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Correlating *ex vivo* and *in vivo* osteogenic assays for quality control of clinically destined cGMP grade hBM-MSCs

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ABSTRACT

Among diverse approaches in translational medicine, there is growing interest in use of autologous human mesenchymal stem cells (hMSC) to enhance bone regeneration. Working within a European consortium, we aimed to evaluate the osteogenic potency of primary bone marrow- (BM) hMSC cultures after isolation and expansion using clinical grade procedures (cGMP-BM-hMSC). Conforming to this context, we aimed to standardize a functional assay that was rapid, reproducible and readily applicable.

Historically, numerous genes have been associated with the cascade of genetic changes required for osteogenic differentiation. Though expression of these genes in differentiated hMSC *ex vivo* rarely predict *in vivo* bone formation, recent studies using immortalized hMSC, have shown some correlations. Aiming to comply with a clinical protocol, we wished to explore whether such gene expression changes could also be applicable in different contexts: (1) using primary hMSC; (2) with growth medium supplemented by platelet lysate (PL) instead of fetal bovine serum (FBS) and (3) after induction of *ex vivo* osteogenic differentiation for one week instead of two weeks.

Testing six cGMP-BM-hMSC samples for osteogenesis by *ex vivo* cellular matrix mineralization stainings, only four were positive for alizarin Red S (ALZ) and only three for Von Kossa (VK). Four of these donors generated ≥15% new bone area in histological sections from bone formation assays in immunodeficient mice after 6 weeks. Notably, the *ex vivo* phenotype of matrix mineralization staining at two weeks only correlated well with *in vivo* bone formation in 50% of the samples. When testing a panel of twelve osteogenic biomarker genes after one week of osteogenic induction, a subset of genes showed statistically significant changes in expression level that correlated with *ex vivo* mineralization. No direct correlation

between expression and bone formation was found for any individual gene. Nonetheless, cluster analyses of five consistently expressed genes correlated with the hBM-MSC bone forming potential in an ectopic murine model, suggesting this approach may prove helpful for assessing the osteogenic differentiation potential within clinical trials.

INTRODUCTION

MSCs: definition and biological proprieties

Musculoskeletal disorders and disease are more common than heart disease, respiratory disease or cancer[1] and there is particularly interest in the development of new therapeutic approaches based on stem cells. The fundamental difference between the two main categories of stem cell is that embryonic stem cells appear to be totipotent and are able to generate every type of tissues, while the adult ones are multipotent and generate cells belonging to the same germ layer from which they originated themselves. Mesenchymal stem/stromal cells (MSC) are among the most commonly used adult stem cells in regenerative medicine. In 1974 MSCs were isolated from bone marrow (BM) stroma by Friedenstein et al[2], but in the last years they have been isolated from other sources, such as: adipose tissue (AT), dermis, hair follicles, umbilical cord blood (UCB), peripheral blood, heart, liver, spleen and dental pulp[3]. The MSCs isolated from these different sources have similar properties and morphology, but differences were identified between UCB-MSCs and BM-MSCs and between AT-MSCs and BM-MSCs. In particular the UCB-MSCs do not seem to have the ability to differentiate into the adipogenic lineage, however they differentiate into osteogenic lineages within a shorter time than BM-MSCs. In addition the UCB-MSCs did not show contact inhibition and proliferated faster than BM-MSCs. No remarkable difference in morphology was observed between AT-MSCs and BM-MSCs, but the adipose derived cells had a faster doubling time and higher proliferation potential relative to BM-MSCs[4].

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) has proposed a minimal set of criteria to define human multipotent mesenchymal stromal cells (MSCs), including plastic-adherence ability, specific surface marker expression and differentiation potential[5]. The

immunophenotypic evidence for MSC is mainly based on characterization *in vitro* and it is not possible to identify them through a single surface marker. A flow cytometry characterization must show 95% of the MSC population to express CD73 (ecto-50-nucleotidase), CD90 (Thy-1), and CD105 (endoglin), and no more than 2% of the cells may express CD34 (hematopoietic progenitor and endothelial cell marker), CD45 (pan-leukocyte marker), CD11b or CD14 (monocyte and macrophage markers), CD19 or CD79a (B cell markers), and HLA-DR (marker of stimulated MSCs). MSCs also are recognized by the monoclonal antibody CD49a, STRO-1 and CD200 (OX-2). The 95% of MSCs *in vivo* express low-affinity nerve growth factor receptor, (LNGFR; now clustered as CD271) and HLA-DR (marker of stimulated MSCs)[6]. Such antigenic marker surface may also differ depending on the source from which the MSC derive, in fact the CD106 was detected only in the BM-MSCs, the CD49d is expressed only in AT-MSC and the expression of chemokine receptors, as CCR1, CCR7, CXCR4, CXCR6, appears to be increased in AT-MSCs compared to BM-MSCs[7].

These adherent colony-forming fibroblastic cells have a self-renewing capacity and generate cells with multilineage potential, corresponding to osteoblasts, adipocytes and chondroblasts under *in vitro* culture-differentiation condition[8]. Despite this great potential for proliferation and differentiation, there is substantial variation, among different marrow samples, in the number of population doublings, in the number of CFU-F colonies per total mononuclear cells and in the size of the individual colonies. Furthermore, when these cells were maintained in culture for 12 passages[9], they senesced and lost their multipotentiality as demonstrated by loss of their ability to differentiate into adipocytes and chondrocytes. Interestingly, they retain their ability to differentiate into osteoblasts[9] and donor age did not affect

osteogenic differentiation potential[10]. In recent literature it has been demonstrated that in rare instances and under specific contexts hMSCs may have the additional potential to differentiate along non-mesodermal lineages, in particular they are able to differentiate into hepatocytes *ex vivo* and *in vivo*[6], into neuron-like cells *ex vivo*, into astrocytes and neurons after *in vivo* implantation into the mouse brain[4]. These cells did not differentiate spontaneously during the culture and maintained a normal karyotype and telomerase activity[11].

Besides their differentiation potential, MSCs modulate the immunological response[12] and are able to migrate into inflammation sites. In the context of regenerative damaged tissues and remodeling processes, the ability to direct the MSC to target tissue is required[13]. The homing of stem cells is directed by chemotaxis mechanisms and involves CC chemokine receptors (except CCR10), CXC and CX3CR1 receptors, localized on MSCs surface and the cytokines produced by injured sites. The human MSCs showed significant chemotaxis responses to several factors, including PDGF, VEGF, IGF-1, IL-8, SDF1-a, bone morphogenetic protein (BMP)-4 and BMP-7[14]. The fact that MSCs express a variety of chemokine receptors suggests that have the potential to home into various tissues, e.g. CCR9 is used to enter into intestines or CCR1 to enter into inflamed joint tissues[15]. Once at the damaged tissue MSCs extravasate, a process involving transient disassembly of the endothelial junctions and penetration through the underlying basement membrane[16].

MSCs clinical application

In its broadest sense, use of cells for therapy has been clearly demonstrated from use of bone marrow transplantation that is now routinely used to treat hematological malignancies, nonmalignant hematologic disorders, and solid tumors. More specifically, appreciating the osteogenic differentiation potential of bone marrow derived MSCs, experiments have tested their capacity to treat different animal models of bone disease.

In order to define the osteogenic *in vivo* potential of MSCs, several models of ectopic bone formation have been developed. By incorporation of MSCs into a biodegradable three-dimensional (3D) scaffold, the osteogenic potential of the cells can be combined with the osteoconductive properties of the scaffold, creating a tissue-engineered construct for bone grafts. Because of its excellent osteoconductivity and biocompatibility, calcium phosphate-based bioceramics are a promising candidate as the bone tissue-engineering scaffold. New bone deposition in porous calcium phosphate ceramics seeded with human bone marrow stem cells is well established in xenograft models of subcutaneous implantations into rats or mice[17]. In particular when β-TCP ceramics combined with osteo-induced stem cells were implanted subcutaneously into nude mice, tissue engineered bone formed in the scaffold pores after 4 weeks. In agreement with previous studies by the same group, β-TCP scaffolds alone exhibited no osteoinductive properties and even after 12 weeks, no *de novo* bone was deposited on the subcutaneous scaffolds in the nude mice[17].

Similar promising results have been reported with use of BM-MSCs in animal models for different bone diseases, including: osteogenesis imperfecta and critical sized segmental defects. In particular the mouse model of segmental bone defect

demonstrated the potential of autologous MSC transduced with VEGF and BMP2. After 5 weeks of MSC therapy the tibial bone defect was corrected with new bone growth leading to less deformed skeletal integrity compared to control tibiae[18]. Liu et al have investigated if β-TCP ceramic cylinder loaded with osteogenically induced autologous bone marrow stromal cells can be used to repair load-bearing bone defect in a goat model. At 16 weeks post-operation the union between the implant and the host bone was observed[19]. Furthermore, Nather et al, have shown that after 12 weeks the injection of autologous MSCs can be able to repair a large tibia weight-bearing defect in adult rabbits[20].

Positive results in animal models have favored the development of several clinical trials and encouraging results have been reported for administration of autologous hMSC in various contexts, including to enhance engraftment of heterologous bone marrow transplants[9]. As an example that powerfully demonstrates the correlations between animal models and clinical trials Otsuru et al, in a murine model of OI, demonstrated that the MSCs differentiate to osteoblasts and contributed normal collagen to the bone matrix. Applying similar strategies in a clinical trial in children with osteogenesis imperfecta the same authors demonstrated that the hMSC induced the production of a soluble mediator from a second tissue that activates the proliferation of chondrocytes in the growth plate. The donor-derived cells engrafted in bone and contributed to form normal collagen to the bone matrix, providing an effective cell therapy for the osteogenesis imperfecta[21].

BM-MSCs ex vivo expansion

For bone tissue engineering approaches that may need a therapeutic dose of 10^6 cells/kg, it is not possible to directly isolate this number of cells from the bone marrow, however it is possible to obtain this number of cells via *ex vivo* expansion[22]. Indeed the MSCs constitute about 0.001% to 0.01% of BM cells and they can be enriched by a density gradient separation step in order to help eliminate unwanted cell types[23,24]. The MSC have the ability to proliferate in culture with an attached well-spread morphology whereas associated HSCs and other non-adherent cells are removed with medium changes. The two must commonly use hMSC culture media used are the basal culture medium Dulbecco's modified Eagle's medium (DMEM) and Minimum essential Medium alpha (α-MEM), supplemented with fetal bovine serum (FBS). These two media have different characteristics, particularly DMEM has a smaller range of individual components, but amino acids, vitamins and nutrient concentrations are higher than those found in α-MEM. This high nutrient concentration composition may help explain the higher values for cell proliferation found in DMEM as compared with those observed in α-MEM[25]. Usually the FBS-based media are been used to sustain the MSC *in vitro* expansion and osteogenic induction, and Kuzetsov et al., by using a colony-forming assay, has demonstrated that FBS remains an efficacious additive to induce and sustain BMSC proliferation *in vitro*[26].

However clinical applications of cell therapy represent a complicated challenge, due to the necessity of operating under strict Good Manufacturing Practice (GMP) conditions, to ultimately certify the quality of the cell product. This quality assurance includes not only the determination of the sterility of the cell lot and the compliance with specific clinical needs, but also the identity, purity, safety, and

functionality of the MSC-based constructs. Although FBS-based medium has been a conventional medium for hMSC isolation and expansion, it is nevertheless an undesirable source of xenogeneic antigens and bears the risk of transmitting animal viral, prion and zoonose contaminations. The culture of hMSCs in a clinical-grade medium is required for translation into clinical practice and serum-free medium or medium with a variety of human supplements, such as platelet lysate (PL), are under development to avoid use of FBS.

A more fully defined serum-free medium has been derived consisting of a blend of essential amino acids, inorganic salts and a mix of the recombinant human growth factors PDGF-BB, bFGF, and TGF-β1. Using a GMP-accepted culture medium, as EMEA medium, both spindle-shaped cells and large flat cells have been identified. Moreover, when comparing cell cultured in EMEA and in FBS supplemented media, about the same level of surface markers expression are found[27]. The human bone marrow-derived MSCs isolated and expanded in serum-free medium retained the competence for osteogenic induction and ectopic bone formation. Moreover the percentages of new bone area in the serum-free group and serum group are 25.1% and 21.1%, respectively.

A variety of human supplements have been postulated as alternatives to FBS to provide nutrients, attachment factors and growth factors. Indeed an alternative is to supplement media with human blood-derived platelet lysates, platelet rich plasma, thrombin-activated platelet released in plasma, plasma or sera from autologous or allogeneic donors, to create culture conditions that more closely mimic the human environment. Among these alternatives, platelet preparations are of particular interest as for the easy collection and for the availability in many hospital and transfusion centers[28].

PL-based medium: a novel clinical grade GMP reagent

The proliferation and osteogenic differentiation of MSCs can be enhanced by using culture medium with human PL generated by a simple freeze-thaw procedure of platelets providing growth factors released from the platelet fraction. Principal growth factors include: platelet derived growth factor (PDGF), transforming growth factor beta 1 (TGF-β1), insulin like growth factor (IGF-1) and basic fibroblast growth factor (bFGF).

The concentrations of various growth factors (PDGF-AB, TGF-β1, bFGF, IGF-1) or hormones (estradiol, parathormone, leptin, 1,25 vitamin D3) may vary with PL-donor age. Indeed Lohmann et al., has postulate that PL derived from younger donors enhances expansion rates of MSC and PL from older donors increases activity of senescence-associated beta-galactosidase. PL-donor age did not affect the fibroblastoid colony-forming unit (CFU-f) frequency, immunophenotype or induction of adipogenic differentiation, whereas osteogenic differentiation was significantly lower with PLs from older donors[29]. Since the growth factors concentration showed considerable donor-to-donor variation and to minimize batch-to-batch variation individual PL units are usually pooled[30].

As mentioned above, adding PL in expansion media does not change the MSC immunophenotypic characteristics, namely, expression of CD73, CD90, CD105 and CD106 and lack of expression of haematopoietic markers CD45, CD14 and CD34. For immaturity markers, expression of CD49a was low and CD133 was undetectable with PL or FBS medium[31].

Platelets based medium, besides avoiding the use of animal-derived material, accelerates MSC expansion, reducing the duration of *ex vivo* manipulation in

comparison to FBS[32]. In particular, the population doubling analysis revealed that PRP-cultured MSCs proliferated two fold faster than FCS-cultured MSCs[33]. hMSC grown in PL containing expansion media retained a multipotent differentiation potential towards osteogenic, adipogenic, and chondrogenic lineages. BM cells grown in PL or FBS deposited an extensive mineralized matrix when cultured for two weeks in osteogenic medium, as demonstrated by strong Alizarin red and von Kossa staining. These cells also efficiently differentiated into the adipogenic lineage, as indicated by Nile red staining of lipid droplets in the cytoplasm following culture in adipogenic medium. After chondrogenic differentiation for three weeks, MSCs displayed a typical chondroblast shape, as indicated by the deposition of specific glycosaminoglycans seen after Alcian blue and O Safranin staining[31].

Ex vivo osteogenic differentiation

In order to recreate the microenvironment that favors the differentiation of MSCs to osteoblasts *in vivo*, various *ex vivo* induction protocols based on the addition of different osteogenic components in the culture medium, including vitamin D3[34] and strontium ranelate have been developed[35]. Additionally, osteogenic differentiation can be obtained using well-investigated differentiation factors, such as bone morphogenic proteins (BMPs), dexamethasone, ascorbic acid and β-glycerophosphate.

BMPs are members of the transforming growth factor β (TGFβ1-3) superfamily and have essential roles in a wide range of *in vivo* biological processes. Genetic studies in knockout mice have showed that a critical threshold level of BMP2 was required for differentiation to mature osteoblasts. Furthermore, mice lacking only

BMP2 in the limb mesenchyme formed bone during embryogenesis but exhibited a clear defect in bone mineral density shortly after birth, resulting in frequent fractures that failed to heal[36]. Bone morphogenetic proteins are able to induce ectopic ossification at extraskeletal sites. In particular BMP-2 induces osteoblast differentiation and matrix maturation *in vitro*. BMP-2 inhibited the proliferation of both relatively immature and more mature normal human osteoblastic cells. BMP-2 at 100 ng/ml significantly increased the expression of alkaline phosphatase, type α1(I) collagen, osteopontin, bone sialoprotein, osteocalcin, and decorin in MSCs[37]. It has been postulated that BMP-2, a member of TGF-β superfamily, may regulate the activity of TGF-β in the same superfamily via the stimulation of decorin. In contrast, BMP-2 had very little effect on the osteonectin and biglycan mRNA levels[38].

