recently it was discovered that embryonic stem cells form bone *in vivo* via the process of endochondral ossification. Thereby the cells were differentiated *in vitro* into the osteogenic lineage and bone was formed *in vivo* when implanted subcutaneous in an immune-deficient mouse. To study whether hMSCs are also susceptible to this process and to get a better understanding in the pathways involved in the differentiation processes, we performed a series of experiments.
Methods: HMSC were obtained from bone marrow aspirates from patients who had have given informed consent. After several population doublings to obtain a sufficient amount of cells, the hMSCs were seeded onto a porous Collagen GAG scaffolds. These constructs were cultured *in vitro* for four weeks in the following media; proliferation medium, chondrogenic medium and chondrogenic medium switched to osteogenic medium after three weeks. During *in vitro* culture samples were taken for *in vitro* analysis and micro-array studies. Next, the constructs were implanted subcutaneous for eight week in immune-deficient mice. After explantation the constructs were embedded in paraffin and sections were made which were stained with H&E for light-microscopic evaluation, also the *in vitro* constructs were evaluated.
Results: Sample analysis is ongoing but preliminary data confirms that chondrogenic primed hMSCs form bone *in vivo*. As can been seen in Figure 1. On the outside of the scaffold (black arrows) bone formation can be observed and within the embedded bone matrix also bone marrow is present. Currently we are evaluating samples the other samples from multiple donors and comparing histological sections with 3D Micro-CT images obtained from the *in vivo* samples. A micro-array analysis of the *in vitro* samples will also be performed to investigate the differences between the endochondral and the intramembranous process in this model.
Conclusion: Our hypothesis was correct that bone formation can occur via the endochondral route following *in vitro* chondrogenic priming of human mesenchymal stem cells.

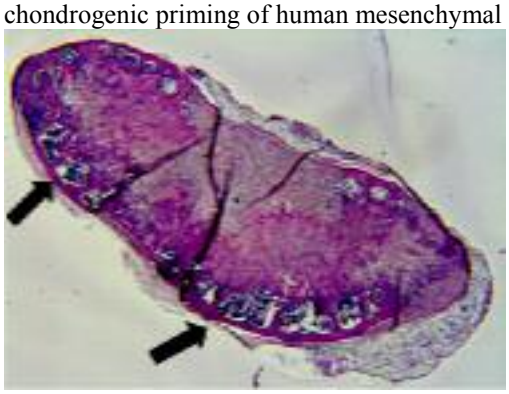

Figure 1. In vitro bone formation by hMSCs via the endochondral pathway

DEVELOPMENT OF A SINGLE-STEP PROCEDURE FOR A HUMAN BONE GRAFT CONTAINING BONE ALLOGRAFT AND AUTOLOGOUS MESENCHYMAL STEM CELLS

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Aims: Development of an implantable bone allograft, which is seeded with autologous human stem cells. This procedure must comply with the followig requirements: no recombinant or animal-derived materials are used, autologous human materials are preferred, minimal manipulation of cells, fast approach which enables harvesting and grafting in a single operation. In order to reach these goals first we developed a procedure for seeding and proliferation human mesenchymal stem cells (MSCs) on the surface of lyophilized bone allografts.
Methods: Human lyophilized bone fragments were pre-incubated with fibronectin, albumin, fetal calf serum or collagen I solutions which were used immediately or lyophilized before use. Human bone marrow derived mesenchymal stem cells were seeded onto the constructs and cultured in standard media. We reproducd the experiments with human dental pulp derived MSCs. Cell attachment and proliferation was evaluated by confocal microscopy 3, 10 and 18 days after seeding.
Results: The untreated allograft failed to attract significant numbers of cells and even those that were attached failed to proliferate during 18 days. Coating the bone with fibronectin or collagen increased the attachment, however, proliferation and long-term survival was still very low. Pre-incubating the bone with human albumin, especially when it was lyophilized onto the bone surface resulted in improved stem cell attachment and the cells proliferated until they covered the full surface of the bone. Fetal calf serum showed similar effects than that of albumin. Placing the constructs into a rotating bioreactor further improved the proliferation of the cells. MSCs from human bone marrow or dental pulp showed comparable results.
Conclusion: We developed a new procedure, which allows significantly better stem cell attachment and proliferation on the surface of the allografts. Increasing the stem-cell friendliness of the lyophilized human bone allogaft may improve its biocompatibility and incorporation time The protocol uses ingredients of human origin and autologous fresh cells, which allows the development of a seeding bioreactor for routine clinical use.

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HUMAN DENTAL PULP STEM CELLS AS MODELS FOR STUDYING POTENTIAL NERVE REGENERATION

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Objectives: A major focus of bioengineering is the appraisal of the biological response of cells responsible for detection and repair of in situ tissue damage. With exposure to biological, chemical and mechanical insults, tooth injury (and its associated pulpal nerve damage), initiates a cascade of signaling cues to progenitor stem cells. A subpopulation of these cells (dental pulp cells), possess the potential to differentiate into odontoblasts which can be isolated and characterized in the presence of appropriate growth factors. These odontoblasts are in close proximity to pulpal nerve endings (which possess cation transient receptor channels, TRP) involved in the response to pain-evoking stimuli .The objective of this was to develop a characterized dental pulp stem cell model in order to investigate odontoblast differentiation and potential functional links to the transient receptor channels (TRP) .

Materials & Methods: Pulp tissue was obtained from fresh human intact 3rd molar teeth extracted for orthodontic treatment (informed consent of the patients, and ethical approval). The teeth were cut around the cemento-enamel junction, separating the crown and the root to expose the pulp chamber. Whole pulps were removed apically (6 patients), and minced into fragments.

Explants were cultured in Dulbecco's Modified Essential Medium (DMEM) supplemented with growth factors including fibroblast growth factor. Following cell outgrowth expansion (up to 28 days), cells were examined for their differentiation potential as indicated by alkaline phosphatase (ALP) activity and by immunofluorescence microscopy studies using a combination of antibodies, including GFAP, β – III tubulin, p75, Nestin, S100, and SOX-2. Explants cultured in non supplemented DMEM were used as controls.

Results: All cell explants proliferated for the time period studied (Fig 1). Cell differentiation was observed in the absence/presence of conditioned medium (Fig 2). Cells were cultured from explants (Fig 3a) and mineralization nodule-like structure observed after 28 days in culture. Induction towards neural, astrocytic and odontoblastic growth pathways was observed following immunostaining of dental pulp stem cells with β – III tubulin positive staining indicative of a neuronal phenotype (Fig 3c). Transmitted light images (Fig 3d)

Conclusion: Human dental pulp cells have been successfully isolated and differentiated in vitro into "odontoblast-like" cells. Expression profiles of these cells are currently under investigation for their potential use in nerve regeneration and as models for studying pain.

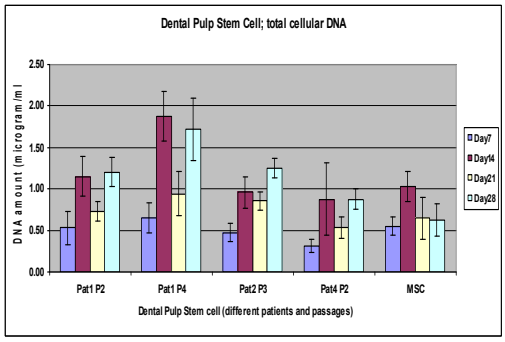

Fig 1

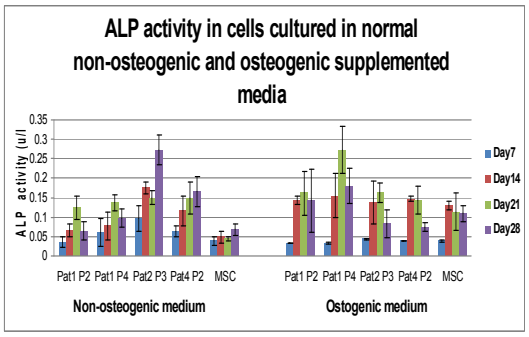

Figure 2 ALP activities of stem cells cultured in normal and conditioned osteogenic medium

Fig 3 (a)

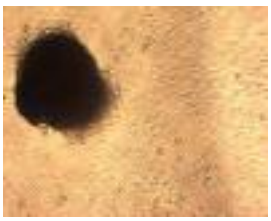

Fig 3 (b)

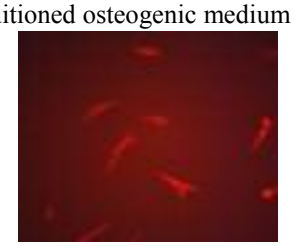

Fig 3 (c)

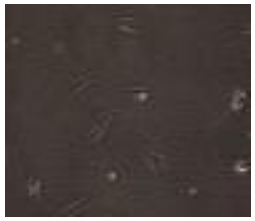

Fig 3 (d)

REPAIR OF HUMAN MANDIBLE BONE DEFECTS BY THE GRAFTING OF DENTAL PULP STEM CELLS AND COLLAGEN SPONGE BIOCOMPLEXES: A SUCCESSFUL CLINICAL STUDY

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Aims: New bone formation represents a major challenge for the repair of injuries in the Oro-Maxillo-Facial (OMF) complex. The use of stem cells is a promising approach for the repair of various tissues and organs. In particular, dental pulp, housing neural crest-derived stem cells, should be the most appropriate for OMF bone repair. Moreover, these cells are easily accessible, show a limited morbidity after collection and quickly proliferate and differentiate into osteoblasts.
Methods: In this clinically-oriented study we used Dental Pulp Stem Cells (DPSCs) for bone tissue repair in patients selected according to the Internal Committee Permission Guidelines (Internal registry: experimentation #914-Bone repair using stem cells) and after written informed consent. The four third molars of the patients needed extraction because they presented with bilateral bone reabsorption of the alveolar ridge distal to the second molar secondary to impaction of the third molar on cortical alveolar lamina which allow alveolar bone loss, without walls, of at least 1.5 cm in height (vertical loss). This clinical condition does not permit bone repair with conventional techniques and leads to a loss of adjacent second molar. Before grafting, in order to assess the presence of DPSCs in the patients, their maxillary third molars were extracted.
Results: Three months after surgery and grafting of DPSCs, alveolar bone of the patients had optimal vertical repair and there was a complete restoration of periodontal tissue back to the second molars, as assessed by clinical probing and X-rays. Histological observations clearly demonstrated the complete regeneration of the lamellar bone at the injury site. Optimal bone regeneration was evident one year after grafting.
Conclusions: This clinical study demonstrates that DPSCs injected in a medical device of collagen, completely restore human mandible bone defects and indicates that this cell population could be used in the future for the repair and/or regeneration of many other tissues and organs.

EFFECTS OF CULTURE CONDITIONS ON THE PROLIFERATION CAPACITY OF BONE HUMAN FETAL CELLS

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Background: Human primary fetal bone cells (hFBC) display a high capacity of replicating, differentiating and forming bone tissue. Even though growth factors that regulate bone formation and osteoblastogenesis have been identified, the environmental factors influencing the rapid growth rate and the responsiveness to differentiation of human fetal bone cells have not been characterized to date.
Aims. In this study, we aimed to optimize environmental conditions for proliferation and differentiation of bone fetal cells.
Methods: hFBC, obtained from our dedicated, consistent banks of bone cells comprising several fetal donors (from 12 to 16 week gestation), were studied for their ability to proliferate and differentiate into mature osteoblasts.*in vitro*.
Results: Proliferative capacities of fetal cells were assessed with three defined growth conditions.
hFBC proliferated more rapidly in MEM alpha medium, compared to other media (0.5×10^6 cells in DMEM; 0.5×10^6 cells in F-12 Ham's/DMEM and 1.0×10^6 cells in MEM alpha after 19 days of proliferation). Then, we were interested in growth factors that could stimulate cell proliferation rate. Wnt3a seemed to negatively act upon the growth rate in DMEM but not in MEM alpha, whereas PDGF and bFGF had positive effects on proliferation of hBFC regardless of media. Differentiation state was measured by alkaline phosphatase (ALP) enzymatic activity. We demonstrated a three-fold increase of ALP activity induced by a complete osteogenic mix prepared in one of the three media (DMEM, F-12 Ham's DMEM or MEM alpha) containing dexamethasone. This positive effect was enhanced by Wnt5a, whereas it was reduced by Wnt3a.
Conclusion: The association of appropriate culture medium with a selection of growth factors could be interesting for human fetal bone cell proliferation and differentiation *in vitro*.

IN UTERO TRANSPLANTATION OF ADULT BONE MARROW DECREASES PERINATAL LETHALITY AND RESCUES THE BONE PHENOTYPE IN THE KNOCK-IN MURINE MODEL FOR CLASSICAL, DOMINANT OSTEOGENESIS IMPERFECTA.

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Aims: Autosomal dominant osteogenesis imperfecta (OI), caused by glycine substitutions in type I collagen, is a paradigmatic disorder for stem cell therapy. Bone marrow transplantation in OI children produced low engraftment, but surprisingly encouraging phenotype improvements. *In utero* transplantation may be even more promising. However, systematic studies of both methods have so far been limited to a recessive mouse model, the oim mouse. The aims of this study were to evaluate the feasibility and the effects of intrauterine transplantation of donor adult bone marrow using the BrtlIV mouse. BrtlIV is a knock-in OI murine model carrying a α1(I)-Gly349Cys substitution with a dominant transmission, and its outcomes resemble moderately severe and lethal OI.
Methods: Fresh bone marrow was obtained from hind limbs of 6 to 8 weeks-old CMV/eGFP CD-1 transgenic mice by flushing cells with phosphate-buffered saline (PBS). After a red blood cell lysis, the cells were filtered through nylon mesh to remove clumps before injection. Pregnant WT females crossed with BrtlIV heterozygous males underwent allogenic intrauterine transplantation (IUT) at embryonic day (E) 13.5-14.5. Each fetus was injected intrahepatically with 6 μL suspension of 5×106 cells through a glass capillary. Mice were analyzed at 2 months, the age corresponding to the severest BrtlIV phenotype with respect to WT. Engraftment was detected at sacrifice by inverted microscopy in tissues of different germ layer origin and was quantified in bone by confocal microscopy and in different tissues by FACS and by Real Time PCR. PQCT, Micro CT and biomechanical analyses on long bones of Brtl and WT mice were perfomed to study the bone density and geometry. Collagen analysis and Raman Microspectroscopy were done to investigate the extracellular matrix composition.
Results: Adult bone marrow donor cells from eGFP transgenic mice engrafted in haematopoietic and non haematopoietic tissues, differentiated into trabecular and cortical bone cells, and synthesized up to 20% of all type I collagen in the host bone. The transplantation eliminated the perinatal lethality of heterozygous BrtlIV mice. At 2 months of age, femora of treated Brtl mice had significant improvement in geometric parameters ($p<0.05$) versus untreated Brtl mice, and their mechanical properties attained wild-type values.
Conclusions: Our results suggest that the engrafted cells form bone with higher efficiency than the endogenous cells, supporting *in utero* transplantation as a promising approach for the treatment of genetic bone diseases.

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REAL TIME MONITORING OF BMP SMADS TRANSCRIPTIONAL ACTIVITY IN BONE DURING FRACTURE HEALING TREATED WITH AN EXTERNAL-FIXATOR

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Introduction: Evidence points to the bone morphogenetic proteins (BMPs) as the most important growth factors in bone fracture healing. To date, introduction of BMPs in clinical fracture care has not been overwhelmingly successful. Despite undisputed clinical potential, BMPs will loose their appeal if preclinical results cannot be reproduced in the patient. The spatiotemporal expression of BMPs and BMP signaling in a murine fracture model has been widely studied. Molecular profiling of fracture tissue has confirmed that BMPs are expressed during the healing process. These studies are limited to the analysis of fixed material.

This study evaluates real time physiological spatiotemporal pattern of BMP signaling in BRE-GFP reporter mice during bone healing in an established tibia fracture model. The direct aim of this study is to generate a murine fracture model that facilitates in vivo monitoring of BMP Smads activity. The general and future purpose of this model is to gain insight in the roles of BMPs and BMP-inhibitors in normal and abnormal bone healing in vivo.

Methods: The murine model used in this study facilitates real time monitoring of BMP Smads transcriptional activity. Transgenic BRE:gfp reporter mice were obtained from the Hubrecht Institute, Utrecht, the Netherlands (courtesy of Professor Christine L. Mummery). BMP-response element (BRE) containing BMP Smad (PSmad) DNA-binding sites – derived from the Id1 promoter– is used to drive the expression of green fluorescent protein (GFP). The expression of GFP reveals sites where BMP Smad-dependent transcriptional activity is present. The GFP signal was measured under general anaesthesia using non-invasive IVIS 200 Spectrum. GFP signal was quantified using Living Image software (Version 3.0, Caliper Live Sciences Inc.).

Nine BRE-GFP mice were used for this pilot. Base measurements of GFP in bilateral hindlimbs were performed on days -1 and 0. Mice were allowed unrestricted activity. A mini-external fixator fixed at the proximal and distal tibia was applied under general anesthesia on day 0. The animals were permitted full weight baring and unrestricted activity after awakening from anaesthesia. The GFP signal of the tibia and fibula in bilateral limbs was measured on days 1, 3, 7, 10 and 14 after application of the external fixator. At day 14, a midshaft tibia fracture was created by controlled blunt trauma in the experimental group (n = 5). Control mice retained the external fixator without creating fracture (n = 4). GFP signal in bilateral hindlimbs was subsequently measured on days 0, 1, 4, 7, 10, 14, 17, 30 and 37.

Results: Baseline measurements ranged from 6.8x10e9 photons to 3.1x10e10 photons between individual mice. After application of the external fixator, the GFP signal of the unloaded tibia and fibula decreased in all mice to on average 71% on day 14 (SD ± 30%, $p < 0.001$). In the contra-lateral non-operated limb, the GPF signal increased to an average 172% on day 14 (SD ± 82%, $p < 0.05$).