Dexamethasone (DEX) is a synthetic glucocorticoid, which has been shown to stimulate MSCs proliferation and to support osteogenic lineage differentiation *in vitro*, together with β-glycerophosphate and ascorbic acid, in a concentration dependent manner[39]. The DEX has been identified to be able to increase the mRNA expression of RUNX2 and ALPL, stimulate the secretion of ALPL *ex vivo*, yet also induces a decrease in the production of OC[40]. Furthermore, DEX treatment increased the amount of calcium phosphates deposited by cells, confirmed as differentiated by expression of the osteoblastic glycoproteins, osteopontin and bone sialoprotein, known to be present in the ECM of bone.

The β-glycerophosphate (β-GP) is an organic phosphate source, which plays an important role in the mineralization process of primary cell cultures *ex vivo* that can modulating alkaline phosphatase (ALPL) activity and influence the expression of osteocalcin[41]. Coelho demonstrated that after 21 days cultures grew in the

presence of β-GP, alone or in combination with DEX and AA, presented ability to form mineralized calcium phosphate deposits. In addition cells cultivated in the presence of β-GP showed a decrease in cell proliferation respect to the cells grew in presence of DEX or AA[42].

Ascorbic acid (AA) plays a key role as a cofactor in the post-translational modification of collagen molecules and increases type I collagen production. In particular some studies have showed that the presence of various concentrations of AA resulted in a dose-dependent synthesis of collagen and suggested that the resulting increase in the accumulation of the extracellular matrix is associated with a higher ALPL activity and ability to form a mineralized matrix[42]. Aside from its role in collagen synthesis, soluble AA modulates the expression of other bone-related genes such as BSP and ALPL[43]. Furthermore, it is well known that ascorbic acid stimulates and modulates the proliferation of various mesenchyme-derived cell types including osteoblasts, adipocytes, chondrocytes, and odontoblasts *ex vivo*[44].

Regarding the interdependence of the combination of dexamethasone, ascorbic acid and β-glycerophosphate in the culture medium, Jun-Beom Park has shown that DEX alone may not be sufficient for the formation of mineralized nodules since this also depended upon the ability of cells to form multilayers *ex vivo*, the presence of AA, and the inclusion of β-GP into the culture medium. The addition of AA and/or β-GP seemed to increase the expression of TGF β, ER-α. An additional consideration is that the osteogenic induction factors can have time-dependent effects; really short-term application of DEX seemed to increase ALPL activity, on the other hand the long-term effect of DEX may resulted in the down-regulation of the expression of this receptor and TGF-β along with the upregulation of OPN expression[45].

Read-out for Bone Differentiation

The combined application of molecular, biochemical, histochemical and ultrastructural approaches have defined stages in the process of hMSC differentiation towards the osteoblast phenotype. A maturational sequence characterized by peak levels of expression of genes involved in osteoblast growth and differentiation has led to the description of three principal periods: a proliferative phase, which is followed by the period of matrix deposition, and lastly the mineralization phase[46].

A number of techniques have been used to examine mineralization *ex vivo*, including: quantitation of, inorganic phosphate and inorganic calcium, electron microprobe and electron diffraction analysis, scanning electron microscopy, X-ray diffraction, infrared (IR) spectroscopy and microspectroscopy. Despite a diversity of techniques for examining the deposition of mineralized matrix, this phenotype usually requires at least two weeks of continuous culture in osteogenic conditions and for our clinic protocols there was need to develop faster assay methods that aimed to be easy, inexpensive and widely standardized. For these purposes it sufficed to use the most commonly adopted methods including: the Von Kossa and Alizarin red S staining, the molecular analyses of m-RNA, microRNA or protein.

An additional approach for assessing mineralization, the colorimetric quantification of an osteogenic biomarker ALPL has been employed. The intracellular ALPL is found to peak early during the *ex vivo* bone formation, but after this starting phase its level starts to decline, probably due to the onset of the mineralization process[47]. Nonetheless the ALPL serum concentration is only an estimation of the rate of

osteoblastic bone formation because its activity was not proportional to observed mineralization levels[48].

After *ex vivo* osteogenic induction it is possible to identify the mineralization process using Von Kossa and Alizarin red S staining, and both methods have been used for diagnosis of calcium deposition and inspection of fine structures by phase contrast microscopy. In particular the Von Kossa's procedure is believed to visualize phosphate and carbonate anions, whereas Alizarin red S reacts with calcium and other cations[49].

Alizarin red S staining has been used for decades to evaluate calcium-rich deposits by cells in culture. It is particularly versatile because the dye can be extracted from the stained monolayer by using acetic acid or Cetylpyridinium Chloride, followed by colorimetric detection at 405nm[50]. This staining is also applicable after *in vivo* bone formation, indeed after the implant of MSCs and scaffold in mice, the grafts are fixed, paraffin embedded and then the Alizarin red S is used to detect the mineralization of the extracellular matrix[51].

In the Von Kossa's technique silver cations replace calcium bound to phosphate or carbonate groups and are then reduced to black metallic silver during exposure to UV light. This staining is well known since 19th century and von Kossa staining has been performed in conjunction with other methods, such as electron microscopy and phase contrast microscopy, to examine and confirm the MSCs mineralization. However, the von Kossa staining is used with a greater frequency as an indicator and semi-quantitative means for measuring mineralization by primary osteoblasts and osteoblast-like cell lines[52].

Despite being a commonly adopted method, the ALZ and von Kossa assays have limited sensitivity and are generally restricted to the latest stages of osteoblast maturation. Nowadays the quantitative RT-PCR is more reliable for evaluating the osteogenic differentiation by measuring the biomarkers levels.

During osteogenic differentiation, MSCs generate osteoprecursors, which progress to generate osteoprogenitors, preosteoblasts, functional osteoblasts and osteocytes; this progression is accompanied by the activation of transcription factors and the expression of bone-related marker genes. To evaluate the expression levels of these osteogenic biomarkers the RealTime-PCR is used. This more sensitive method is able to quantify the fold increase of different biomarkers between osteogenic inducted samples and undifferentiated samples. The quantification of bone-related gene upregulation or down-regulation is based on the possibility to detect or not detect the specific mRNA in the total RNA extracted from the cells. Due to this ability, the different stages of osteogenic differentiation are identifiable by measuring transcription factors related with the cell commitment (e.g. RUNX2, DLX5 or TAZ) or extracellular matrix proteins related with the mineralization process (e.g. Collagen I or Elastin).

The molecular analysis of the ability of MSCs to differentiate into the osteogenic lineage is applicable also at the level of MicroRNAs and proteins. The MicroRNAs (miRNAs) are small noncoding RNAs that act as key regulators of diverse biological processes by mediating translational repression or mRNA degradation of their target genes. At the present stage miRNA profiling is an innovative method and further investigations by PCR is needed to confirm the data obtained. This method has been used by different research teams and e.g. Eskildsen et al has identified that miR-138 modulates osteogenic differentiation of human MSCs[53].

The analysis of osteogenic differentiation is also possible by measuring the levels of intracellular proteins and secreted by MSC. The bone-related protein expression level can be quantified by using proteinChip approach[54] that includes a mass spectrophotometry analysis, or by the western blot[55] followed by chromatographic analysis. These methods, however, turn out to be more complicated than RealTime-PCR and are for the moment less applicable in the clinical setting.

Furthermore in a 3D *in vitro* system, in addition to Von Kossa staining or molecular analysis, the birefringence measurement technique can be used used to verify the arrangement of collagen fibers, a fundamental checkpoint for the mineralization process[56]. However this method required a polarized light microscopy and a detailed preparation of the samples for the quantitative analysis[57].

Despite differing contexts several methods can be applied to analyze the *in vivo* bone formation, both in animal models and human biopsies, including histological and immunohistochemical staining, Raman spectroscopy and bioimpedance. The most commonly used techniques are histological, comprising routine Haematoxylin and Eosin staining of histomorphology , Masson's or Mallory's trichrome stain for bone tissue, and more specific immunohistochemistry staining with antibodies that targets bone proteins such as collagen type I and osteocalcin antibodies to better define the osteogenic cells. These histological stainings require a specific sample processing, including decalcification and a section between 3-5 µm. Moreover the semi-quantification of new bone forming areas is achievable by a scoring method[58] adopted for this thesis.

Existing noninvasive technologies used to monitor bone have evaluated the morphology and calcium content, but provide no direct information on other

properties of the tissue. Vibrational spectroscopy has proved useful in the study of bone development, bone biomechanics, and bone pathologies because it provides this composition information. Raman spectroscopy was based on the study of vibrational proprieties of tissues by visible or near-infrared light, it may suffer from water interference but is applicable to fresh tissue and during *in vivo* measurements. The sensitivity of this method is very high, but the difficulty of acquiring and processing data does not allow its use as a routine screen[59].

Bioimpedance analysis, based on applying a small electric current into the body to measure the specific resistance characterizing the various tissues, is able to evaluate bone density. This technique is increasingly being used to quantify body composition in both the clinical and research settings, however it currently lacks the sensitivity needed to identify bone matrix pathologies like osteoporosis[60].

Relation between *ex vivo* osteogenic differentiation and *in vivo* bone-forming ability

Taking into account diverse sources, hMSC are among the most used adult stem cells in tissue engineering and regenerative medicine, with the advantage that most cases allow an autologous settings where cells can be obtained from the patient to be treated. No formal tests are available to predict the potency of individual bone marrow-derived MSC populations with respect to bone formation *in vivo.* This would be very helpful in order to pave the way for standardized clinical use of MSC in bone repair. In addition, few rate-limiting cellular or molecular correlates of *in vivo* bone formation have been identified for hMSC. Therefore a critical point is to know whether it is possible to identify *ex vivo* functional features

that correlate with the extent of *in vivo* bone formation in order to develop a potency assay that verifies the bone-forming function of human MSC before clinical transplantation. Parameters such as age, gender, medication or disease of the donor and the location of bone marrow harvest can all influence human MSC and lead to functional heterogeneity, making human MSC from some donors ineffective compared to others. Similarly, it is important to consider that the culture conditions are likely to influence the overall cell composition and functionality, with the fitness of cells at the time of transplantation strongly influencing their bone forming potency *in vivo*.

Several research groups have tried to find a correlation between the *ex vivo* and *in vivo* osteogenic potential of human BM-MSC, by evaluating the expression of osteogenic markers *ex vivo* and the subsequent ability of the cells to form bone tissue *in vivo*. For example, Mendes et al., analyzed the activity of alkaline phosphatase and the expression of pro-collagen type I and osteopontin in cultured hMSC that were then used for subcutaneous implantation in a nude mice model. There was no direct correlation between the expression levels of these biomarkers *ex vivo* and the *in vivo* bone formation[61].

In contrast, Jaquiery et al. have suggested that the expression of osteoblasts-related markers *ex vivo* could be used to predict the osteogenic capacity of the cells *in vivo*. In particular, they analyzed expression of BSP, OPN, and OSX mRNA in a 3D culture system and then used the cells for ectopic bone forming assays. They found a significant correlation (p value <0.02) whereby only cells expressing relatively high levels of these genes formed bone *in vivo*, indicating that this assay may be used to predict the osteogenic potential of cells from different donors[62].

To gain a broader overview of genes that may be important for bone formation, researchers used hBM-MSCs that stably expressed the human telomerase reverse-transcriptase (hTERT) gene (hMSC-TERT)[63]. The cells allowed expansive gene expression analysis using the Affymetrix DNA Microarray platform, clonal expansion and reproducible testing of *in vivo* bone formation by subcutaneously implanting hMSC-TERT derived cell lines mixed with an osteoconductive material in immune deficient mice. The overall aim was to identify *ex vivo* molecular markers that were better at predicting the bone-forming capacity of hBM-MSC[64]. Notably, when clonally derived hMSC-TERT populations were tested in this assay, not all of the cloned cell lines were able to form bone and common *ex vivo* markers of osteoblastic phenotype (e.g., ALPL) were not predictive of the *in vivo* bone-forming ability. Significant novel biomarkers predicting the bone-forming potential of the hMSC-TERT cell lines, included a cohort of genes encoding extracellular matrix proteins and genes that tended to have Sp3 transcription factor binding-sites in their promoter. In contrast, clones that did not form bone expressed a large number of immune-response related genes, such as the interferon-gamma inducible gene SLAMF7[64].

Using primary hMSC cultures from different donors, Janicki et al., explored correlations between the donor characteristics, the *ex vivo* growth properties, differentiation potential and associated gene expression profiles relative to subsequent analysis of *in vivo* bone-forming ability. Results from *ex vivo* mineralization assays did not correlate with bone formation, also the range of ALPL activity during osteogenic differentiation was highly variable among the hMSC that could form bone *in vivo*. The authors concluded that their gene expression profiles highlighted the key role played by proliferation *ex vivo* for bone

formation *in vivo*. They proposed that this probably reflected that rate-limiting anabolism and open chromatin structures were determinants and predictors of the therapeutic potency of culture-expanded MSC[65].

The above results, confirmed earlier studies that there is extensive heterogeneity in the potential for osteoblastic differentiation among standard cultured BM-MSCs populations[66]. It was also verified that the surface markers and *ex vivo* mineralization assay did not *per se* indicate whether cultured MSC populations were capable of forming bone *in vivo*. The canonical osteoblastic markers may be specific for the osteoblastic commitment phenotype in general but lack the appropriate correlation to predict *in vivo* bone-formation capacity, which may be dependent on additional cellular characteristics, including the ability to produce a large number of ECM proteins. Thus these markers define the ‘‘minimal criteria’’ for commitment of a cell to the osteoblastic phenotype without distinction among functional subpopulations. These findings suggest that predicting osteogenic potency in primary culture of hMSCs represent a complex challenge, not simply met by identification of a limited number of canonical osteoblastic markers. Hence the urgent need to explore here, whether we can better predict bone forming potential in a clinical context, by identifying multiparametric biomarkers with greater relevance for *in vivo* bone formation.

MATERIALS AND METHODS

Cell Culture

The bone marrow derived mesenchymal stem cells (MSCs) were obtained from the GMP facility at the cell therapy centre of EFS, Toulouse, France, and Ulm University (UULM) (Table 1). After local anaesthesia, bone marrow tissue samples, of consenting donors was harvested from the iliac crest in accord with local ethical committee guidelines. About 10 mL of bone marrow tissue was collected in heparinized syringes and the mononuclear cells were seeded in culture Maintenance Medium (MM) at a density of $5x10^4$ cells/cm^2 in CellSTACK™ (Corning) culture vessels. MM consisted of Minimum Essential Medium (MEM) Alpha without nucleosides (Gibco® Invitrogen, UK), supplemented with 5% Platelet lysate (PL), 1% L-Glutamine (Gibco® Invitrogen, Belgium), 1 IU/mL heparin (Sigma-Aldrich, USA) and 10 µg/mL ciprofloxacin (HIKMA, Portugal). BM-MSCs were passaged only once before shipment as frozen cells. Upon arrival the cells were stored in liquid nitrogen until thawing and cultured in Maintenance Medium[67], consisting of Minimum Essential Medium (MEM) Alpha without nucleosides (Gibco® Invitrogen, UK), supplemented with 8% Platelet lysate (PL), 1% L-Glutamine (Gibco® Invitrogen, Belgium), 1UI/mL heparin (Sigma-Aldrich, USA) and 10 µg/mL ciprofloxacin (HIKMA, Portugal). BM-MSCs were grown in T_{75} flasks (Greiner Bio-one, Germany) at a density of $6\text{-}7X10^3$ cells per cm^2 and incubated at 37°C with 5% CO_2 in a humidified incubator and were replenished with fresh medium three times per week until near confluent.

Ex vivo osteogenic differentiation

After 5-7 days the BM-MSCs were at 80-85% confluence and after wash with PBS 1X w/o Ca^{2+} and Mg^{2+} (PAA) the cells were detached using trypsin/EDTA (Cambrex, Europe) for less than 5 minutes at 37°C. DMEM High Glucose (PAA) with 10% FBS (PAA), 1% penicillin/streptomycin (PAA) was used to stop the trypsin reaction. The cells were centrifuged at 300 x g, for 10 minutes at room temperature (RT). For the osteogenic differentiation experiments cells resuspended in MM were plated at a seeding density of $1X10^4/cm^2$ in 24-well multiwell plates (Greiner Bio-one) for Alizarin Red and Von Kossa staining and in T_{25} flasks (Greiner Bio-one) for RNA extraction. All culture vessels were incubated at 37°C with 5% CO_2 in a humidified incubator. When the cells reached 85-90% confluence (≈4 days), osteogenic differentiation was induced by using Osteogenic induction Medium (OM) consisting of MM supplemented with previously verified osteogenic factors, namely; 10mM β-Glycerophosphate (Sigma-Aldrich), 0.1 mM ascorbic acid-2-phosphate (Sigma-Aldrich), 10 nM Dexamethasone (Sigma-Aldrich) and 100 ng/ml rhBMP-2 (Peprotech, UK) further supplemented with either 10% defined FBS (v/v) or 8% PLP. Two types of osteogenic induction protocol were compared: (1) A two weeks protocol, initially using a modified osteogenic induction medium without rhBMP-2 for the first week, adding rhBMP-2 for the second week only. (2) An accelerated protocol that involved adding the full osteogenic induction medium containing rhBMP-2 to the cells for one week.

Cytochemical staining for mineralization: Alizarin red S and von Kossa staining

Alizarin Red. To demonstrate osteoblastic differentiation, the *ex vivo* mineralization phenotype was tested using Alizarin red S (Sigma) staining. After cell induction toward osteoblast differentiation, the cultures were washed at room temperature in PBS 1X, fixed in ice-cold methanol (100% v/v) and washed twice with distilled water at RT. The wells were then stained with Alizarin Red S (1.5% v/v, pH 4.2) to detect calcium precipitation at RT for 5 minutes. After another two wash in distilled water at RT, the cells were washed with PBS 1X at RT for 15 minutes. Then the stained monolayers were dehydrated using ice-cold methanol (100% v/v) for 2 minutes and were visualized using 10X magnification with an inverted microscope (Zeiss). For quantification of staining, 500 µL of Cetylpyridinium Chloride (CPC) was added to each 24-well and incubated at RT in the dark for 15 minutes[24]. The eluted dye solution was then removed and transferred to a fresh eppendorf tube. After a dilution 1:10 with PBS 1X, the dye was transferred to a transparent 96-well plate and the optical density (OD) measurement was performed as a technical triplicate (200 µL/well) at 562 nm on an ELISA reader (GDV, Roma)[47].

Von Kossa. After two weeks of osteogenic induction the cultures were stained with VK staining in order to visualize phosphate and carbonate anions[49]. The induced monolayers were washed in PBS 1X at RT for 5 minutes and fixed on ice-cold methanol (100% v/v) for 4 minutes. The cells were rinsed twice in distilled water and incubated with 0.8 µm filtered 1% silver nitrate (Sigma) for 30 minutes under a UV lamp[68]. Stained samples were washed twice in distilled water and

visualized by 10X magnification using an inverted microscope (Zeiss). The area of dark positive Von Kossa staining was calculated as a percentage of the total area using Image J software (http://rsb.info.nih.gov/ij/).

RNA extraction and cDNA synthesis

Total cellular RNA was isolated using a single-step method with TRIzol® (Invitrogen) according to the manufacturer's instructions[69]. Briefly, 1 mL of TRIzol® reagent per cm^2 was added to the T_{25} flasks containing either control or osteogenically induced cell monolayers for at least 5 minutes at RT to permit the complete dissociation of nucleoprotein complexes. The samples were harvested and transferred into new Eppendorf tubes, with chloroform added according to the volume of TRIzol® as indicated by the manufacturer. After brief shaking, the samples were then incubated 2-3 minutes at RT and centrifuged at 12000 x g for 15 minutes at +4°C. The upper transparent aqueous phase was transferred into a fresh tube and isopropyl acid was added to precipitate the RNA. The samples were incubated for 10 minutes at RT and centrifuged at 12000 x g for 10 minutes at +4°C. The supernatant was removed and the RNA pellet was washed with 75% ethanol. The samples were mixed and centrifuged 7500 x g for 5 minutes at +4°C. Then the RNA pellet was dried, dissolved in RNase-free water and incubated at 55-60°C for 10 minutes. The RNA was then quantified using a spectrophotometer (Beckman Coulter DU® 730).

First-strand complementary cDNA was synthesized from 1 µg of total RNA using a revertAid H minus first-strand cDNA synthesis kit (Fermentas)[70]. First 1 µg of RNA,

1 µL of oligo dT primers and nuclease free water until the final volume of 12 µL were put into a new PCR tube, briefly centrifuged and incubated at 65°C for 5 minutes. After the eppendorf tube was centrifuged, it was returned to ice and 4 µL of 5X Reaction buffer, 1 µL of RiboLock RNase Inhibitor (20 U/µl), 2 µL of 10 mM dNTP Mix and 1 µL of RevertAid M-MuLV Reverse Transcriptase (200 U/µL) were added. The next step was to mix and incubate the sample for 60 minutes at 42°C, following an incubation for 5 minutes at 25°C. The reaction was terminated by heating at 70°C for 5 minutes. The single strand cDNA was quantified by spectrophotometer (Beckman Coulter DU® 730) in order to use 10 ng of cDNA in each Real-Time PCR well.

Quantitative Real Time PCR analysis of osteogenic biomarker expression

Quantitative real-time PCR was performed using the Applied Biosystems StepOne™ Real-Time PCR System and the Fast SYBR® Green Master Mix reagent[71]. The quantification of gene expression for each target gene and reference gene was performed in separate tubes. Forward and reverse primers were designed using http://test.idtdna.com/site web site and to be specific for mRNA rather than genomic DNA, it was ensured that they spanned an intron sequence, thus avoiding the possibility of confusion from primer amplification of spliced mRNA. Expression of the target gene was normalized to that of the endogenous reference β-actin gene.

The $2^{-\Delta\Delta Ct}$ (cycle threshold) method, was used to calculate relative expression levels of the target genes defined by the primers summarized in Table 2[71].

Cluster analysis

The objective of the cluster analysis was to see whether we could use the expression patterns from our osteogenic biomarker genes to identify the hBM-MSC populations with similar bone-forming potential. Ideally, there would be enough similarities and distinctions to discriminate between bone forming and non-bone forming hBM-MSC populations. Different clustering algorithms will give different results on the same data, with the choice of distance measure and linkage method being two main factors. Two different methods are described here, one using Pearson uncentered correlation with centroid linkage and the other using Euclidian distance with single linkage, each having particular advantages and disadvantages. Use of both methods served to illustrate that our gene expression patterns could correlate with bone-forming potential in a robust way and one method proved better than the other at discriminating dissimilarity between bone forming from non-bone forming hBM-MSC populations. Pearson uncentered correlation distance is an index of the strength of linear association between two variables calculated from the sample values and their standard deviations with sample means set to zero in the expression for uncentered correlation. This was deemed appropriate because the values represent a fold-increase in gene induction above a zero reference state determined by the control samples. Centroid linkage calculates a vector point from an average of all the items contained within the cluster and thus has the advantage that the effect of outlier values is minimized. Euclidian distance is simply the geometric distance in multidimensional space. It is a commonly chosen type of distance that is applicable here because we are clustering raw data for continuous variables that all have the same scale. The advantages over correlation-based distance

measurements, is that the Euclidian distance takes the magnitude of changes in gene expression levels more into account. Therefore it preserves more information about the data. Single linkage refers to a nearest-neighbour method that considers the distance between two clusters to be the shortest distance from any member of one cluster to any member of the other cluster. The single link clustering method has the advantage of being versatile, emphasizing dissimilarity, but in some contexts has drawbacks if the data had many points that bridge between clusters.

Open source cluster analysis software was used to organize and analyze the data for all the genes examined and also a cohort of genes, selected according to their statistically significant expression relative to four selected phenotypes, namely; Alizarin Red positivity, Von Kossa positivity, high Ki-67 expression and *in vivo* bone forming ability. Values for the fold-gene induction for each of the genes were entered as a tab delimited text file, and clustered using Pearson uncentered correlation with centroid linkage and the other using Euclidian distance with single linkage [72]. TreeView provided interactive graphical analysis and global view diagrams[73]. For the five genes that were significantly expressed in all the four contexts, the degree of correlation between nodes was correlated to the relative amount of bone formed to determine whether the donors with similar patterns of gene expression had correspondingly similar bone forming potential.

In vivo bone formation assay in immune deficient mice

Following approval from the local ethical committee of the University of Modena and Reggio Emilia, the bone forming potential of the cGMP-BM-hMSC from each donor was tested using four implantation sites shared between two 8-week old NOD.CB17-Prkdc ^{scid}/J mice. Previously described methods, were modified to maintain the same cell to scaffold ratio as proposed for a clinical trial[74]. Briefly, animals were anaesthetized with 3.6% isofluorane and the dorsal skin was shaved and cleaned. Incisions of ≈1 cm in length were performed on upper and lower dorsal regions on the back of each mouse. Blunt dissection was used to form a 3 cm long pocket and the graft was implanted therein. A total of 1,6X10^6 cells were mixed with 40 mg (≈40 granules) of hydroxyapatite β tricalcium phosphate (HA-βTCP 20:10, 1-2mm, Biomatlante, Vigneux de Bretagne, France) and implanted subcutaneously into the upper and lower dorsal flank of each mouse. The control samples were transplanted with 40 mg HA-βTCP scaffold granules alone. The incisions were closed with sutures of ethicon vicryl rapide 5-0. The xenografts were recovered from sacrificed animals six weeks post implantation and transferred to 4% neutral buffered formalin for two days. Then two PBS washes were followed by decalcification for 2-3 weeks in buffered (15%) EDTA (pH 7.4). The decalcified HA-TCP implants were embedded in paraffin and 3 µm sections were stained with Hematoxylin and Eosin (H&E) for assessment of bone tissue formation. The percentage of bone tissue formed per total area was quantified by two independent observers estimating the percentage of pink stained osteoid tissue relative to the total amount of unstained scaffold and diversely stained surrounding stroma[58].

Statistical analysis

For the RealTime-PCR analysis a two-tailed *t* test was applied to analyse the significance of each triplicate measurement for each of the initially selected genes. In addition, a two-tailed t test was applied to analyse the significance of each of the differentially expressed genes between the control and the ostoegenically-induced groups. Gene expression data that had a *p* value of less than 0.05 was considered to be statistically significant.

RESULTS

αMEM media was suitable for ex vivo osteogenic induction

Our first goal of our experiments was to choose favorable medium conditions for osteogenic induction. Using a consistent hMSC source (Donor #1) we compared DMEM plus 10% defined FBS; αMEM plus 10% defined FBS and αMEM supplemented with 8% of PL, first testing their performance during a two weeks osteogenic induction protocol. Although we failed to see calcium deposition evidenced by Alizarin Red S (ALZ) staining when inducing osteogenesis with DMEM with FBS (Fig. 1B), or αMEM plus FBS (Fig. 1D), there was strong ALZ positivity when using αMEM plus PL (Fig. 1F). In addition to ALZ staining we noted weaker growth and poorer morphology when cells were differentiated in DMEM. According to these results, we used an osteogenic induction medium based on αMEM media.

PL was better than FBS in for early induction of osteogenic differentiation

The next purpose was to test the two weeks (2W) osteogenic induction protocol comparing PL versus FBS in the osteogenic induction medium using six cGMP hBM-MSC populations derived from independent donors. Confirming the preliminary data, the poor osteogenic differentiation seen with αMEM FBS (Fig. 2B) was greatly improved when using αMEM PL (Fig. 2D), moreover these cells lost their fibroblastic morphology and efficiently deposited calcium.

We next explored whether we could obtain measurable osteogenic differentiation as determined by ALZ within one week. In both osteogenic induction protocols the control samples with cells maintained in maintenance medium containing FBS (Fig. 2E) or PL (Fig. 2G) did not show any significant spontaneous differentiation.

After only one week of induction, the weak ALZ staining seen with FBS in the osteogenic medium (Fig. 2F) was intensified when using PL (Fig. 2H). Nonetheless, there was considerable donor heterogeneity among the six hMSC populations and the ALZ signal at one week was not necessarily significant. Thus although there were indications of some osteogenic differentiation within one week, the ALZ protocol was not a suitable method for obtaining an accurate measurement at one week.

Donor heterogeneity for osteogenic gene induction

Having obtained a protocol for osteogenic differentiation that worked efficiently over a one and two week period, we then explored expression of 19 osteogenic biomarker genes. Of these, 7 genes were not measurable with any reliability, presumably because their level of inducibility was very low, or they were not significantly induced during the early phases of osteogenesis. Of the 12 remaining genes, their expression in the individual donors at two weeks (Fig. 3) or one week in OM was highly variable (Fig. 4). The one gene that did not vary to this extent, CADM1, was nonetheless the most weakly induced gene with a maximum measurable induction of only 5-fold in donor #6 ($p < 0.05$).

Given the wide variation in gene expression patterns, we sought to determine the most relevant and useful osteogenic biomarker genes by selecting donors on the basis of a particular phenotype, including; matrix mineralization, metabolic activation in response to OM and the potential for *in vivo* bone formation.

Donor heterogeneity for cytochemical staining

In order to characterize the ability of every single donor to deposit mineralized bone matrix after two weeks of osteogenic induction and to verify the heterogeneity revealed by previous mentioned pilot study, the samples derived from six donors were stained with two different matrix mineralization assays.

Alizarin Red S. Among our ability to detect matrix mineralization, after two weeks of osteogenic induction, the BM-MSCs were stained with ALZ and two different phenotypes were identified. In particular the red stained samples, derived from 4 donors (#1, #2, #3, #4), showed an extensive matrix mineralization and were identified as ALZ^+ (Fig. 5A). These ALZ^+ samples showed OD values between 5 and 13 and were statistically significant relative to the control samples ($p<0.00003$) (Fig. 5B). The other two samples derived from donor #5 and #6 had a mineral deposition no greater than the negative controls (Fig. 5A). These samples showed OD values less than 0.5 (Fig. 5B) and only the sample derived from the donor #5 was statistically significant ($p<0.015$). Therefore the samples derived from donor #5 and #6 were defined as ALZ^-.

Von Kossa. The next purpose was to explore whether we could obtain similar results by measuring the phosphate anions by Von Kossa (VK) staining. This method is more sensitive than ALZ and the quantification of VK positive areas was suitable by using Image J software. In particular the samples derived from 3 donors (#1, #2, #3) showed a diffuse calcium phosphate areas, identified by black particles of silver ions (Fig. 6A), and so were identified as VK^+. In the other three samples (derived from donors #4, #5 and #6) the presence of non-mineralized areas were identified as VK^- (Fig. 6A). No VK positive staining was observed in the un-differentiated controls samples. The percentage of VK positive areas in

photographs of representative osteogenic induced and control samples at 10X enlargement was evaluated by using the Image J software. The VK^+ samples had showed statistically significant ($p<10^{-17}$) positive areas comprised from 8 to 16 percent (Fig. 6B). In the other hand the VK^- samples showed statistically significant ($p<0.00001$) dark areas under 0.5 percent (Fig. 6B).