After a midshaft fracture was created, the GFP signal significantly decreased on days 1 and 4 in the experimental group (n = 5) to 57% (SD ± 30%, $p < 0.05$) and 60% (SD ± 38%, $p < 0.05$) respectively. A subsequent increase was measured on days 7 (92%; SD ± 31%, $p = 0.29$), 10 (108%; SD ± 44%, $p = 0.35$), 14 (92%; SD ± 46%, $p = 0.37$), 17 (97%; SD ± 39%, $p = 0.441$), 30 (82%; SD ± 23%, $p < 0.05$) and 37 (105%; SD ± 54%, $p = 0.42$). This increase was statistically non-significant when compared to the baseline measurement before creating the fracture. Measurements of GFP signal of non-fractured tibia with external fixator in the control group (n = 4) showed a non-significant upward trend with respect to baseline measurements (100% on day 1(SD ± 15%, $p = 0.48$); 81% on day 4 (SD ± 13%, $p < 0.05$); 133% on day 7 (SD ± 27%, $p < 0.05$); 130% on day 10 (SD ± 35%, $p = 0.10$); 101% on day 14 (SD ± 33%, $p = 0.48$); 119% on day 17 (SD ± 39%, $p = 0.20$); 155% (SD ± 14%, $p < 0.05$); 130% on day 37 (SD ± 36%, $p = 0.10$)).

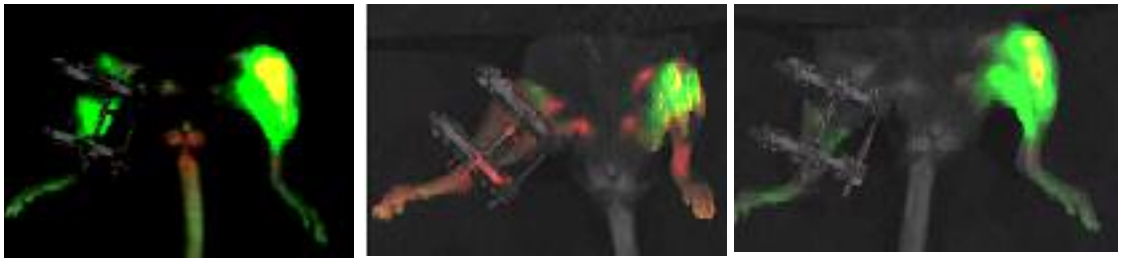
Conclusions: Pilot studies suggest that initial application of an external-fixator without creating a midshaft tibia fracture results in decreased signaling of mechanically induced BMP transcriptional activity due to weight-"sharing" between tibia and fibula and the fixator.

After creating fractures of tibia and fibula, mechanical cues for BMP signaling initially disappear and measurements show a decrease in BMP transcriptional activity on days 1 and 4. Subsequently, BMP signaling -indispensable for fracture healing- 'takes over' and GFP signals increase again.

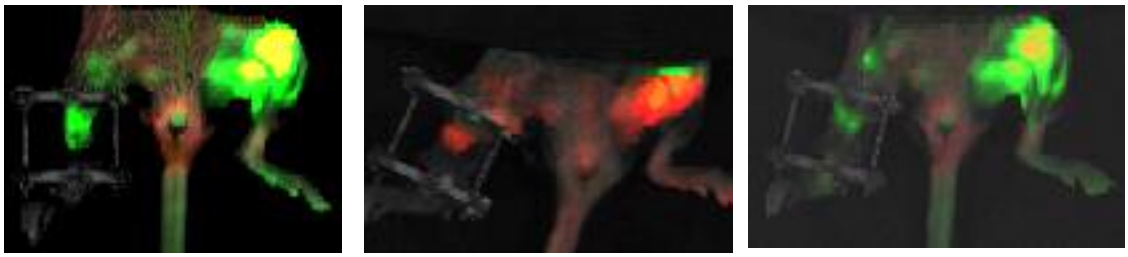
In this study, real time monitoring of BMP signaling during application of an external fixator and subsequent fracture healing, suggests that BMPs do not only mediate bone formation in context of repair, but also has a role in maintaining adult bone.

Figure 1:

Base | Day 1 –post fracture– | Day 4 –post fracture–

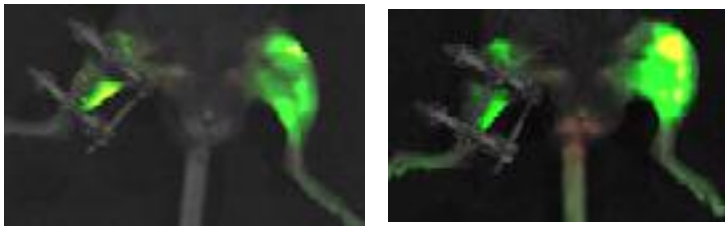


Mouse RL

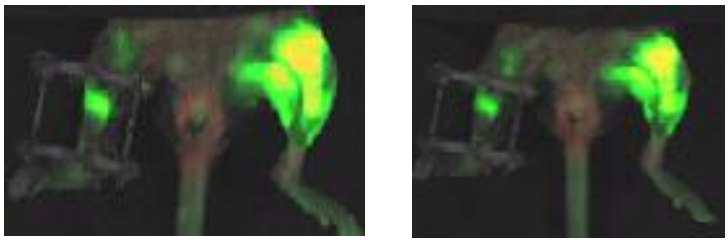


Mouse R

Day 10 –post fracture– | Day14 –post fracture–



Mouse RL



Mouse R

Green Fluorescent Protein (GFP-) signal after application of an external fixator and creating a fracture of the midshaft tibia and fibula to evaluate BMP transcriptional activity during bone fracture healing in vivo; One can appreciate that after a midshaft fracture was created, the GFP signal decreased on days 1 and 4. A subsequent increase was measured on days 10 and 14.

This experiment of in vivo monitoring of BMP transcriptional activity during fracture healing suggests that after creating fractures of tibia and fibula, mechanical cues for BMP signaling initially disappear and measurements show a decrease in BMP transcriptional activity on days 1 and 4. Subsequently, BMP transcriptional activity -indispensable for fracture healing- 'takes over' and GFP signals increase again.

TREATMENT OF NONUNION IN LONG BONES USING PLATELET GEL AND PACKED AUTOLOGOUS STROMAL CELLS

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Aims: In nonunion the bone loses its ability to repair and forms a pathological callus. Therefore, stable fixation and a biological support are needed to achieve union. The combined use of homologous bone, platelet gel, and packed autologous stromal cells represents a biological stimulus for bone regeneration, as we have shown in experimental models and randomized clinical trials. In fact, platelets contain several growth factors, such as PDGF, TGF-beta, IGF I, and VEGF that influence positively the survival, differentiation, and proliferation of bone cells. These effects are also exerted on stromal cells, which transform into osteoblasts, osteocytes, and thus bone tissue.
Methods: Since February 2003 we have treated 22 lower limb nonunions (10 femurs and 12 tibiae) and 11 upper limb nonunions (4 humerus, 2 radii and 5 ulnas) using homologous lyophilized bone, platelet gel and packed autologous stromal cells. The mean age was 43 years (range: 26 -70). Twelve nonunions were hypertrophic, whereas 21 were atrophic. The choice of treatment depended on the type of lesion, its location, and size of defect. In 7 cases we used lyophilized homologous intercalary bone grafts to fill large defects.
Results. Healing occurred in 60-90 days in the hypertrophic lesions and in 90-180 days in the atrophic ones.
Conclusions: The data presented in the literature suggest that growth factors promote bone repair. Our preliminary results show the usefulness of platelet gel and packed autologous stromal cells as an adjuvant in the treatment of pathologic lesions, such as nonunion of long bones, which are often difficult to address.

SHOCK WAVES ON HUMAN PLATELET-RICH PLASMA (PRP) AND OSTEOBLASTS

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Aims: Shock Waves (SW) involves mechanical disturbances that can result in biological stimulus to many different cell lines. The aim of this study was to assess the interaction of different cell populations, osteoblasts and platelets, after the stimulation of only one of them.
*Methods***:** Human platelet-rich plasma (PRP) was exposed to a single SW treatment (0.16 mJ/mm^2, 500 impulses) and then added to mouse calvaria osteoblast (OB) cultures. Cell activity was evaluated by Western-blot and real time-PCR experiments.
*Results***:** We found an increase of Growth Factors involved in OB proliferation and differentiation. In addition, the increase of the markers of osteoblast maturation (RUNX2, Collagen I and Osteocalcin) was demonstrated. Our findings suggest that the recruitment of platelets is a critical step in bone reparation process and is enhanced by SW treatment. In fact, Insulin Growth Factor-1 and IGF-Binding Protein-3 are proposed to play a chemotactic and mitogenic role in differentiation and proliferation of OB. Shockwaves can produce activation of platelets and drive OB to express genes for osteogenesis.
*Conclusions***:** Previous studies showed SW positively influence OB proliferation modulating membrane permeability and intracellular signalling. In this study, we found that these effects can also be achieved by the stimulation of other cells. Therefore, it is probable that during applications in vivo the stimulation of shockwaves could be the sum of the interaction of different cell lines.
In recent years the application of PRP has emerged as a potential solution in bone and tendon injuries. The present results demonstrate that PRP could be added to SW to increase the osteoblastic activity.