Molecular characterization of ALZ^+ and VK^+ phenotypes

Initially a panel of 19 genes was explored, but seven genes had too low expression level for reliable measurement. In particular: tafazzin (TAZ), msh homeobox homologue 2 (MSX2), osterix (OSX), parathyroid hormone receptor 1 (PTH1R), secreted frizzled-related protein 1 (SFRP1) and secreted phosphoprotein 1 (SPP1) did not showed appreciable fold increase respect to control samples. Subsequently, a panel of 12 genes was analyzed, targeting the process of commitment until the deposition of mineralized phase in the matrix, including: alkaline phosphatase (ALPL), osteocalcin (BGLAP), biglycan (BGN), cell adhesion molecule 1 (CADM1), type I collagen α1 (COL1A1), type I collagen α2 (COL1A2), decorin (DCN), Distal-less homeobox 5 (DLX5), elastin (ELN), Ki-67, runt-related transcription factor 2 (RUNX2) and tetranectin (TNA). Subsequently, we needed to understand whether we could detect the same genes among different experimental contexts, defined by (1) the medium composition used for the osteogenic induction medium; (2) the different donors whose MSC showed different degrees of osteogenic performance depending on the assay chosen to characterize osteogenic differentiation. Therefore, we aimed to better understand the heterogeneity of six hBM-MSCs derived from independent donors, and after

the matrix mineralization staining; the positive stained samples were tested for the twelve osteogenic biomarkers panel. In order to understand the molecular characteristics of the ALZ^+ samples after two weeks of osteogenic induction using either PL or FBS in the induction medium, RealTime-PCR was employed (Fig. 7A). The upregulation of ALPL, COL1A1, COL1A2, DCN, ELN and RUNX2 was statistically significant after two weeks of both PL or FBS based induction protocols. In particular the upregulation of ALPL, BGLAP, COL1A1, COL1A2, DCN, DLX5, ELN and RUNX2 when using PL showed statistically significant ($p<0.03$) fold increase values between 5 and 27 with respect to the control samples. Instead when differentiating the hBM-MSC with an FBS based osteogenic induction protocol for two weeks, the expression of ALPL, COL1A1, COL1A2, DCN, ELN, Ki-67 and RUNX2 showed statistically significant ($p<0.0002$) upregulation with upregulation ranging from 2.4 to 17 fold relative to the control uninduced hBM-MSC. Notably after two weeks of induction, either with PL or FBS addition, the induction level of BGN and CADM1 was below to 2-fold and was not statistically significant. Comparing the two osteogenic media we noted that after two weeks of treatment the expression of ALPL, COL1A2, DCN, RUNX2 were more up-regulated when using PL and the upregulation of BGLAP and DLX5 were only statistically significant with this medium. In contrast, the FBS-based induction protocol seems to be more efficient for upregulation of COL1A1, ELN, Ki-67 and TNA. These data, taken together, suggested that osteogenic induction in terms of biomarker gene expression was more effective in an induction medium containing PL rather than FBS.

Having established that we could readily measure the osteogenic biomarker gene induction at two weeks using our diverse differentiation media we subsequently,

tested the same biomarker gene panel at one week using same the four hBM-MSC strains derived from the ALZ^+ donors #1, #2, #3 and #4, and both using PL or FBS based osteogenic induction medium (Fig. 7B). The use of PL or FBS in the osteogenic induction medium led broadly similar results but there were differences. Notably, the expression of BGLAP, COL1A2, DCN, and TNA was more upregulated when using PL based media. In particular statistically significant (p value<0.008) fold increases between 2.5 and 22 were measured. The ELN and RUNX2 expression was efficiently upregulated with FBS induction, with fold increase between 8 and 14 (p value<0.0001). The expression of ALPL showed a statistically significant 3-fold increase relative to the control samples only when using the PL-based differentiation protocol ($p<0.00001$). In contrast, the fold increase of BGN, CADM1, COL1A1 and Ki-67, was between 0.5 to 20 and statistically significant ($p<0.01$) at one week of induction only when using FBS.

Therefore, these data taken together, confirmed that 1) it is possible to detect the osteogenic differentiation of the hBM-MSC by using the molecular analysis of bone-related biomarkers, 2) the induction protocol based on addition of PL in the osteogenic media promoted efficient *ex vivo* differentiation as measured at both one week or two week time points after treatment. Moreover, after one week of osteogenic induction the PL was better than FBS in causing up-regulation of BGLAP, COL1A2 and DCN ($p<0.001$) and this was sustained so that after two weeks of induction BGLAP and DCN were significantly more upregulated ($p<0.03$) with PL than with FBS.

With the aim of characterizing the molecular profile of VK^+ hBM-MSC populations, after two weeks and one week of PL based osteogenic induction the mRNA expression of the twelve osteogenic biomarkers were analyzed. The gene

expression of ALPL, BGLAP, COL1A2, DCN, DLX5, ELN and RUNX2 was statistically significant ($p<0.02$) with fold-increases between 3 and 46 (Fig. 8A). After one week of induction the m-RNA level was also analyzed and statistically significant fold-induction values ($p<0.02$) between 3 and 38 were measured for ALPL, BGLAP, COL1A2, DCN, ELN and RUNX2 (Fig. 8B).

The molecular analyses of ALZ^+ and VK^+ phenotypes strongly suggested that a subset of osteogenic biomarkers was constantly upregulated either with one week or two weeks induction protocols. In particular, after two weeks of PL based induction, these phenotypes showed a statistical significant increase in ALPL, BGLAP, COL1A2, DCN and DLX5 levels. Indeed we were pleased to find that after one just week of induction the hBM-MSC were able to show significant induction of ALPL, BGLAP, Col1A2, DCN, ELN and RUNX2.

Proliferation/osteogenic induction switch-off: Ki-67 expression

Given the importance of proliferation in the early stages of differentiation, and the consequent activation of the cell cycle, we analyzed the expression of Ki-67. The Ki-67 antigen (pKi-67) is a nuclear protein essential for cell cycle progression and is identified in all phases of the cell cycle except the G_0-phase[75]. This nuclear protein is used diagnostically as a proliferation marker in different cancers[76] and *ex vivo* studies confirmed its role during the mitosis process. The proliferation rate after one week and two weeks of osteogenic induction using our PL based protocol was analyzed. After two weeks of osteogenic induction cell proliferation was reduced and the level of Ki-67 induction was only statistically significant in the samples derived from donors #1, #3 and #4 (Fig. 9A) where fold-increase values

between 4 and 66 were measured. hBM-MSC populations from the other three donors had fold increase values below 2.5, equivalent to the control samples (Fig. 9A). After one week of osteogenic induction cells from donors #1, #3, #4 showed statistically significant fold-increases in Ki-67 expression between 8 and 102 fold (Fig. 9B). In marked contrast, the samples derived from donors #2, #5 and #6 showed Ki-67 fold increase values below 1 (Fig. 9B). These data revealed a donor heterogeneity for Ki-67 expression upon osteogenic induction and a reduction in Ki-67 expression during differentiation. Samples derived from donors #1, #3 and #4 were identified as Ki-67^{+}, while, the samples derived from donors #2, #5 and #6, were defined as Ki-67^{-}.

Molecular characterization of Ki-67^{+} phenotype

In order to determine the molecular profile of Ki-67 positive hBM-MSC strains derived from donors #1, #3 and #4 the panel of twelve osteogenic biomarkers was then analyzed. The molecular analyses of Ki-67^{+} samples after two weeks of osteogenic induction showed a statistically significant ($p<0.02$) upregulation of ALPL, BGLAP, COL1A1, COL1A2, DCN, DLX5, ELN, RUNX2 and TNA. The fold of increase values of these genes was between 2.5 and 29, whereas instead BGN and CADM1 only had expression values that were near-equivalent to the control samples (Fig. 10A). After one week of PL-based osteogenic induction we observed a statistically significant ($p<0.0002$) upregulation of ALPL, BGLAP, COL1A1, COL1A2, DCN, ELN, RUNX2 and TNA genes, with upregulation ranging between 2.5 and 22-fold (Fig. 10B).

Ectopic *in vivo* bone formation assay

All NOD/SCID mice subjected to xenograft implants were healthy after the procedures and small incisions after operation healed in 7 days with no infections or complications including post-operatively bleeding, haematoma or seroma accumulation. After six weeks post-implant the hBM-MSCs and HA-βTCP xenografts were recovered and diffuse blood vessel formation was observed. In order to evaluate the *in vivo* bone forming ability the xenografts were decalcified and stained with Hematoxylin and Eosin. With relevance to what has been described in the literature[17], we established that for a 6-week xenograft assay reliable bone formation needed to occupy over 5% of the total tissue area. By using a direct microscope with 10X high-power field magnification, we observed that the donors #1, #2, #3 and #6 were able to form new bone quantified as comprising from 15% to 21% of the total tissue area. We categorized these hBM-MSC strains with good ectopic bone forming potency as belonging to *in vivo* bone forming positive (BF^+) donors (Fig. 11A-C, 11F). In the other two samples, derived from donors #4 and #5, no significant bone formation was observed with respect to the controls, and these hBM-MSC strains were categorized as belonging to donors that were bone forming negative BF^- (Fig. 11D-E). HA-βTCP granules without cells were also implanted to control for any host-cell-derived osteoinductive response (Fig. 11G) however in these samples we only observed stromal cells surrounding the scaffold without any evidence of bone formation. The BF^+ implants were characterized by: lacunae containing mature osteocytes, bone-lining cells, vessels, bone marrow drafts (Fig. 11B and 11F) and pink areas of collagen fibrils. High stromal cellularity lacking bone-like tissue organization was observed in BF^- implants, besides a different tissue architecture respect to the control implants was

observed. The new bone formation percentage respect to the total area of implants by a scoring system was quantified, and a range of fifteen to twenty-one percent ectopic bone area was confirmed for hBM-MSC from donors #1, #2, #3 and #6 (Fig. 11H). The bone formation of BF^- implants, if present, did not exceed five percent, confirming the early histological observations.

Molecular characterization of BF^+ phenotype

In order to determine which osteogenic biomarker genes were inducible ex vivo in the hBM-MSC derived from bone forming donors, the expression of twelve osteogenic biomarker genes was measured after two weeks and one week of PL based osteogenic induction. After two weeks ALPL, COL1A1, COL1A2, DCN, DLX5, ELN and RUNX2 were significantly upregulated ($p<0.05$) with increases ranging from 6 to 37 fold. (Fig. 12A). Therefore for cells showing a BF^+ phenotype the osteogenic biomarker genes induced at one week were similar to those induced at two weeks, in particular: ALPL, COL1A1, COL1A2, DCN, ELN, RUNX2 and TNA showed a statistically significant ($p<0.02$) upregulation ranging between 4 and 22-fold (Fig. 12B).

Cluster analyses

Considering differentiation using PL based osteogenic induction medium, the cluster analysis obtained from all 12 osteogenic biomarker genes at one week with an algorithm that used uncentered Pearson correlate distances and centroid linkage, produced a heat map (Fig. 13A) that showed a varied level of gene induction ranging from zero (black pixels) to positive (red pixels). DCN, RUNX2

and ELN were the most consistently expressed genes among all the donors. With respect to how the cluster analysis associated the donors to each other, donors #5 and #6 were closely clustered, and this was consistent with their showing similarity in three *ex vivo* phenotypes ALZ$^-$, VK$^-$ and Ki-67$^-$. Despite similarity *ex vivo*, there were contrasting *in vivo* phenotypes; donor #5 did not form bone, whereas donor #6 did. At two weeks (Fig. 13B) the heat map indicated an overall increase in gene upregulation (more red pixels) and in particular ALPL was now upregulated for all donors. With respect to how the cluster analysis of the two week data associated the donors to each other, there was a clustering of donors #4 and #5 that shared the common phenotype of both being VK$^-$ and BF$^-$. The next node of similarity clustered donors #1 and #3, both positive for all phenotypes. The next node of similarity indicated donor #6 was linked to donors #4 and #5, however the donors in this cluster had a different bone forming phenotype. Either the one or two week clusters generated using all twelve osteogenic biomarker genes failed to generate a dendrogram linking the donors in a way that correlated with bone forming potential.

We reasoned that an informative cluster analysis correlating *ex vivo* gene expression and *in vivo* bone formation would only be possible using genes that proved globally significant in all contexts. Multiparametric assessment of osteogenic potential *ex vivo* and the *in vivo* phenotype of bone formation was used to select hBM-MSC from donors that were functional for each phenotype. From these specific hBM-MSC strains we calculated the average fold-increase of OM-induced gene upregulation and this minimized the influence of donor-donor variation when identifying which osteogenic biomarker genes were correlated to a particular phenotype. It emerged that five genes showed a significant upregulation

in response to OM in all the four phenotypes; ALPL, COL1A2, DCN, ELN, RUNX2 (Fig. 14A) and these we selected as the only genes that remained globally relevant for both *ex vivo* and *in vivo* contexts. Indeed five genes were preferred for discriminating bone forming ability since an abundance of clustering variables would reduce the probability of the donors being very clearly dissimilar. Given that we have a subset of only five genes it was relevant that there was little collinearity among their patterns of expression, and looking at the pattern of expression we found that only the RUNX2 and ELN genes were co-induction in a similar manner among the six donors. Too many highly correlated variables would risk overrepresentation in the clustering solution.

Using the gene expression data obtained after osteogenic induction for only one week and restricting the cluster analysis algorithm to the five globally relevant genes produced a very different dendrogram to that seen using twelve genes (Fig. 14B). Most important, bone-forming donors were shown to be more similar to each other than the non-bone forming donors. Not only were the two highest bone forming-donors #2 and 3 identified as the most similar (correlation coefficient 0.967), but also the known colliniearity in ELN and RUNX2 expression among the donors was identified by the dendrogram (correlation coefficient 0.907). The hierarchical clustering of the donors was correlated with the amount of ectopic bone made by each donor. Linear regression analysis of the similarity between donors (correlation coefficient) versus the amount of bone made by the relevant pair of donors (Mean %Ectopic bone formed) showed strong correlation (r^2= 0.812) (Fig. 14C).

Given our interest in discriminating between bone forming and non-bone forming donors we extended our cluster analysis to determine whether alternative

algorithms could enhance dissimilarity. Choosing Euclidian distance and single linkage clustering we obtained a dendrogram that also associated bone forming donors (Fig. 15A) with a linear regression analysis that showed a stronger correlation between bone forming ability and donor similarity (r^2= 0.911). Notably, the differences between bone forming donors and non-bone forming donors were more appreciable.

Applying the same approach to the data obtained when hBM-MSC were osteogenically induced for two-week produced a globally relevant set of only four genes (Fig. 16A), a cluster analysis dendrogram that did not discriminate donor bone forming potential (Fig.16B). This highlighted the advantages of characterizing gene expression changes *ex vivo* during the early phases of osteogenesis.

It could be expected that data obtained during the early phases of osteogenic induction, characterized by extracellular matrix synthesis is more likely to be reproducible and consistent with early events in vivo, allowing gene expression data to be more relevant.

In contrast using the five globally significant genes for expression data after one week of osteogenic differentiation, a remarkable hierarchy for similarity among all the six donors was evident. The two most similar donors were #2 and #3, then with a decreasing degree of similarity the order of the remaining donors was #1, #6, #4 and #5 (Fig. 15B). The node score of 0.967600 meant that donors #2 and #3 were very significantly alike for the cluster of 5 globally significant genes. The second node (coefficient 0.799633) representing the second-strongest correlation, suggested that donor #1 was quite similar to #2 and #3. Notably these three donors were distinguished by being positive for the Von Kossa staining method

and the best bone formers. The amount of ectopic bone estimated for donor #2 was 18.81% and for donor #3 was 21.28%. So the average amount of bone resulting from these two donors was 20%. Notably, donor #1 formed 15.33% bone and continuing this analysis, for all the nodes it emerged that the coefficient of correlation obtained from the cluster analysis correlated with the relative amount of bone estimated from the six week ectopic bone forming assay (Fig. 16). Thus it was possible to obtain data after just one week of osteogenic differentiation *ex vivo* that had predictive potential for bone formation.

DISCUSSION

It is well established that cell culture conditions, including the basal medium, play a fundamental role in cell proliferation and differentiation. It is broadly appreciated that after 21 days of osteogenic induction, in the presence of ascorbic acid, β-glycerophosphate and dexamethasone, hMSC in cultures with α-MEM based medium are characterized by mineralized deposits with numerous globular structures containing calcium and phosphate. In contrast, by DMEM based osteogenic media, mineral deposits appeared later (day 28) and the amount of mineral deposition measured by ALZ and VK staining was significantly lower than that observed in the α-MEM[25]. In agreement with these data, our experiments showed α-MEM based osteogenic medium accelerated our ability to detect the mineralization phenotype; the presence of mineralized deposits within two weeks only occurred upon induction with the α-MEM medium. This possibly reflected higher concentrations of ascorbic acid (0.05 g/L) in α-MEM compared to DMEM[44].

We also observed more mineralization when using PL rather than FBS during osteogenic induction of the BM-MSC. This was consistent with observations made by Chevallier et al., demonstrating that PL based conditions favored matrix mineralization of cultures in an osteogenic medium for three weeks[77]. Similarly Goedecke et al[33], established that PL drove osteogenic differentiation of BM-MSC more efficiently than the FBS using a two weeks osteogenic induction protocol. Our results confirmed this within a similar two week induction period yet further demonstrated that within one week, even in the presence of PL, matrix mineralization was not detectable using ALZ. This result emphasized the importance of chronological events and met the expected timing, that requires a first phase of matrix synthesis before a following phase of mineral deposition[46].

To evaluate our ability to measure osteogenic differentiation *ex vivo* we first needed to cope with the problem of donor heterogeneity, given that some donors may provide hMSC that do not differentiate osteogenically. Therefore, we chose to first test matrix mineralization before exploring the gene expression of potential biomarkers. The cell populations obtained from the six donors were placed in OM for two weeks and indeed showed inter-donor heterogeneity and even ALZ^+ but VK^- phenotypes for cells from the same donor[78]. Thus, two different cytochemical stainings for matrix mineralization could each report conflicting results within a cell population from the same donor. Notably, Alizarin red S and the von Kossa stainings specifically identify different aspects of the mineralization complex. Whereas Alizarin red S detects calcium and other cations, VK detects phosphate and carbonate anions[49]. These results revealed limitations for use of cytochemical methods alone in the evaluation osteogenic potency.

In addition to matrix mineralization other authors have emphasized that an initial proliferative response to osteogenic induction may be a crucial phenotype. Janicki et al. showed that the mitotic inhibitor mitomycin C not only irreversibly blocked DNA synthesis and inhibit proliferation but also prevented mitomycin C pretreated MSC from forming bone when implanted with β-TCP into immune deficient mice[65]. Partially agreeing with the above hypothesis, two of our hMSC populations responded to OM with high upregulation of Ki-67 and subsequently formed good bone *in vivo*. However, we also found two bone-forming examples that did not show Ki-67 upregulation in response to OM and *vice versa*. These contrasting results revealed that measurement of a proliferative or cell cycle induction response during the early phase of osteogenic induction was not by itself necessarily helpful for predicting osteogenic potency.

The above phenotypes indicated donor specific differences in the *ex-vivo* ability of hBM-MSC to express an osteogenic phenotype. They were not all capable of mineralization and donors responding to OM with an upregulation of Ki-67 gene expression, did not necessarily correspond to the set of donors positive for mineralization. Thus from *ex vivo* assays alone, only two out of six donors provided hBM-MSC that were positive for osteogenic mineralization and an active cell cycle metabolism in response to OM.

To understand whether the *ex vivo* data had any relevance for bone forming ability, hBM-MSC from each donor were tested using an ectopic bone formation assay *in vivo*. In contrast to finding that only two donors were consistently positive for our *ex vivo* assays, the cells from four donors were able to form bone well. We therefore explored whether the pattern of gene expression was informative and helpful in distinguishing donors that had poor or good bone forming potential.

Bone formation is a complex process that involves cascades of cellular and molecular events, including matrix deposition of collagen type I and a large number of extracellular non-collagenous proteins followed by matrix mineralization. Based on this understanding, the osteoblastic phenotype *ex vivo* has been defined by the ability of cells to express a number of biomarkers known to be relevant for bone formation, for example, production of ALPL, Col I, RUNX2 and BGLAP, as well as the ability to form mineralized matrix *ex vivo*[63]. After two weeks of induction, most of the significantly up-regulated genes (>4-fold, $p< 0,01$) in cells positive for the ALZ^+, VK^+ and Ki-67^+ cell phenotype included two transcription factors, genes involved in bone matrix assembly and the relatively late osteoblast marker as BGLAP.

Alkaline phosphatase (ALPL) is a glycoprotein and functions as an ectoenzyme attached to the cell membrane by a hydrophobic glycosyl-phosphatidylinositol (GPI) anchor. This molecule is found in plasma cell membrane of hypertrophic chondrocytes and osteoblast. Different regulatory factors increase ALPL expression, such as Sp3, progestin, granulocyte colony-stimulating factor, cAMP, retinoic acid[79] and phosphates. It plays a pivotal role in the maintenance of mineralization processes after birth and ALPL genetic mutations are involved in the hypophosphatasia and in osteoporosis[79]. Is well know that ALPL activity need not be proportional to observed mineralization levels[80] and significant combined age- and gender-related differences in ALPL activity have been revealed[81]. PL addition to MM induces in BM-MSC an ALPL upregulation of approximately 2-fold after 15 days[82]. In agreement with these observations, we have found that this early osteogenic biomarker was only slightly up-regulated in BM-MSC expressing either an ALZ^+, VK^+ or Ki-67^+ cell phenotype (between 3 and 5-fold increase). These findings taken together showed expression levels of ALPL alone would not be useful as a marker of bone formation.

The activation of transcription factors accompanies BM-MSC differentiation and the expression of RUNX2 and DLX5 was evaluated. RUNX2 is the master regulator of osteoblast differentiation during both endochondral and intramembranous ossification and regulates the expression of genes that characterize the osteoblast phenotype, including BGLAP, OPN, Col type I and bone sialoprotein (BSP). Moreover, Runx2 identifies and binds the intronic enhancer of ALPL determining its consequent expression in a nuclear matrix-targeting-dependent manner. RUNX2 helps to enhance commitment towards osteogenesis by inhibiting the differentiation of MSCs towards the adipogenic

lineage. In the RUNX2 knockout mice, there was complete lack of endochondral and intramembranous bone formation, in effect, the osteoblasts compartment was lost. Indeed the fact that in literature it is known that RUNX2 levels are increased after exposure to dexamethasone, makes this gene an internal control for showing that our osteogenic induction protocol was functioning. Gene expression analysis of BM-MSC with ALZ^+, VK^+ and Ki-67^+ phenotypes consistently showed about a 10-fold upregulation of RUNX2 after one week in OM and about a 20-fold increase after two weeks of osteogenesis, indicating its pivotal role.

The other transcriptional factor, DLX5, usually expressed in the later stages of osteoblast differentiation, is often coincident with the expression of osteocalcin and acts as a positive transcriptional regulator of osteoblast differentiation and function[83]. The DNA binding homeodomain of DLX5 bind to consensus TAATT sequence found on the promoter region of osteogenic genes, including RUNX2, OSX, OC and ALPL[84]. Furthermore, DLX5 is the key mediator of BMP-induced RUNX2 expression, by binding the RUNX2 P1 promoter. These findings strongly suggested the important role of DLX5 in the development of mineralized tissues. Our data confirmed that after two weeks of osteogenic induction, in ALZ^+, VK^+ and Ki-67^+ phenotypes, we could measure statistically significant upregulation of both DLX5 and BGLAP. This was not the case after one week of osteogenic induction, emphasizing that not all our candidate biomarker genes may be useful. Genes that regulate later phases of osteoblast differentiation, may not be expressed at a level that is significant when BM-MSC have been exposed to OM for just one week. Our data showed similar time-course kinetics to that described by others[84].

Another widely used marker of osteogenic differentiation is type I collagen. Type I collagen is expressed most highly in osteoblasts and represents approximately

95% of the entire collagen content of bone and about 80% of the total proteins present in bone. In particular, in bone matrix collagen fibers are bound with crystals of hydroxyapatite and play an important role in the bone's ability to absorb energy[85]. Collagen type 1 is constituted by a heterotrimer of two α1 chains and one α2 chain [α1(I)2α2(I)], encoded by two distinct genes, COL1A1 and COL1A2 located in two different chromosomes, chromosome 17 and 7 respectively. The expression of both of these collagen genes is regulated by RUNX2, and in addition, by interlukin-1 (IL-1) and IL-4, growth factors like TGF-β1[86,87] and insulin like growth factor-1[88]. There is much confusion regarding the role played by two distinct chains of collagen type 1, in particular, some authors have investigated the upregulation of COL1A1[77] in response to osteogenic stimuli, others considered expression of the gene for the COL1A2[89] chain, while in other studies, authors merely mention collagen type 1[90] without being more specific. We chose to explore the expression level of each collagen type 1 chain, given that Miles et al was able to highlight a particular role for the two different collagen type 1 constituent chains. In particular, the major effect of the α2 chain in the heterotrimer was to increase the hydrophobic side-chain interactions, driving out the structured water, thus increasing the molecular binding of the molecules in the fiber[91]. Given our interest in deriving a prompt gene expression assay that could be performed after just one week in OM, it was important that differences in COL1A1 and COL1A2 gene expression were observed. Notably, COL1A2 was expressed at similar levels regardless of whether the differentiation protocol was for one week or two weeks, and was significant for all phenotypes. In contrast, COL1A1 expression was much more variable, the relative level of gene expression could go up or down when comparing the one week to two week OM treatment and it was not necessarily

significant for all phenotypes, with very low levels found when selecting donors on the basis of Von Kossa staining.

Our results were in broad agreement with the proposal that extracellular matrix components were fundamental to form bone *ex vivo* and *in vivo*[64,92]. In particular the small leucine-rich proteoglycans (SLRPs) and elastin (ELN) have been described as some of the major non-collagen components of the ECM[93]. It is well-known that ELN plays a fundamental role during the mineralization of the bone matrix phase. In particular the peptides resulting from the degradation of ELN molecules activated a transduction pathway involving an inositol triphosphate (IP3) and diacylglycerol (DAG) signaling cascade, that determines the increase of the intracellular free calcium. This free calcium is in turn used for the mineralization process. Furthermore the elastin peptides, in association with TGF-β1, induced an increase of RUNX2, ALPL and BGLAP gene expression[94]. Such post-translational regulation helps explain why correlation of gene expression and bone formation is complex and unlikely to be determined by individual genes, certainly the inducibility of ELN gene expression in OM did not *per se* correlate with bone formation. With respect to the interactions mentioned in the above studies, we confirmed the statistically significant contemporaneous upregulation of ALPL, BGLAP, ELN and RUNX2 genes after one week in OM.

The SLRP superfamily currently consists of 13 known members that can be divided into 3 distinct subfamilies, and BGN and DCN belong to the class I type of SLRPs. BGN and DCN are the predominant proteoglycans expressed in bone and skin respectively[95]. Their structural features have importance in facilitating protein-protein interaction with a range of other matrix components including Col type I, with a role in the mineralization phase in the formation of calcified tissues. BMP-2

stimulation could induce the expression of these two proteoglycans[96] and our experiment confirmed this, but the expression of DCN showed a greater fold induction than BGN (e.g. $p < 0.00008$ versus $p < 0.5$) either after two weeks or one week of *ex vivo* treatment. It is also well know that they can bind TGF-β, modulating the proliferation and survival of hBM-MSC[97,95].

We used an ectopic model of bone formation to investigate the *in vivo* bone formation ability of hBM-MSCs on HA-βTCP when both were co-implanted subcutaneously into immunodeficent SCID mice. With the aim of achieving good bone formation, we noted that from five donor cell populations that formed bone, one was much poorer than the others, in particular although one could see lacunae with osteocytes, the areas where bone was deposited were very sporadic, covering less than 10% of the associated tissue area and there was little to no evidence that a marrow-like tissue supporting haematopoietic development next to the newly formed bone areas. This was in contrast to the greater amount of bone tissue (over 10%) and marrow-like areas seen for the other donors, thus it was decided to distinguish the one poor bone forming donor as distinct from the other four good bone forming donors. Thus we observed good bone formation from four out of six donors (67%). A similar degree of heterogeneity for bone formation and dependence on bone marrow donor was observed by Kuznetov et al., 1997[74] who reported that about 50% of the substrains derived from four individual donors, were positive for bone formation and strains originating from one of the donors consistently formed more abundant bone often accompanied by areas of hematopoiesis[98]. Our findings confirmed the suitability of using PL as a pre-osteoblastic inducer during *ex vivo* MSC expansion[77]. The molecular analyses of the hBM-MSC from donors selected on the basis of having a good *in vivo* bone

forming potential (BF^+) showed that after two weeks in OM, the level of gene expression for ALPL, COL1A1, COL1A2, DCN, DLX5, ELN and RUNX2 was significantly upregulated. In contrast, when measuring the induced genes after one week in OM, the significantly upgregulated genes were ALPL, COL1A1, COL1A2, DCN, ELN, RUNX2 and TNA. Thus as seen for the *ex vivo* phenotypes, most of the genes that were significantly measurable at two weeks were also measurable at one week, allowing us to consider that a one-week measurement might remain relevant for predicting bone forming potential.

With the aim of drawing some correlation between observations regarding osteogenesis *ex vivo* and bone formation *in vivo*, a number of factors needed to be considered. Firstly, heterogeneity among the donors with respect to hBM-MSC function meant that we needed to perform a multiparametric analysis with independent assays for mineralization and cellular activation in response to OM, in order to explore the induction of gene expression in adequately responsive cells. Secondly, we noted that correlations between our *ex vivo* phenotypes of mineralization or cell cycle activation, did themselves not necessarily agree with bone forming potential. hBM-MSC from donor #4 was positive for Alizarin Red staining but did not form bone. hBM-MSC donor #6 was negative for Alizarin Red staining yet subsequently could form bone *in vivo*. With respect to VK staining for mineralization, this may be a more stringent assay, since all VK^+ donors went on to form bone, however donor #6 again was negative for VK staining yet formed bone. Clearly hBM-MSC from donor #6 confirm that mineralization *ex vivo* is not directly correlated or needed for the cells to form bone[64] and presumably *in vivo* conditions overcome *ex vivo* limitations for allowing these cells to express a mineralized phenotype. Although it was suggested that a proliferative response to

OM was a key factor for predicting bone forming ability[65], we observed that donor #4 hBM-MSC had the highest Ki-67 induction, yet did not form bone, whereas, donor #6 hBM-MSC showed very little Ki-67 induction, but certainly had good bone formation.

Given the above discrepancies we investigated whether it was possible to find a cohort of significant genes defined via *ex vivo* osteogenesis assays there were also relevant to the bone forming potential of hBM-MSC populations. Of the six significantly upregulated genes at one week in PL-based OM, five remained consistently significant when identifying the significantly upregulated genes in the four donors that formed bone. These globally relevant genes were; ALPL, COL1A2, DCN, ELN and RUNX2. Notably, none of these globally relevant genes were by themselves predictive of bone forming ability.

To explore further whether selection of hBM-MSC for gene analysis on the basis of functional phenotypes may be useful for defining genes useful for predicting bone formation, we resorted to cluster analysis. More specifically, we wished to explore whether genes upregulated by OM could correlate with bone formation among the six hBM-MSCs donors. Initially, to gain an overview from all the osteogenic biomarker genes, we performed a cluster analysis using the data obtained after both one and two weeks of induction. Simply using the complete 12 biomarker panel did discriminate the *in vivo* bone formation ability. The flaw in the above approach was that not all of the genes used in the cluster analysis were significant in all contexts. To be informative the cluster analysis needed to be restricted to variables that were significant for both *ex vivo* and *in vivo* phenotypes[99].

Our multiparametric data revealed a common subset of four globally relevant genes after two weeks in OM and five globally relevant genes after one week in OM, all upregulated in a statistically significant manner in the four phenotypes.

Applying cluster analysis using the five globally relevant genes obtained after only one week of osteogenic induction, (ALPL, COL1A2, DCN, ELN and RUNX2) instead of all twelve biomarker genes, we obtained a very different dendrogram pattern. The first object clustering concerned donors #2 and #3 that in turn clustered with donor #1. Next the stepwise hierarchy of similarity continued with donor #6, thus the dendrogram prioritized the four hBM-MSC populations from donors that were capable of good *in vivo* bone formation.

In contrast, applying cluster analysis using the four globally relevant genes obtained after two weeks of osteogenic induction, (ALPL, COL1A2, DCN and DLX5) the first cluster concerned donors #4 and #6, both VK^-, but differing in bone forming ability. The dendrogram pattern indicated three paired object clusterings, including donor #2 with donor #5 that only shared the common phenotype of low Ki-67 induction. Using this subset of osteogenic biomarker genes there was no correlation with bone formation.

Further, using only the dendrogram data that discriminated donors with a bone forming phenotype, we asked whether the cluster analysis was correlated to the bone forming potency of the different hBM-MSCs populations. The similarity between donors (the correlation value for each hierarchical cluster node) was plotted against the average amount of bone formed by the members of each cluster. This agglomerative analysis confirmed a significant correlation between bone formation in a 6 week murine model and the correlation coefficients between

pairs of clustered donors. Thus the induction of a cohort of five biomarker genes, measurable in cells cultured in OM for just one week was deterministic of the bone forming potential of the same population of cells after 6 weeks *in vivo*. Given that different cluster algorithms may help improve discrimination between clusters, we used Euclidian distances instead of Pearson's correlations to more stringently take into account gene induction levels. The dissimilarity between bone forming donors and non-bone forming donors was emphasized more clearly. The molecular signature described here may be useful for understanding the inter-donor heterogeneity for hBM-MSC bone-forming potential, information that could improve clinical trials involving cell therapy for bone regeneration. However, despite these encouraging preliminary results, Formann (1984)[99] recommends a sample size of at least 2^m where *m* equals the number of cluster variables to obtain confidence that the clustering approach we have adopted here is more broadly applicable, which would mean extending our analysis to at least 32 donors before declaring the predictive potential to be robust.