POLY-L-LACTIC ACID/HYDROXYAPATITE ELECTROSPUN NANOCOMPOSITES INDUCE CHONDROGENIC DIFFERENTIATION OF MESENCHYMAL STEM CELL

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Aims: Cartilage and bone tissue engineering has been widely investigated but is still hampered by cell differentiation and transplant integration issues within the constructs. Scaffolds represent the pivotal structure of the engineered tissue and establish an environment for neo-extracellular matrix synthesis. They can be associated to signals to modulate cell activity. In this study, considering the well reported role of hydroxyapatite (HA) in cartilage repair, we focused on the putative chondrogenic differentiation of human mesenchymal stem cells (hMSCs) following culture on membranes of electrospun fibers of poly-L-lactic acid (PLLA) loaded with nanoparticles of HA.
Methods: The PLLA/HAp nanocomposite was prepared by electrospinning. Membranes microstructure was evaluated by SEM. hMSCs were seeded on PLLA/HA and bare PLLA membranes and cultured in basal medium, using chondrogenic differentiation medium as a positive control. Cell attachment and engraftment was assessed 3 days after seeding and MSC differentiation was evaluated by immunostaining for CD29, SOX-9 and Aggrecan under a confocal microscope after 14 days.
Results: PLLA/HAp membrane obtained was composed by fibers (average diameter of 7μm) with nano-dispersed hydroxyapatite aggregates (average diameter of 0.3μm). 3 days after seeding, MSCs were well adhered on the PLLA/HAp fibers with a spindled shape. After 14 days of culture, SOX-9 positive cells could be detected in the PLLA/HA group.. Cartilage specific proteoglycan immunostain confirmed the presence of neo-extracellular-matrix production. Co-expression of CD29, a typical surface marker of MSCs and SOX-9 suggested different degrees in the differentiation process.
Conclusion: We developed a hydroxyapatite functionalized scaffold with the aim to recapitulate the native histoarchitecture and the molecular signaling of osteochondral tissue to facilitate cell differentiation toward chondrocyte. PLLA/HA nanocomposites induced differentiation of hMSCs in a chondrocyte-like phenotype with generation of a proteoglycan based matrix. This nanocomposite could be an amenable alternative scaffold for cartilage tissue engineering using hMSCs.

BIOACTIVE 3D TISSUE-ENGINEERING PRODUCT FOR CARTILAGE/BONE REPAIR

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Aims: The aim of the project is to develop a bioactive 3D tissue-engineering product (TEP) for cartilage and bone repair in sports and occupational traumas and degenerative osteoarthritis. Different types of scaffolds, nano-size fibre mats and collagen gels are tested for compatibility with mesenchymal stromal cells (MSC) to form cartilage-like and bone-like matrices. Nano-size fibres should induce differentiation and keep the MSCs inside and fibroblasts from surrounding matrix outside to prevent fibrotic tissue formation.
Methods: MSCs from different source (commercial, adipose tissue, bone marrow) are used for differentiation into osteoblasts with dexamethasone, ascorbic acid and β-glycerophosphate, and into chondrocyte pellets with TGFβ3. Cells maintenance of their osteoblast and chondrocyte phenotype are monitored by gene (real time PCR) and protein (immunohistochemistry) expression of bone related markers including Runx2, Alkaline phosphatase (ALP), osteocalcin (OCN) and collagen I (Col1A1, Col2A2) and cartilage related markers as SOX9, Collagen II and collagen X. Von Kossa staining of osteoblast calcium and Safranin O staining of cartilage proteoglycans is also used to characterize the differentiation process. Polycaprolactone (PCL) and poly-l-lactide (PLA) micron-size scaffolds with chitosan/polyethylene oxide and PLA nano-size fibres are used for 3D matrix. Collagen I and II gels are used for planting of MSCs.
Results: Both commercial and bone marrow MSC differentiate into osteoblast and chondrocytes as shown with real time quantitative PCR, immunohistochemistry and histochemical staining using specific markers. MSCs were attached to PLA nanofibers and to the edge of the PCL scaffold in collagen gel. Chitosan/polyethylene oxide nanofibers were able to form MSC aggregates, similar to chondrogenic pellets, around the fibre mats within 24 hours when cells were placed in media containing TGFβ3.
Conclusions: The goal is to induce MSCs to stable osteoblasts and chondrocytes and achieve a matrix in a biodegradable scaffold with biomechanical properties of bone and articular cartilage. Scaffolds will be build according to the lasagna principle, seeding individual micro-nano-composite mats (PCL-chitosan/PEO) with cells and then piling up these TE composites to form layered scaffolds with optimal cell density inside. The possible osteoblast activity also on chitosan/PEO is investigated.

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ABILITY OF POLYURETHANE FOAMS TO SUPPORT PLACENTA-DERIVED STEM CELLS PROLIFERATION AND DIFFERENTIATION INTO THE OSTEOBLASTIC LINEAGE

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Aims: In bone tissue reconstruction, the use of engineered constructs created by cells cultured onto porous scaffolds is an appealing alternative to clinical therapies. Stem cells hold great promise in this contest, but there is the need to identify a safe source of stem cells easily accessible and exempt from ethical debate. Human placenta represents a possible source of stem cells, as it is readily available without invasive procedures and because of the phenotypic plasticity of many of the cell types isolated from this tissue [1]. Aim of this work is the evaluation of the proliferation and possible differentiation to osteoblastic phenotype of amnion mesenchymal cells (AMCs) and chorion mesenchymal cells (CMCs), isolated from human placenta, cultured onto polyurethane foams (PUFs) coated or not with α-tricalcium phosphates (α-TCP).
Methods: A PU foamed matrix with slow degradation rate (PU-EF) was synthesized by polymerization of MDI prepolymer with a polyether-polyols mixture with high hydrophilicity (600%eq water uptake), using water as expanding agent, as previously described [2, 3]. The PU-EF was characterized for porosity and average pore size by micro CT (Skyscan 1172, Aartselaar, Belgium), and for density. PU-EF discs (Ø = 6 mm, h = 2 mm) were fixed in circular slots in a polymeric mesh, and coated by immersion in α-TCP suspension under magnetic stirring [4].
AMCs and CMCs were isolated as previously described [5] from two human term placentas obtained after maternal consent. The two populations of AMCs (AMC1 and AMC2) and CMCs (CMC1 and CMC2) were cultured onto uncoated and αTCP-coated PU-EF samples (Ø = 6 mm, h = 2 mm) up to 20 days, testing two seeding densities ($2.5*10^5$ and $5*10^5$ cells/well) and two different culture media: the mesenchymal stem cell growth medium EMEM (Lonza, Basel, Switzerland), and the OsteoDiff Medium (NH, Miltenyi Biotec, Bergisch Gladbach, Germany). Cells cultured onto tissue culture wells were used as control. Cells morphology was investigated by SEM (TM-1000 Tabletop, Hitachi), whereas scaffold colonization and cells differentiation were histologically evaluated by staining with hematoxilin-eosin, Alizarin Red and Von Kossa staining.
Results: Physical characterization of PU-EF allowed to evaluate the foam density (0.127±0.003 g/cm3), while by the micro CT analysis PUF porosity (≈90 %) and average pore size (268 μm) were calculated. By SEM, a good cells colonization both onto the matrices and the coated PUF for all the tested cell types was observed. The cells were well adherent within the PUF pores, indicating the absence of cells suffering. SEM analysis seems to not highlight a different behaviour for the two populations of cells isolated from the two placentas (population 1 *vs* population 2), indicating the absence of variability between the populations, while CMC cells seemed to colonize the scaffolds better than AMC cells, in particular when cultured with EMEM culture medium. Cells cultured onto coated foams seem to aggregate around α-TCP particles, while the EDX analysis performed on EF matrix detected the presence of calcium phosphates. The histological analyses results were in agreement with SEM observation, and, in addition, the NH OsteoDiff Medium appeared to support cells differentiation towards the osteogenic phenotype better than EMEM, as detected with Alizarin Red and Von Kossa stainings.
Conclusions: The tested PUF and composites samples seem to be a valid scaffold to support proliferation and differentiation of placenta-derived stem cells, in agreement with the results obtained from a previous study with human MSCs [3]. Furthermore, the α-TCP coated PUF seems to support, under appropriated stimuli, cells differentiation to osteoblastic phenotype, thus providing a good source of active bone forming cells.