Notably developing an hBM-MSC potency assay is important in the clinical context because in an autologous setting it is not possible to have an internal patient control of hBM-MSC functionality. Therefore, the outcome of treatment may be hard to interpret without any indication as to whether the cells were functional before to the implantation.

With respect to the functions desired, the hBM-MSC should show potential for differentiation toward the osteogenic lineage with an assay that is relevant to the in vivo outcome of bone formation. To date, *ex vivo* assays of osteogenic differentiation do not necessarily correlate well with subsequent bone formation and our ability to demonstrate that such a correlation may be possible within the

context of clinical grade protocols is new. In addition, further multiparametric functions include prompt adhesion to the scaffold, adequate cell survival during the operative procedures and an ability to promote a tissue response that leads to long-term survival of the bone repair.

Given that analysis is to be applied to a single patient, future aims could include developing a way of screening hBM-MSC donors in order to predict bone formation at an individual level. For this it would be helpful to extend the analysis with perhaps more than 5 biomarker genes to determine a “fingerprint” gene expression pattern that characterizes cells with a good bone forming potential. A worthy ultimate goal would be to develop an assay that licenses the hBM-MSC as “fit for purpose” with respect to differentiation potential. Additional tests will be required for confirming good performance in association with the selected biomaterials.

This thesis provides valuable foundations for a functional characterization of pre-implantated hBM-MSC that will need to be further validated with appraisal of clinical trials comparing the results of *ex vivo* analysis with long-term patients follow-up.

TABLES AND FIGURES

FIGURE 1

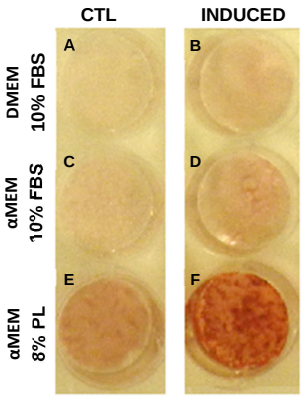

Figure 1. Two weeks osteogenic induction with DMEM FBS, αMEM FBS and αMEM PL based differentiation media. A, C, E: control samples, respectively with DMEM FBS, αMEM FBS or αMEM PL; B, D, F: induced samples. (B) When the osteogenic induction was achieved by using OM based on DMEM supplemented with 10% of defined FBS, no calcium deposition was evidenced by Alizarin Red S (ALZ) staining. (D) Comparable results was obtained by using αMEM plus FBS to osteogenic induce the hBM-MSCs. (F) Contrary to the two previous OM media, when the osteogenic induction was achieved using αMEM plus 8% of PL medium, a strong ALZ positivity was observed. (A-C-E) The samples with cells maintained in maintenance medium did not show any significant spontaneous differentiation.

FIGURE 2

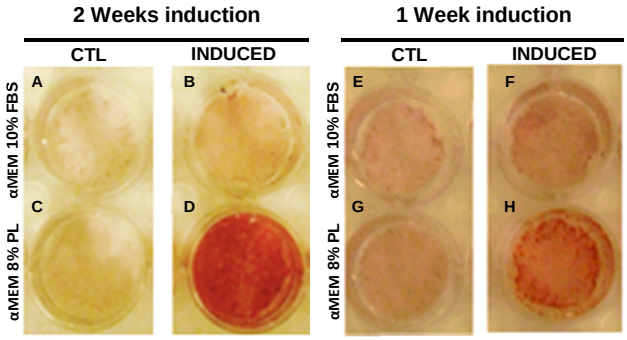

Figure 2. Two weeks and one week osteogenic induction protocol: Alizarin Red S staining. A, C control samples; B, D induced samples respectively with FBS or PL after two weeks of induction. E, G control samples; F, H induced samples respectively with FBS or PL after one week protocol. (B-D) Confirming the previous preliminary data, after two weeks of induction a poor osteogenic induction was saw with αMEM supplemented with 10% of defined FBS, and was greatly enhanced when using αMEM plus 8% of PL. (F-H) After only one week of induction, the weak ALZ staining seen with FBS in the osteogenic medium was intensified when using PL based medium. (A-C-E-G) The samples with cells maintained in maintenance medium did not show any significant spontaneous differentiation.

FIGURE 3

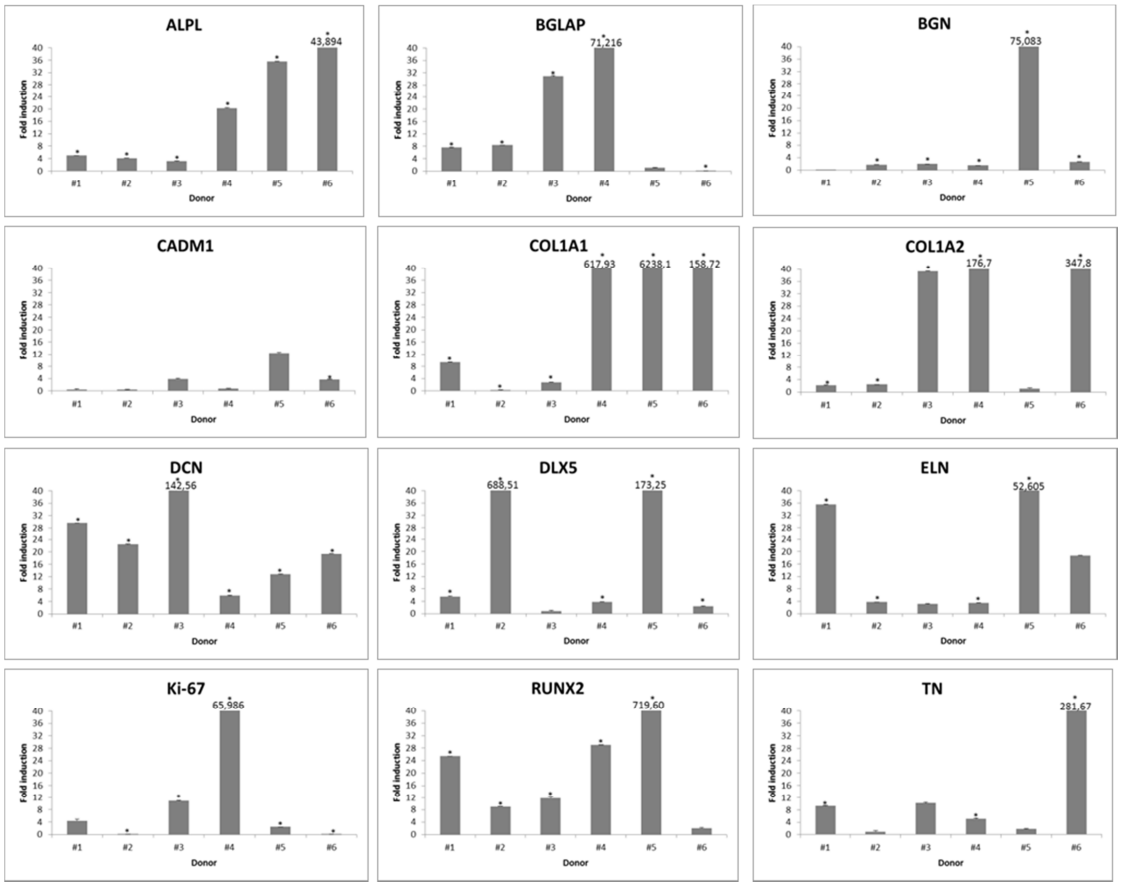

Figure 3. Two weeks osteogenic biomarkers expression in six hBM-MSCs donors.

FIGURE 4

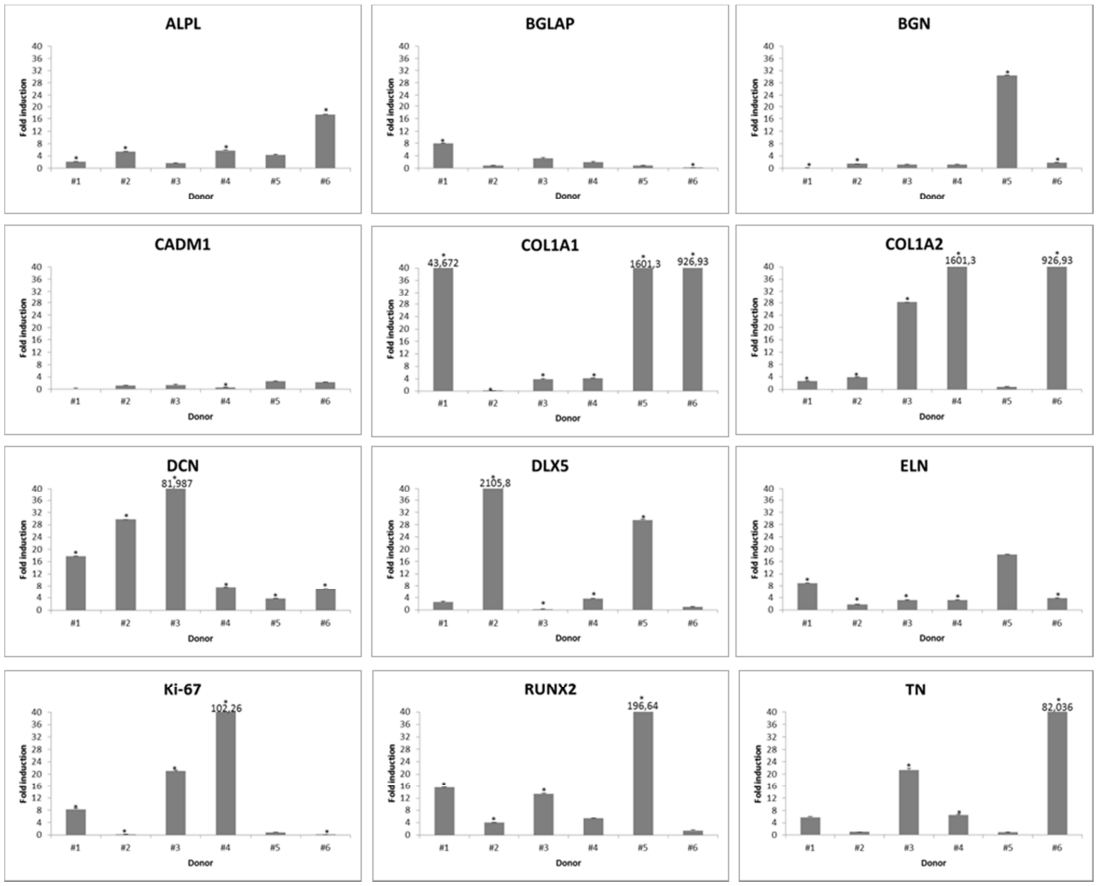

Figure 4. One week osteogenic biomarkers expression in six hBM-MSCs donors.

FIGURE 5

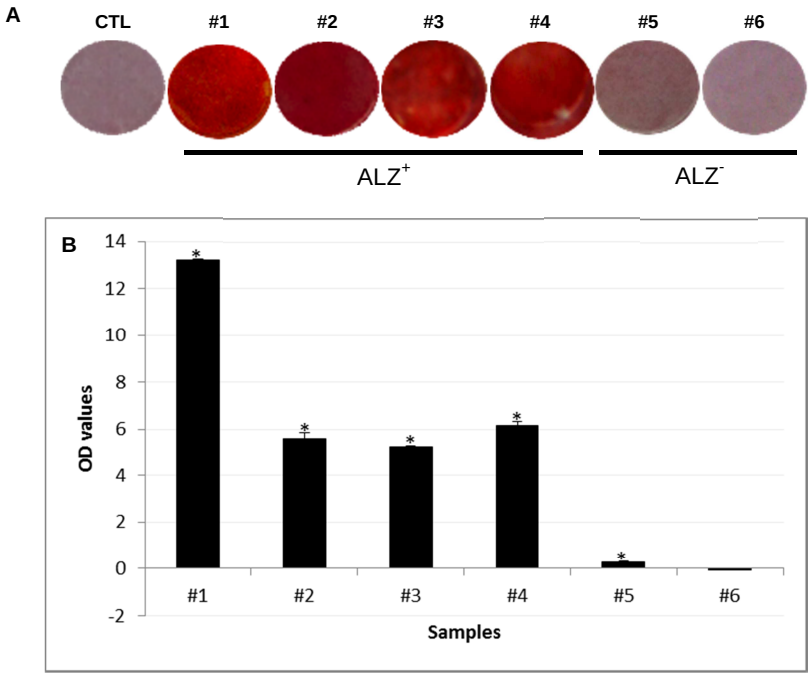

Figure 5. Alizarin red S staining after two weeks osteogenic induction of six GMP hBM-MSCs samples. After two weeks of osteogenic induction, the hBM-MSCs were stained with ALZ and two different phenotypes were identified. (A) Representative photograph of stained wells. The samples derived from donors #1, #2, #3 and #4 showed an extensive matrix mineralization and were identified as ALZ^+; instead the other two samples, derived from donor # 5 and # 6, showed a calcium deposition comparable with that seen in the negative controls. (B) Alizarin red S semi-quantification analysis achieved by using Cetylpyridinium Chloride method. The ALZ^+ samples showed statistically significant OD values between 5 and 13, instead the ALZ^- samples showed OD values less than 0.5.

FIGURE 6

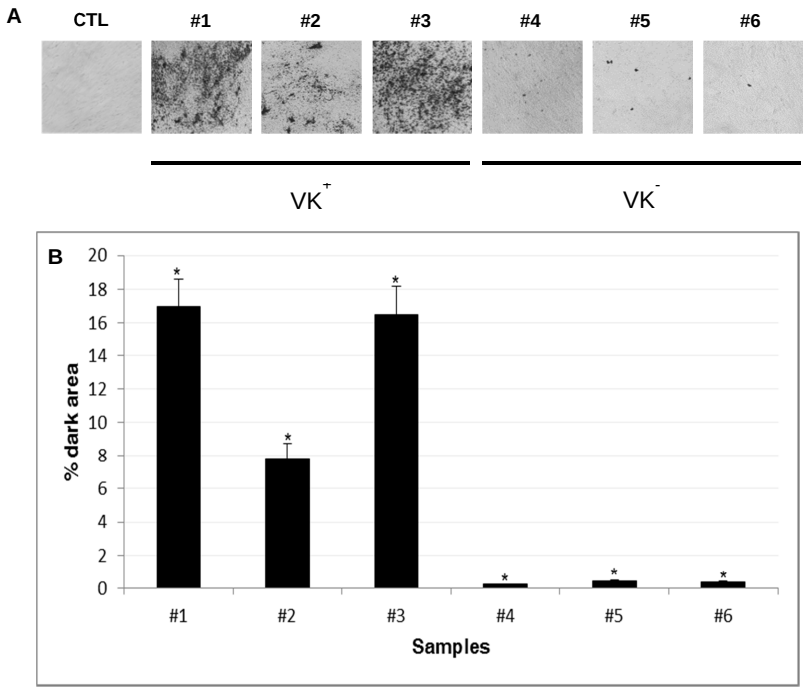

Figure 6. Von Kossa staining after two weeks osteogenic induction of six GMP hBM-MSCs samples. (A) Representative photomicrograph (10X) of stained samples after two weeks of osteogenic induction. The samples derived from donors #1, #2 and #3 showed a diffuse calcium phosphate areas, on the contrary in the samples derived from donors #4, #5 and #6 few black particles of silver ions were identified. No VK positive staining was observed in the un-differentiated controls samples. (B) evaluation of VK positive areas in representative photographs of osteogenic induced and control samples by using the Image J software. The samples derived from donors #1, #2 and #3 had showed positive areas comprised from 8 to 16 percent, instead the samples derived from donors #4, #5 and #6 showed dark areas under 0.5 percent.

FIGURE 7

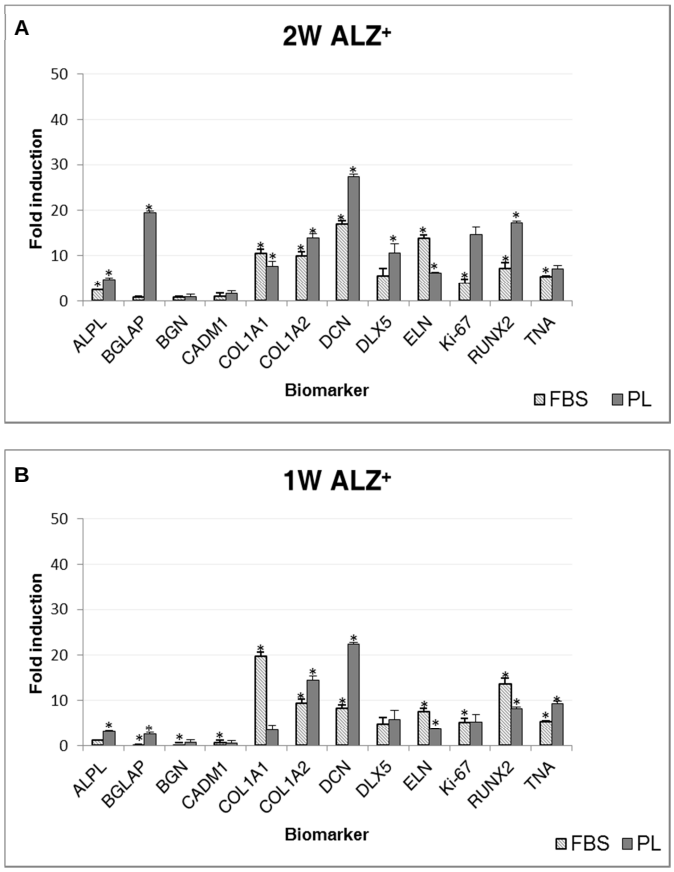

Figure 7. Molecular characterization of ALZ$^+$ samples after two weeks and one week of PL or FBS based osteogenic induction. (A) The upregulation of ALPL, COL1A1, COL1A2, DCN, ELN and RUNX2 were statistically significant after two weeks of both PL or FBS based induction protocol. Notably after two weeks induction, either with PL or FBS addition, the expression level of BGN and CADM1 was below to 2-fold increase and was not statistically significant. (B) The expression of ALPL, BGLAP, COL1A2, DCN, and TNA was more upregulated by using PL based media, in the other hand the expression of BGN, CADM1, COL1A1, ELN, Ki-67 and RUNX2 was efficiently upregulated after one week of FBS based osteogenic induction.

FIGURE 8

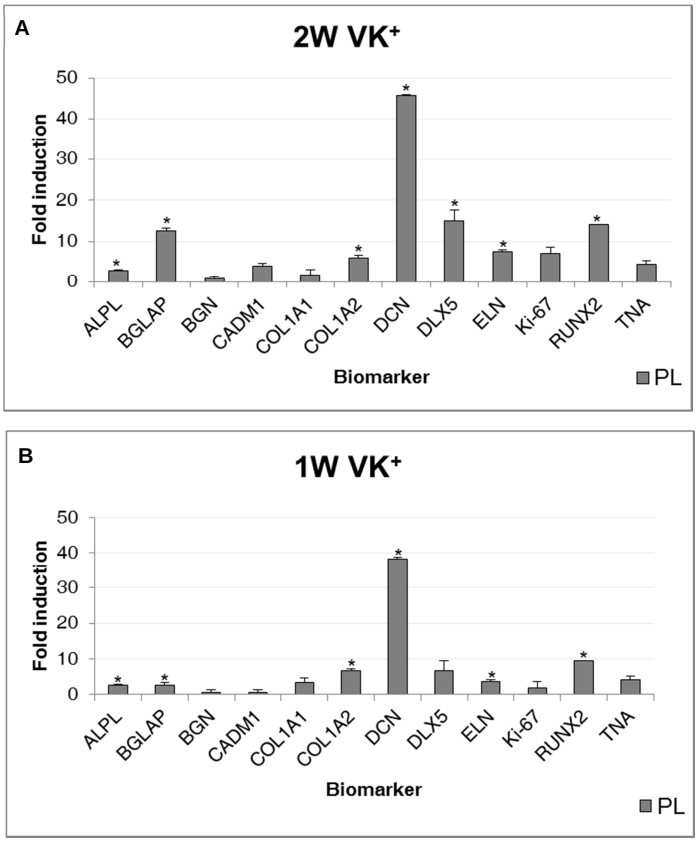

Figure 8. Molecular characterization of VK^+ samples after two weeks and one week of PL based osteogenic induction. (A) After two weeks of osteogenic induction the VK^+ samples showed a statistically significant upregulation of ALPL, BGLAP, COL1A2, DCN, DLX5, ELN and RUNX2. (B) The same osteogenic biomarkers tested after one week of induction has showed the statistically significant upregulation of ALPL, BGLAP, COL1A2, DCN, ELN and RUNX2.

FIGURE 9

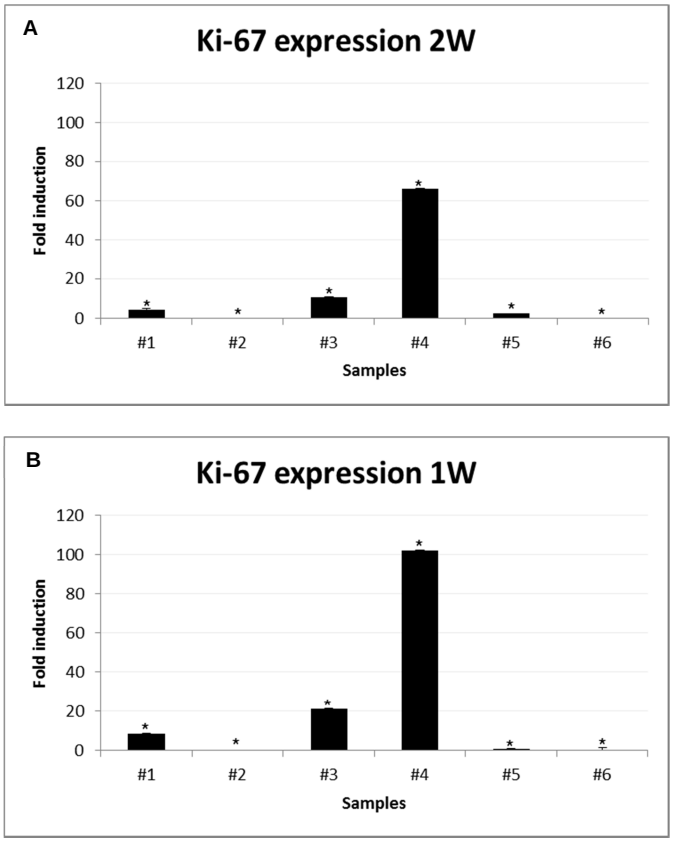

Figure 9. Ki-67 expression after two weeks and one week of PL based osteogenic induction. (A-B) The samples derived from donors #1, #3 and #4, either after two weeks or one week of osteogenic induction, had showed statistically significant upregulation of Ki-67 cellular cycle activation marker. Therefore, the samples derived from donors #2, #5 and #6 showed Ki-67 fold increase values below 2.5 after two weeks and below 1 after one week of osteogenic induction.

FIGURE 10

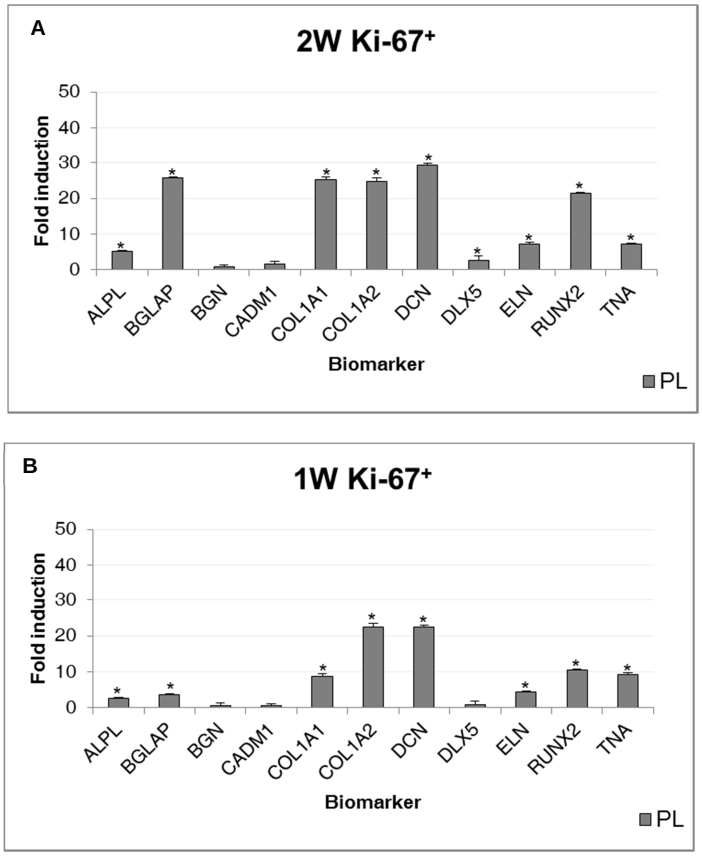

Figure 10. Molecular characterization of Ki-67$^+$ samples after two weeks and one week of PL based osteogenic induction. (A) The molecular analyses of Ki-67+ samples, derived from donors #1, #3 and #4, after two weeks of osteogenic induction showed a statistically significant upregulation of ALPL, BGLAP, COL1A1, COL1A2, DCN, DLX5, ELN, RUNX2 and TNA. (B) After one week of osteogenic induction by adding PL was founded a statistical significant upregulation of ALPL, BGLAP, COL1A1, COL1A2, DCN, ELN, RUNX2 and TNA genes.

FIGURE 11

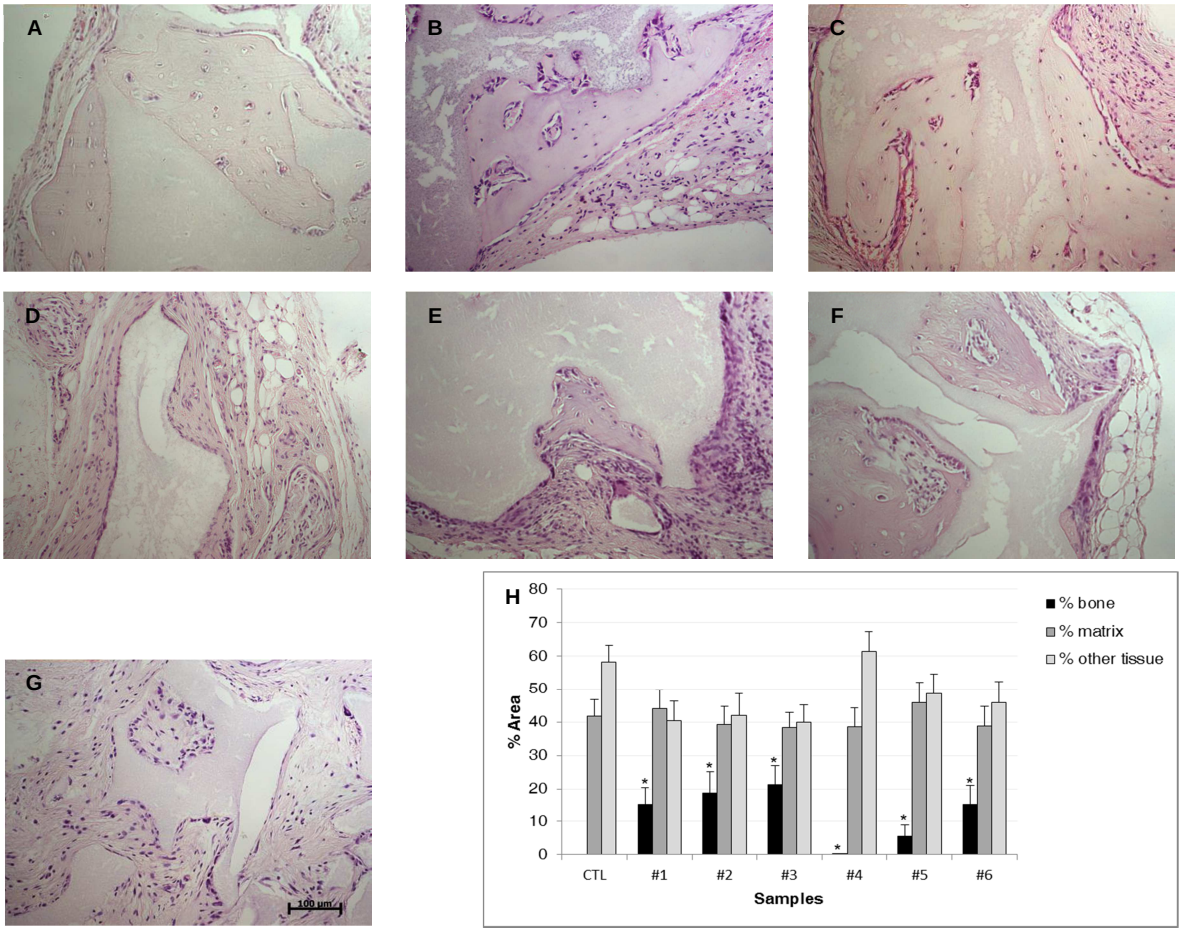

Figure 11. *In vivo* bone formation in ectopic murine model. (A-F) H&E staining of xenografts constituted by the hBM-MSCs from donor #1 to donor #6 mixed with MBCP granules; (G) the internal control implanted with MBCP granules alone; (H) scoring system quantification of stained samples. The donors #1, #2, #3 and #6 are able to form greater ectopic bone, with values comprised from 15% and 21% of total area. In the samples derived from donors #4 and #5 no significant bone formation was observed with respect to the controls one.

FIGURE 12

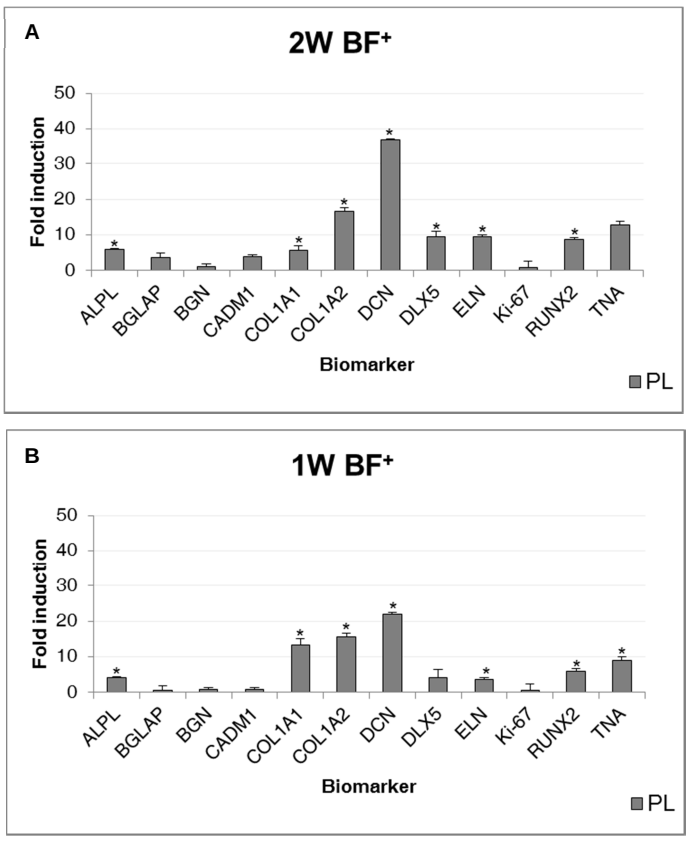

Figure 12. Molecular characterization of BF$^+$ samples after two weeks and one week of PL based osteogenic induction. (A) After two weeks of osteogenic induction the BF+ samples showed a statistically significant upregulation of ALPL, COL1A1, COL1A2, DCN, DLX5, ELN and RUNX2. (B) The same osteogenic biomarkers tested after one week of induction has showed the statistically significant upregulation of ALPL, COL1A1, COL1A2, DCN, ELN, RUNX2 and TNA.