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COLLAGEN/NANO-B-TCP CROSS-LINKED SCAFFOLDS FOR HUMAN MESENCHYMAL STEM CELLS USED IN BONE TISSUE ENGINEERING

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Aims: The aims of this study were: to prepare and characterize cross-linked 3D porous nanocomposite scaffolds, based on collagen type I (COL I) and β-tricalcium phosphate nanopowder (β-nTCP) and to investigate their bioactivity in a mesenchymal stem cell culture.
Methods: Human mesenchymal stem cells (hMSC) were obtained from iliac crest by enzymatic digestion and subcultivated in α-MEM with 20% FBS. Cell morphology, colony forming capacity (CFU-F), expression of surface antigens and their ability to differentiate in osteoblasts were investigated.
Cell morphology was analyzed by phase-contrast microscopy. CFU-F assay was performed by Giemsa staining. Flow-cytometry analysis was performed to characterize the surface antigen expression of CD34, CD45, CD73, CD90, CD105, CD133. Also, fluorescence microscopy was used to identify CD44, CD106. Osteoblast differentiation was performed by identification of specific markers osteocalcin (OC), osteopontin (OP) (fluorescence microscopy). Also, matrix mineralization was analyzed by Alizarin Red S staining and quantitative measurement of calcium content in cell culture medium was performed using Quantichrom Calcium assay kit.
Composite scaffolds were prepared by sonicating a mixture of COL type I solution (0.8%, w/w) and β-nTCP, in dry weight ratios of 1:1 and 1:2.6 and freeze-drying. COL/β-nTCP scaffolds were cross-linked by treatment with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC). Physico-chemical and biochemical properties of the cross-linked scaffolds were analyzed.
hMSC viability in presence of scaffold was analyzed by MTT assay, SEM and confocal microscopy (Live/Dead assay). Also, the differentiation of MSC was assessed by osteoblast marker identification: COL I, alkaline phosphatase (ALP) and chondroitin sulphate (CS).
Results: During expansion in monolayer, hMSC exhibited a spindle-shape and fibroblast-like morphology. Seeded at low concentration, hMSC formed different size colonies after 10 days of culture. Analysis of surface antigens shown that majority of cells expressed specific hMSC markers: CD44, CD73, CD90, CD105, CD106. Negative expression of surface antigens for CD34, CD45 and CD133 suggested the absence of any contamination with hematopoietic stem cells and endothelial stem cells. Expression of OC, OP, calcium release and mineralizing nodule formation in the extracellular matrix of hMSC confirme the osteoblast differentiation capacity of isolated hMSC.
SEM micrographs of COL/β-nTCP composite scaffolds showed their 3-D structure having unevenly sized pores. The pore size reducing was due to the cross-linking process. Still, porosity values are high enough (> 80 %) to allow cell infiltration. EDC cross-linking also lowered the biodegradability of the scaffolds in the presence of bacterial collagenase and the calcium quantity released in physiological conditions over 5 days period.
Confocal microscopy and MTT assay shown a high viability of hMSC seeded into composite scaffolds. Also, expression of ALP and CS demonstrate the osteblast differentiation potential.
Conclusions:

- Enzymatic digestion of small trabecular bone pieces is a simple and productive methods to isolate cell which express hMSC phenotype: adhesion capacity, CFU-F capability, expression of specific markers CD44, CD73, CD90, CD105, CD106 and lack of hematopoietic stem cells markers CD34, CD45 and CD133 and osteogenic differentiation property (OP and OP expression);
- Both composite variants allowed adhesion and proliferation of viable hMSC and stimulate osteoblast differentiation;

COL/β-nTCP scaffolds exhibited essential properties for materials used in bone tissue engineering and could be potential candidates for bone substitutes using as system delivery for hMSC.

A NOVEL CANDIDATE GENE THERAPEUTIC AND MESENCHYMAL STEM CELL-DERIVED CHONDROCYTES AS POSSIBLE TOOLS AGAINST INTERVERTEBRAL DISC DEGENERATION

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Introduction and Aims: Most humans of civilized populations experience back pain, one of the main cause of disability below fifty years and thereby of economic losses, which main cause is degeneration of intervertebral disc (IVD). Despite its huge health and economic impact, pathogenesis of IVD degeneration has been scarcely ravelled so far and, consequently, most current therapeutic treatments for back pain are only addressed to attenuate the symptom.
Recently, advances in molecular biology allowed to identify several gene alterations underlying IVD degeneration, opening new avenues to treat the degenerative pathways through gene therapies. IVD degeneration is characterized by increased degradation of collagenous structures of the IVD matrix by locally produced neutral endoproteinases called matrix metalloproteinases (MMPs). Most MMPs are secreted as latent proenzymes or zymogens. Plasmin, a serine protease activated by urokinase tissue-type plasminogen activator (uPA) and urokinase-type plasminogen activator receptor (uPAR), seems to play a major role in the activation of many of the key MMPs. Importantly, Plasmin has been found accumulated together with some distinct MMPs in animal models of disc lesion.
Main aim of this study is to assess the ability of two novel potential therapeutics to inhibit IVD degeneration and to regenerate tissue. The first, is an anti-uPAR antisense oligonucleotide (ODN) – scientific patent now pending – we propose as candidate post-transcriptional gene therapeutic able to inhibit activation of MMPs responsible for matrix structural protein degradation resulting in IVD degeneration. The second, is a cell therapy tool to reconstitute damaged tissues consisting of Mesenchymal Stem Cells (MSCs), which transplantation has been recently demonstrated to be effective in counteracting disc degeneration in small animals, following their differentiation to chondrocytes.
Methods: An anti-uPAR antisense ODN is applied to cellular model of IVD degeneration. ODN effectiveness is evaluated on the basis of biochemical, functional and molecular analysis. MSCs are isolated from the bone marrow, adapted to *in vitro* culture, characterized by flow cytometric analysis, and committed to chondrogenic differentiation by plating in the presence of chondrogenic medium: IMDM supplemented with 10% FBS and 10ng/ml transforming growth factor-β1 (Sigma, St. Louis). Briefly, following their identification by morphologic analysis, scanning electron microscopy (SEM) and specific molecular markers (Type II and IX Collagen, SOX9), differentiated chondrocytes are applied to our cellular models of disc degeneration and evaluated for their therapeutic effectiveness on the basis of specific phenotypic and molecular parameters.
IVD degeneration has been proven to be associated with increased expression of several MMPs, whose major activation pathway could involve the uPA-uPAR system. Before analysis of antisense ODN-mediated uPAR inhibition in cultured cells isolated from intervertebral discs of patients, we have evaluated MMP and uPA-uPAR expression in our experimental model. Two strategies have been designed: 1) gene specific quantitative RT-PCRs, to monitor the levels of MMPs and uPA-uPAR mRNAs, 2) gel zymography, to check for MMP activity.
*Results***.** Here, we present for the first time, results of the effectiveness of our anti-uPAR antisense ODN in terms of exogenous inhibition of targeted gene expression and induction of relevant phenotypic effects obtained in experimental models of pathological processes.
Conclusions: Although very preliminary, our results are in keeping with the working hypothesis that anti-uPAR aODN as well MSC, alone or in combination, are suitable as possible therapeutics aimed to inhibit IVD degeneration or to reconstitute the degenerated tissues.

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THE PRESENCE OF TRANSFORMING GROWTH FACTOR -B_1 INDUCED THE ENDOCHONDRAL PHENOTYPE SWITCHING ON CULTURED HUMAN ARTICULAR CHONDROCYTE

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Aims: Articular chondrocyte' s culturing is required to perform cartilage resurfacing in tissue engineering-based repair approaches, and the use of serum-free medium (SF) during the expansion phase can opportunely mimic the physiological (avascular) cartilage 3D environment. Nonetheless chemically defined medium is unable to hinder the normal in-vitro dedifferentiation of articular cells (chondrocyte acquires a fibroblast-like shape). The presence of Transforming Growth Factor-β_1 (TGF- β_1) in the medium regulates the expression of cartilage proteins and also affect the re-differentiation availability of the chondrocyte. We therefore evaluated if TGF- β_1 administration sustain a better chondrogenic phenotype during cell expansion in SF medium.
Methods: To this purpose human articular chondrocyte, released by repeated enzymatic digestion, were expanded in a serum-free medium (SF), with or without TGF- β_1 (TGF). After 5 duplications the chondrogenic potential was tested by means of alginate and micromass 3D culture for immunocytochemisty, TUNEL and qualitative/quantitative mRNA analysis. Cell aliquots, for both amplification conditions, were also replated in osteogenic medium. Expression's markers of osteogenic lineage was assessed by RT-PCR and the mineralization level was revealed after Alizarine Red staining. In all 2D culture conditions was tested the activity of MAP Kinase by means of immunoprecipitation analysis.
Results: Results on 3D culture system showed that TGF-expanded cells displayed a partial loss of typical cartilage matrix component (Aggrecan, type-II Collagen and proteoglycan elements); on the contrary, they were characterized by a massive increase in RAGE (Receptor of Advanced Glycation End products), IHH (Indian Hedgehog), type-X Collagen and by the presence of apoptotic cell, paralleling a reduction of BCL-2 levels. The transcript analysis by Real Time-PCR underlined this data and interestingly showed high levels of Osteopontin (OP) and Osteocalcin (OC) after the amplification phase. Moreover, after the osteogenic induction, TGF-expanded chondrocyte massively enhanced their mineral deposits respect to the SF cultured cells. They also revealed increased mRNA levels of several osteogenic markers [such as ALP (Alkaline Phosphatase), Cbfa-1 (osteogenic transcription factor) and Glvr-1 (Pi transporter active during the onset of mineralization)], evidencing a clear predisposition to phenotype switching, from the articular to the fully mature endochondral phenotype. The involvement of MAPK was confirmed by the analysis of p38 activity. Once induced by osteogenic medium, only SF-expanded cells displayed a strong kinase activity on the specific substrate (ATF-2), whereas the response was completely abolished in TGF-expanded cells.
Conclusions: Immunocytochemistry and transcript analysis showed that cells expanded in the presence of TGF-β_1 and transferred in 3D cultures acquired a pre-hypertrophic phenotype. However, if the same cells were osteoinducted, they strongly mineralized and displayed an increase levels for several osteogenic markers, characteristics not shared by SF-expanded cells. Obtained results suggest an involvement of MAP Kinase pathway in these processes.
Cartilage lesions, inflammation and osteoarthritis (OA) are known to enhance TGF-β_1 in damages tissues. The cytokine presence in the lesion microenvironment may represent a crucial factor to address the phenotype of the chondrocyte used in cell-based cartilage resurfacing attempts, possibly igniting undesired mineralization on articular surfaces.

AUTOLOGOUS CHONDROCYTE-BASED THERAPY FOR THE REPAIR OF CARTILAGE DAMAGES

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Aims: Clinical application of chondrocyte culture: the development of a process suitable for the production of engineered chondrocytes for autologous implantation for the repair of hyaline articular cartilage.
Methods: Cell manipulations were performed in a production facility located inside Rizzoli Orthopaedic Institute, Bologna, Italy, and including clean rooms of different classification up to A in B work places, according to the EU guidelines of current "Good Manufacturing Practices" (cGMP).
Cartilage biopsies harvested from a non-bearing area of the knee were enzymatically digested to isolate chondrocytes. Primary cultures were expanded in monolayer up to 3-4 passages and cells seeded at defined density onto matrices derived from hyaluronic acid (three-dimensional) or collagen I/III (bi-dimensional).
At the time of release, engineered constructs were analyzed for sterility, cell viability and the expression of different phenotypic markers.
Results: Starting from September 2006, 25 patients undergoing Autologous chondrocyte implanation were treated (12 knees, 2 ankles and 11 hips).
Quality control analyses revealed that the engineered cells were sterile (free of aerobic and anaerobic bacteria, fungi, mycoplasma and endotoxins), viable (89-98%) and expressing typical hyaline cartilage molecules, collagen II and aggrecan. Collagen I, typical of fibroblasts, and osteocalcin, a bone formation marker, were not present.
Conclusions: The chondrocyte-based process that we developed is safe, repeatable and thus suitable for clinical application for the repair of articular cartilage damages.

MULIPARAMETRIC IMMUNOHISTOCHEMICAL APPROACHES TO DETECT BONE PROTEINS ON PARAFFIN EMBEDDED SPECIMENS IN A MOUSE MODEL

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Aims: Immunohistochemistry (IHC) represents an elective approach to reveal proteins and intra/extra-cellular structures in the context of tissues. Immunofluorescence (IF) provides suggestive images of multiple molecules detection by fluorochrome staining while losing complex histological patterns of the specimens. In addition, the relevant autofluorescence of some tissues can originate aspecific stainings. All these aspects are particularly true for bone. Thus, our study wants to standardize a multiparametric IHC for paraffin embedded bone with the aims to overcome procedural fall pits linked with tissue damage, incomplete demineralization and aggressive antigen retrieval approaches.
Methods: Femora and tibia specimens are harvest from FVB/n mice, fixed in formalin and then decalcified for several days until a good demineralisation is reached. Specimens are then paraffin embedded and 5 □m sections are processed by IHC assays. Antigen retrival is done by chondroitinase incubation at RT. The antibodies have been tested to characterize the main bone and marrow components, such as osteoblast (OB), osteocyte (OC), endothelial cells (EC) and osteoclasts (OCL). Anti-Collagen I, -Osteocalcin, -N-cadherin, -Osteopontin, -Cathepsin-K, -VE-Cadherin, -CD-31, -CD-9 antibodies were introduced. IHC stainings were also performed targeting 5-Bromo2'-deoxy-Uridine (BrdU) and Green Fluorescent Protein (GFP) in a GFP transgenic mouse model (Dominici et al. 2006). Tartrate resistant acid phosphatase (TRAP) was also introduced to further detect the osteoclast's compartment.
Results: The EDTA-based decalcifying solution is able to completely demineralise bone specimens which can be then easily cut by microtome without damage. The antigen retrieval adopted is able to rescue a series of antigens without damaging the bone and marrow structures. For instance, several retrieval approaches have been introduced.
The staining shows OB and OC positive for Collagen I, Osteocalcin as main osteopoietic cell markers. OB,contributing also to the hematopietic stem cell niche, can be visualized by N-Cadherin and osteopontin stainings. Osteoclasts, responsible of bone resorption and remodelling, are visualized by TRAP assay and by anti-Cathepsin-K antibody. Marrow sinusoids and other vessels are positive for CD-31 and VE-Cadherin staining. The anti-BrdU staining successfully reveals proliferating cells either in OB compartment or in marrow. The anti-GFP staining, frequently used to detect cells for tracking studies (Dominici et al. 2004, 2008), is successfully tested providing information about the possible engraftment of transplanted cells and their homing into bone and marrow.
Conclusion: IHC combined with light-microscopy is able to provides information about complex tissue such as bone. The standardization of these IHC staining can be useful to study bone physiology, proliferation of bone cells under particular conditions, such as drugs exposure or physical stress, and bone regeneration.

TELOMERASE DEFICIENCY LEADS TO DECREASED BONE MASS AND IMPAIRED MESENCHYMAL STEM CELLS (MSC) AND OSTEO-PROGENITORS FUNCTIONS IN TELOMERASE DEFICIENT (TERC-/-) MICE

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We have previously demonstrated that telomere shortening leads to in vitro replicative senescence of human mesenchymal stem cells (MSC) and telomerase over expression leads to telomere elongation, extended life span and enhanced bone formation (Simonsen et al (2002), Nature Biotechnology 20:592). In order to study the role of telomerase in MSC biology in vivo, we studied the phenotype of telomerase deficient mice caused by absence of telomerase RNA component (TERC-/-). G3-TERC-/- displayed diminish bone mass already at 3 month of age and during 12 month follow up period (BMD (gm/cm2): G3-TERC-/- 0.04173 vs. wild type (WT) 0.05941, $p < 0.0005$). u-CT signifies compartment specific changes in bone mass of both G3-TERC-/- mice (n=6, 32 weeks old), and WT littermates (n=6, 32 weeks old), G3-TERC-/- exhibited decreased bone mass, deterioration of bone architecture depicting reduced trabecular thickness (KO, 0.04496 ± 0.004776 vs WT, 0.05415 ± 0.004015, $p = 0.009$) and bone volume fraction (BV/TV, KO; 0.0775 ± 0.0218 vs WT, 0.11315 ± 0.02904, $p = 0.039$). The decreased bone mass was associated with reduced total number of CFU-F and alkaline phosphatase positive (AP+) colonies compared to WT ($P<0.005$). Moreover, MSC revealed lower proliferation rate, accumulation of senescent cells, increased DNA damage (high levels of □H2AX staining) and decreased osteoblastic (OB) differentiation marker expression compared with WT. More interestingly neo-natal calvaria cells also showed more less the same marred differentiation pattern in vitro, except for few expression markers (Runx2, OPN & ON), and was further reinforced by in vivo data allied with the reduced proliferation and senescence in the G3-TERC-/- calvaria cells. Similar impairment of in vitro OB differentiation of TERC-/- mouse embryonic fibroblast (MEF) was observed together with decreased ectopic bone formation capacity when implanted subcutaneously in immune deficient mice. Osteogenic super-array analysis of group of G3-TERC-/- bone samples revealed decreased expression of several genes, like Tgfb1, Runx2, Vdr, Bmp4, collagens, Igf1, Asgh, Bgn, Akp2, Twist1, Phex & Vcam1, Known to be involved in osteoblast commitment, proliferation and maturation. Our data demonstrate that telomerase plays an important role in maintenance of stem cell functions and activation of telomerase in MSC or in a compartment specific manner can be a novel strategy for abolishing age-related bone loss.

NEUROPEPTIDE Y1 RECEPTOR AS A POTENTIAL CANDIDATE FOR BONE REGENERATION THERAPIES

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Therapeutic application of Neuropeptide receptors drugs has arisen as a promising area of research. The vast implication of Neuropeptide Y1 receptor in a diversity of centrally physiological actions has prompted Y1 receptor as a novel therapeutic target namely for obesity, eating or anxiety disorders treatment and tumor therapy. The increasing evidences showing the involvement of Y1 receptor signalling in the local control of bone remodelling suggest that an anti-receptor strategy may be a useful approach to prevent and/or reverse bone loss. Nevertheless, the mechanisms underlying the action of Y1 receptor signalling on osteoblast lineage activity are still unknown.

With this purpose, MC3T3-E1 pre-osteoblast cells were treated with a range of concentrations of NPY_{1-36} or co-treated with NPY_{1-36} + Y1-R antagonist (BIBP3226) for 24 hours.

Receptors expression profile analysis showed that the regulation of Y1-receptor expression is possibly associated to Y2 receptor in osteoblast cells. Furthermore, cell proliferation assays showed that the pharmacological blockade of Y1 receptor leads to a dose-dependent increase of the total DNA content of osteoblasts cells. To investigate whether this effect involve ERK activation**,** western blot analysis of ERKp expression were performed. The results showed the involvement of Erk/MAP kinases in the proliferative effect mediated by the blockade of Y1 signalling.

To confirm whether the variations of the proliferation rates associated to Y1 receptor signaling were not associated with cell apoptosis, Cell Metabolic Activity (MTT) and DNA fragmentation (TUNEL) assays were conducted. Accordingly, no significant changes in cell metabolic activity neither apoptotic cells were observed.

Taken together, these results show that Neuropeptide Y1 receptor as a potential candidate for bone regeneration therapies.

SILENCING OF BRG1, THE ATPASE SUBUNIT OF SWI/SNF CHROMATIN REMODELING COMPLEX, INDUCES SENESCENCE OF MESENCHYMAL STEM CELLS

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Aims: Chromatin state is fundamental for gene expression. In fact, euchromatin is permissive for transcription, while heterochromatin is repressive. Chromatin remodeling factors can modify the balance between euchromatin and heterochromatin by acting as main regulators of gene expression. Self-renewal, proliferation and differentiation properties of stem cells are controlled by key transcription factors. However, their activity is modulated by chromatin remodeling factors that operate at the highest hierarchical level. Studies on these factors can be especially important to dissect molecular pathways governing the biology of stem cells. SWI/SNF complexes are ATP-dependent chromatin remodeling enzymes that have been shown to be required for cell cycle control, apoptosis and cell differentiation in several biological systems. The aim of our research was to investigate the role of these complexes in the biology of mesenchymal stem cells (MSCs). To this end, in MSCs, we silenced the ATPase subunit of SWI/SNF (*BRG1*) by adenovirus carrying a shRNA.
Methods: BRG1 silencing is evaluated by RT-PCR and Western blot analysis. *BRG1* silencing effect in MSCs biology is analized by RT-PCR, Western blot analysis, Immunocytochemistry assays, FACS analysis and TRAP assay.
Results and Conclusion: Silencing of *BRG1* expression induced a significant increase ($p > 0.05$) of senescent cells. This was associated with decrease of apoptosis. Of interest, *BRG1* downregulation induced an increase of heterochromatin as detected by micrococcal nuclease assay.
At the molecular level these phenomena were associated with activation of *RB2/P130*- and *P53*-related pathways. Senescence was associated with reduced expression of some stemness-related genes.
Our previous researches showed that BRG1 upregulation by ectopic expression induce also senescence processes. Together these data suggest BRG1 belongs to a class of genes having a tightly regulated expression. For these genes even subtle alterations in their expression may disrupt the normal function of cells. One explanation for why certain genes may require precise control is if they regulate or are involved in balancing disparate downstream pathways possessing mutually opposing activities. This may be the case of *BRG1*, which can modulate gene expression in either positive or negative manner.
Further studies will be devoted to analyze in depth the molecular pathways associated with senescence.

BOTH N- AND C-TERMINAL FRAGMENTS OF PARATHYROID HORMONE-RELATED PROTEIN RESTORE THE ALTERED OSTEOGENIC DIFFERENTIATION INDUCED BY HIGH GLUCOSE IN MESENCHYMAL CELLS FROM THE MOUSE BONE MARROW

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Type 1 diabetes mellitus is associated with bone loss by poorly characterized mechamisms. Parathyroid hormone (PTH)-related protein (PTHrP) is abundant in bone, where it exerts autocrine/paracrine actions to modulate bone remodeling. Our recent findings strongly suggest that this protein has an important role in the reduced osteoblastic function in diabetes-related osteopenia. In the present in vitro study, we examined the effects of high glucose on growth and differentation of osteoprogenitors from mouse bone marrow (BM), and the possible modulation by PTHrP. Both its N-terminal fragment, homologous to PTH, and the PTH-unrelated C-terminal fragment were examined in this regard. BM cells, isolated from tibiae and femurs of CD-1 mice (13 weeks old), were cultured in either osteogenic medium (α-MEM with 10% FBS, 50 μg/ml ascorbic acid and 10 mM β-glycerolphosphate) or adipogenic medium (α-MEM with 10% FBS and 1 mM troglytazone), at a density of $1.5x10^6$ cells/P-6 well for 2-3 weeks. Then, they were exposed or not (control) to either PTHrP (1-36) or PTHrP (107-139) (100 nM) for the first 6 h of each consecutive 48-h incubation cycle (up to 7 cycles) or continuously, without (normal glucose, NG, 5.5 mM) or with high glucose (HG, 25 mM). Total colony-forming units (CFUs) (by cristal violet), alkaline phosphatase (ALP)+ colonies (CFU-ALP), and cell matrix mineralization (by alizarin red) were then assessed. BM cells grown in osteogenic medium with HG yielded less CFUs and small-size CFU-ALP colonies (-60%), as well as a reduction in both the mineralized surface (-40%) and the number of total and mineralized nodules (-30%). This was associated with reduced protein levels (by immunoblotting) of β-catenin, the molecular node of canonical Wnt signaling to increase bone mass decreasing osteoclastogenesis. On the other hand, presence of HG induced an increase in the number of adipocytes (77/field vs 15/field NG) in BM cells cultured in adipogenic medium, and an augmented PPARγ2 gene expression in these cells in osteogenic medium. Continuous or transient (6-h) exposure of the latter cells in HG medium to either PTHrP peptide normalized the aforementioned alterations in CFUs and mineralization and in β-catenin levels. This intermittent exposure to these PTHrP peptides was also enough to significantly decrease the high number of adipocytes and PPARγ2 mRNA levels observed in HG media. In conclusion, these in vitro data indicate that both PTHrP (1-36) and PTHrP (107-139) can similarly restore the deficient osteogenic differentiation of BM stromal cells induced by a HG environment.

DLK1/PREF-1/FA1 INHIBITS CHONDROCYTE DIFFERENTIATION THROUGH AKT-DEPENDENT PATHWAY

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Aim: Delta-like 1 (dlk1) gene knock-out mice display perinatal lethality (more than 50%), growth retardation, and a variety of tissue specific defects, including skeletal abnormalities. In this study, we try to figure out whether this gene has effects on the development of cartilage, and what's the molecular mechanism of the regulations during chondrocyte differentiation.
Methods: Firstly, we studied Dlk1 expression pattern during the development of mouse embryonic cartilage tissue by immunohischemistry. Then we choose the human and mouse mesenchymal stem cells as the models *in vitro*, either by stably overexpressing *Dlk1* gene in the cells, or by treating the cells by secret form of Dlk1 protein (FA1) in the differentiation mediums, we checked the effects of this gene during the chondrogenic differentiation. And the molecular signal transduction pathway was checked by Western blots.
Results: Dlk1/FA1 was detected throughout the epiphyses and the proliferating zone of the epiphyseal growth plates during mouse fetal stage E14.5- E18.5, but no staining was observed in prehypertrophic or hypertrophic chondrocytes. Human mesenchymal stem cells overexpress dlk1 exhibited impaired chondrogenesis when cultured in micro-mass conditions and stimulated by insulin and TGF-β1. Similar inhibitory effects on insulin-induced chondrogenic differentiation were observed in mouse prechondrogenic ATDC5 cells overexpressed dlk1. The inhibitory effects were through secret form of this protein -FA1. The inhibition is FA1 dose-dependent and occurred throughout the different stages of differentiation. Insulin treatment of ATDC5 cells was associated with activation of Akt and ERK1/2 (p42/p44 MAPK), but not p38. The presence of dlk1 completely blocked the phosphorylation of Akt activation by insulin but did not affect the activation of p42/p44 or that of P38 MAPK.
Conclusions: dlk1 is a novel negative regulator for chondrogenic cell differentiation. Moreover, suppression of Akt activation by dlk1/FA1 is a possible mechanism for dlk1-mediated inhibitory effects on chondrogenic differentiation.

TEMPORAL GENE EXPRESSION OF HUMAN BONE MARROW STROMAL CELLS DURING OSTEOBLAST DIFFERENTIATION

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Aim: To identify expression signatures unique for specific stages of osteoblast differentiation in order to improve our knowledge of the molecular mechanisms underlying the onset of orthopaedic defects.
Methods: We performed a microarray analysis on the whole transcriptome of human bone marrow stromal cells (hMSCs) obtained from the femoral canal of patients undergoing hip replacement. By defining different time-points within the differentiation and mineralization phases of hMSCs, temporal gene expression changes were visualised. Importantly, the gene expression of adherent bone marrow mononuclear cells, being the undifferentiated progenitors of bone cells, was used as reference. In addition, only the cultures able to form mineral nodules at the final time-point were considered for the gene expression analyses. To obtain the genes of our interest, we only focused on genes:
i) whose expression was significantly upregulated;
ii) which are involved in pathways or biological processes relevant to proliferation, differentiation and functions of bone cells;
iii) which changed considerably during the different steps of differentiation and/or mineralization.
Results: Among the 232 genes identified as differentially expressed by microarray analysis, we selected 76 molecular markers related to specific steps of osteogenic differentiation. These markers are grouped into various gene families according to their involvement in processes which play a key role in bone cell biology such as angiogenesis, ossification, cell communication, development and in pathways like TGF beta and Wnt signaling pathways.
Conclusions: Taken together, these results allow us to monitor hMSC cultures and to distinguish between different stages of differentiation and mineralization. This work will enhance our understanding of bone development and will enable us to recognize molecular defects that compromise normal bone function as occurs in pathological conditions. As such this protocol will provide us with novel therapeutical targets.

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DUAL FUNCTION OF TNFRSF19 IN OSTEOGENESIS AND ADIPOGENESIS

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Aim: Wnt signaling is important in determination of cell fate of human mesenchymal stem cell (hMSC). Previously, we established hMSC cell lines stably expressing Wnt co-receptor LRP5 with an activated mutation T253I or an inactivated mutation T244M. Both in vitro and in vivo data demonstrated that T253 cells transducer high level of Wnt signaling, have increased bone formation but decreased adipogenesis capacity. T244 cells exhibit opposite effects. The inverse relationship between commitment of hMSC to osteoblast or adipocyte lineage was further confirmed by quantitation of bone marrow composition in iliac crest bone biopsies from patients with T253 mutation. This study aimed at finding out molecules and/or signaling pathways downstream Wnt which responsible for such inverse commitment.
Methods: Microarray was used to compare the gene expression patterns between T244 and T253. Those genes upregulated differentially between two cell lines by Wnt3a were selected for bioinformatic analysis of their promoters. Promoter of selected gene was cloned into luciferase reporter vector and their promoter activity was analyzed by dual luciferase assay. Knock down and overexpressing of selected gene followed by real-time RT-PCR, ALP activity quantitation and Oil-red O staining was performed to examine gene's function during in vitro osteogenesis and adipogenesis.
Results: Fifteen genes differentially expressed between T244 and T253 cells were selected to retrieve their promoters of all of transcripts for bioinformatic analysis. We found tumor necrosis factor receptor superfamily member 19 (TNFRSF19) transcript 2 has six Wnt responsive elements (WRE) within defined promoter region. Besides, several C/EBP binding sites were found in promoters of transcript 1 and 2. Dual luciferase assay indicated that promoter of transcript 2 can respond to Wnt3a stimulation but not promoter of transcript 1. Besides, both promoters' activity could be inhibited by C/EBP transcription factors. During Wnt3a treatment, transcript 2 was dramatically and continuously upregulated by Wnt3a up to 7 days. But transcript 1 was less upregulated and remained stable after one day. Knocking down TNFRSF19 in T253 cells or overexpressing of this gene in T244 cells did not change the ALP expression but significantly reduced ALP activity in T253 cells or increased ALP activity in T244 cells respectively. During in vitro adipogenesis, C/EBP transcription factors were upregulated, therefore TNFRSF19 expression was reduced at early adipogenesis. Knocking down TNFRSF19 in T253 cells or overexpressing this gene in T244 cells dramatically increased adipogenesis in T253 cells or decreased adipogenesis in T244 cells.
Conclusion: We revealed a novel function of TNFRSF19 in mesenchymal tissue development. TNFRSF19 transcript 2 may have specific function in mediating canonical Wnt signal to osteogenic marker ALP possibly at translational level. However, during adipogenesis, TNFRSF19 was suppressed by upregulation of C/EBP transcription factors. Therefore, TNFRSF19 may be involved in determining cell fate of hMSC through Wnt and C/EPB.

PARTIAL SILENCING OF METHYL CYTOSINE PROTEIN BINDING 2 (MECP2) IN MESENCHYMAL STEM CELLS INDUCES SENESCENCE ALONG WITH INCREASE OF DAMAGED DNA

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Aims: Mesenchymal stem cells (MSCs) are of interest because of their multiple roles in the physiology of organisms. Several evidences demonstrate that ageing is in part associated to stem cell senescence that occurs as results of extrinsic or intrinsic agents that cause DNA damages. Ageing of MSCs can have profound consequences on the body physiology. Among regulators of chromatin status the MECP2 protein plays a key role by binding methylated CpG dinucleotides. MECP2 protein mediates gene silencing by causing changes in chromatin structure through the interaction with co-repressors. Although it was considered a global transcriptional repressor, recent studies have provided evidences that MeCP2 function extends beyond gene silencing. In fact, MECP2 may be considered a transcriptional modulator rather than a transcriptional repressor. Rett syndrome (RTT) is one of the most common genetic causes of mental retardation. Mutations in MECP2 gene are found in up to 90% of classic RTT patients. In RTT patients MECP2 inactivation can impair epigenetic mechanisms regulating stem cell biology this in turn could alter the physiological development of tissues and organs.
For these reasons, we decided to investigate the biology of bone marrow MSCs in RTT patients in order to verify if mutation in MECP2 gene can result in alteration of stem cell biology (Squillaro et al., 2008).
Our studies evidenced that MSCs from RTT patient showed precocious signs of senescence compared with healthy controls.
To confirm and extend this research we took advantage of adeno-siRNA technique to silence MECP2 in MSCs from healthy donors.
Methods: MeCP2 silencing is evaluated by RT-PCR and Western blot analysis. MeCP2 partial silencing effect in MSCs biology is analized by RT-PCR, Western blot analysis, Immunocytochemistry assays, FACS analysis and TRAP assay.
Results: Downregulation of MECP2 induced a decrease of cell proliferation and apoptosis along with trigger of senescence in MSC cultures. Reactive oxygen species (ROS), which are normal byproducts of cell's metabolism, are a chronic persistent damaging agent that greatly contributes to aging. 8-oxo-2'-deoxyguanosine (oxo8dG) that increases during cellular senescence is the major product of ROS action. Partial silencing of MECP2 augmented significantly the percentage of oxo8dG positive MSCs.
Senescence induced by partial silencing of MECP2 appears to rely upon impairment of DNA damage repair mechanisms. In fact, following transduction of MSCs with Ad-siRNA-MECP2, we observed a downregulation in the expression of several genes belonging to base and nucleotide excision repair, mismatch repair, and double strand break repair. In agreement with this result, we observed a reduced ability of MSCs transduced with Ad-siRNA-MECP2 to repair double strand breaks.
Cell cycle arrest and senescence induced by MECP2 silencing appear to be governed by activation of RB pathway. In fact, following transduction of MSCs with Ad-siRNA-MECP2, we detected an upregulation of RB gene expression along with a decrease of hyperphosphorylated inactive form of RB2/p130. The activation of RB-pathways seems to be confirmed by the observation that the cyclin kinase inhibitor $P16^{INK4A}$, which prevents RB phosphorylation and inactivation, showed an upregulation of mRNA and protein expression.
*Conclusions***:** We have identified a chromatin modifier whose expression must be tightly regulated to avoid alteration in cell's homeostasis. In detail, its downregulation induces senescence phenomena. Senescence seems to occur throught canonical RB- and P53-related pathways.
Our research could provide also a news insight on RTT syndrome. Senescence phenomena could be involved in triggering RTT syndrome-associated deseases.

TWO-DIMENSIONAL AND THREE-DIMENSIONAL FIB/SEM IMAGING IN BONE AND BIOMATERIALS RESEARCH

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Objective: The objective of this work is to introduce FIB/SEM in the context of bone and biomaterials research and to experiment with its ability to produce 2D and 3D data. The FIB/SEM combines both focused ion beam and scanning electron microscope in a single instrument in which the electron and ion beams are focused in the same point of the sample, called the eucentric position. This feature provides us with a powerful tool for simultaneous SEM imaging and FIB manipulation of a biological sample. The obvious limitation of traditional electron microscopy of being able to image separately the surface (SEM) and intracellular properties (TEM) can thus be overcome by the FIB/SEM. It gives us the opportunity to explore a cm- to mm-scale sample in SEM mode and to manipulate and picture the sample in micro- to nano-scale in FIB/SEM mode to reveal its below-the-surface properties. For that reason the FIB/SEM can be very valuable in needle-in-a-haystack applications, such as imaging a cell in a specific state (e.g. resorptive osteoclast), and offers an alternative to costly and time consuming serial sectioning procedures.
Method: Monocyte-macrophage cell line RAW 264.7, pre-osteoblast cell line MC3T3-E1 and mouse hematopoietic stem cells from the bone marrow were cultured on bovine bone slices and on poly-96L/4D-lactide disks and on CellVessel bioabsorbable poly-96L/4D-lactide multifilament scaffolds (Scaffdex, Tampere, Finland). FIB/SEM imaging and milling was done with Helios Dual-Beam (FEI Company, Eindhoven, The Netherlands). Automatic Slice and View software (FEI Co.) was used for collecting the data for 3D modeling.
Results and Conclusion: FIB/SEM imaging, combined with EDS analysis, can be successfully applied in imaging of the traditionally problematic hard material/soft material interfaces e.g. bone/osteoblast and implant/new bone interfaces due to the good cutting properties of the focused ion beam milling in comparison to the conventional TEM sample preparation with a microtome. However, the strong charging experienced due to the non-conductive elements of the sample, e.g. bone and polymeric biomaterials, present a big challenge in making the use of FIB/SEM more common within this field of application.

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