FIGURE 13

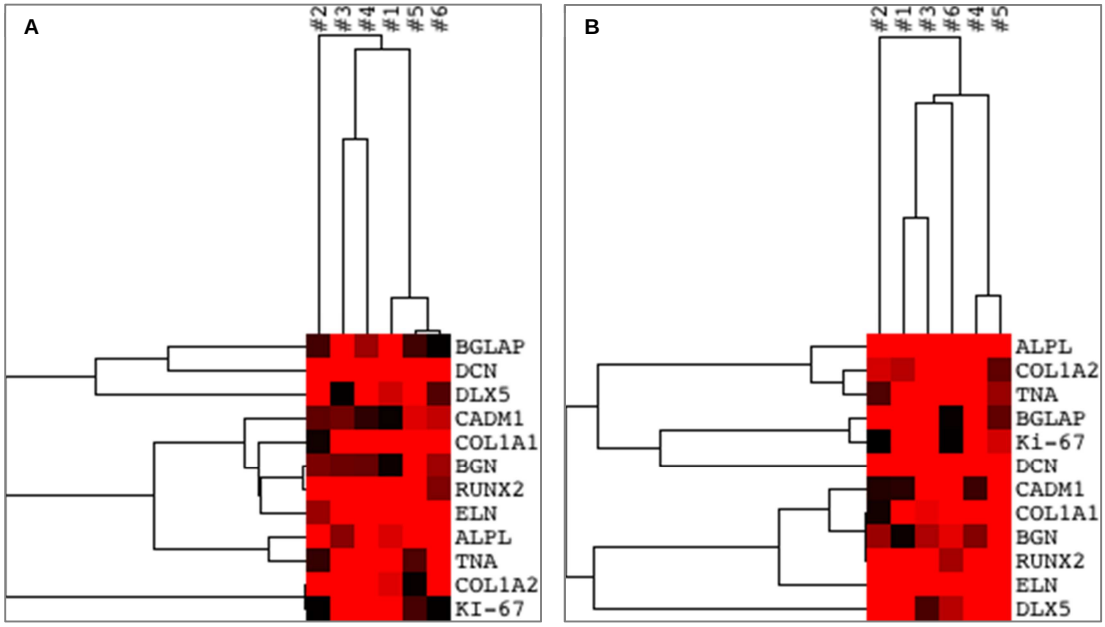

Figure 13. Cluster analysis of twelve osteogenic biomarkers genes (A) after one week of PL induction; (B) after two weeks of induction. The red spots represent high gene expression level and the dark spots correspond to low gene expression. After one week of OM, the uncentered Pearson correlate distances and centroid linkage, produced a dendrogram that showed DCN, RUNX2 and ELN as the most consistently expressed genes among all the donors, instead after two weeks (B) ALPL was now upregulated for all donors. Either the one or two week clusters generated using all twelve osteogenic biomarker genes failed to generate a dendrogram linking the donors in a way that correlated with bone forming potential.

FIGURE 14

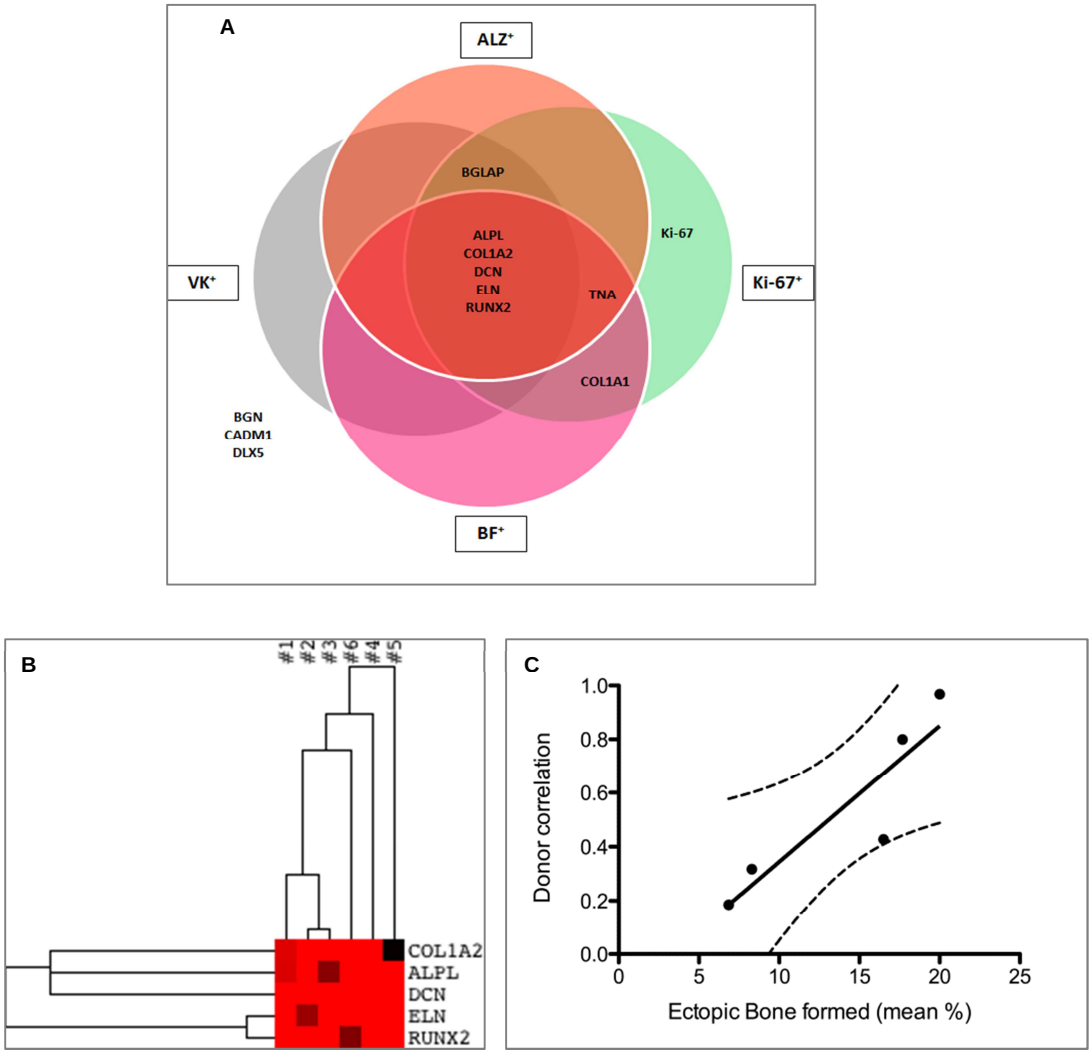

Figure 14. Cluster analyses after one week of osteogenic induction. (A), A Venn diagram is drew to identify the globally significant gene upregulated. (B) The uncentrered centroid cluster analysis of global statistically up-regulated genes after one week of PL-based induction showed that bone forming donors were more similar to each other than the non-bone forming donors. (C) Linear regression analysis of the similarity between donors versus the amount of bone made by the relevant pair of donors showed strong correlation (r2= 0.812).

FIGURE 15

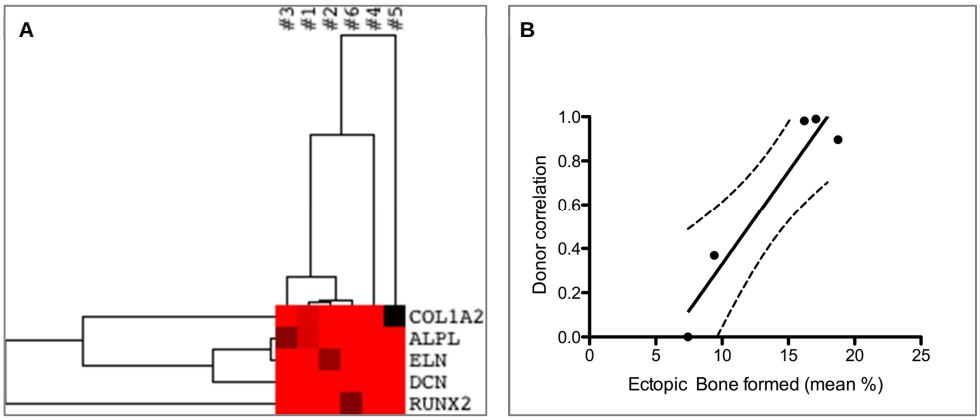

Figure 15. Euclidian distance and single linkage cluster analyses after one week of osteogenic induction. (A) Choosing Euclidian distance and single linkage clustering we obtained a dendrogram that also associated bone forming donors with a linear regression analysis that showed a stronger correlation between bone forming ability and donor similarity (r2= 0.911). (B) The correlation between donor and ectopic bone formation showed a more appreciable differences between bone forming donors and non-bone forming donors.

FIGURE 16

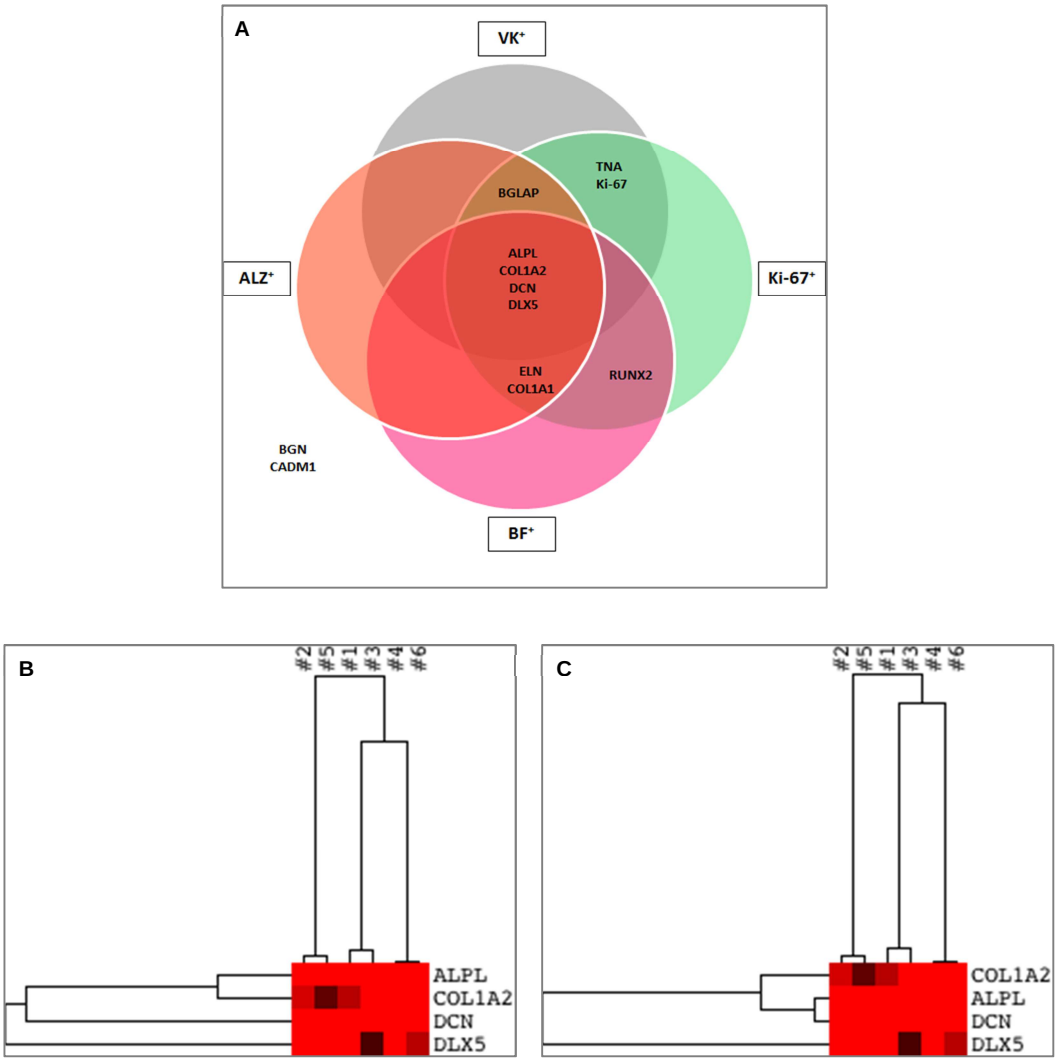

Figure 16. Cluster analyses after two weeks of osteogenic induction. (A), Representative Venn diagram is drew to identify the globally significant gene upregulated. (B) Uncentrered centroid cluster analysis and (C) Euclidian distance and single linkage clustering of statistically up-regulated genes in ALZ^+, VK^+, $Ki\text{-}67^+$ and BF^+ samples after one week of PL. The resulting two dendrogram from the two weeks analyses did not discriminate donor bone forming potential.

Donor	Age	Gender
#1	54	M
#2	51	F
#3	24	F
#4	42	M
#5	24	F
#6	21	M

Table 1. Donor information

Gene	Primer sequence	Amplified length
β Actin	5'-ACCTTCTACAATGAGCTGCG-3' (sense)	148 bp
	5'-CCTGGATAGCAACGTACATGG-3' (antisense)	
ALPL	5'-GATGTGGAGTATGAGAGTGACG-3' (sense)	142 bp
	5'-GGTCAAGGGTCA GGAGTTC-3' (antisense)	
BGLAP	5'-CAGCGAGGTAGTGAAGAGAC-3' (sense)	144 bp
	5'-TGAAAGCCGATGTGGTCAG-3' (antisense)	
BGN	5'-TGGAGAACAGTGGCTTTGAAC-3' (sense)	134 bp
	5'-GTTGTGGTCTAGGTGGAGTTC-3' (antisense)	
CADM-1	5'-CCAGCGGTATCTAGAAGTACAG-3' (sense)	146 bp
	5'-TCACCCAAGTTACCATCACAG-3' (antisense)	
COL1A1	5'-CCCCTGGAAAGAATGGAGATG-3' (sense)	148 bp
	5'-TCCAAACCACTGAAACCTCTG-3' (antisense)	
COL1A2	5'-AGGACAAGAAACACGTCTGG-3' (sense)	146 bp
	5'-GGTGATGTTCTGAGAGGCATAG-3' (antisense)	
DCN	5'-AAAATGCCCAAAACTCTTCAGG-3' (sense)	146 bp
	5'-GCCCCATTTTCAATTCCTGAG-3' (antisense)	
DLX5	5'-CTTATGCCGACTATAGCTACG C-3' (sense)	124 bp
	5'-CCATTCACCATTCTCACCTCG-3' (antisense)	
ELN	5'- CCTGGCTTCGGATTGTCTC-3' (sense)	148 bp
	5'- CAAAGGGTTTACATTCTCCACC-3' (antisense)	
Ki-67	5'- AAAAGAATTGAACCTGCGGAAG-3' (sense)	129 bp
	5'- AGTCTTATTTTGGCGTCTGGAG-3' (antisense)	
MSX2	5'- CGGTCAAGTCGGAAAATTCAG-3' (sense)	149 bp
	5'- GGATGTGGTAAAGGGCGTG-3' (antisense)	
PTH1R	5'- ATGCTCTTCAACTCCTTCCAG -3' (sense)	126 bp
	5'- CTTTCGCTTGAAGTCCAGTG -3' (antisense)	
RUNX2	5'-TTC ACC TTG ACC ATA ACC GTC-3' (sense)	148 bp
	5'-GGC GGT CAG AGA ACA AAC TAG-3' (antisense)	
SFRP1	5'- AAGTGTGACAAGTTCCCCG-3' (sense)	127 bp
	5'-TGGCCTCAGATTTCAACTCG-3' (antisense)	
SPP1	5'- CAGTGATTTGCTTTTGCCTCC -3' (sense)	149 bp
	5'- ATTCTGCTTCTGAGATGGGTC -3' (antisense)	
SP7	5'- GCCAGAAGCTGTGAAACCTC-3' (sense)	141 bp
	5'-GCTGCAAGCTCTCCATAACC-3' (antisense)	
TAZ	5'- CGAATTCCTGCGTTTCAAGTG -3' (sense)	147 bp
	5'- GTGATTTTCTGTCCAAAGCGG -3' (antisense)	
TN	5'-TCCTCCTCTGCCTCTTCTC-3' (sense)	136 bp
	5'-GTGTCCAGACGGCTCTTG-3' (antisense)	

Table 2. RealTime-PCR Primers Sequences

	DONORS / PHENOTYPES	#1	#2	#3	#4	#5	#6
Ex vivo	**ALZ^{+}**	✓	✓	✓	✓	X	X
	VK^{+}	✓	✓	✓	X	X	X
	KI-67^{+}	✓	X	✓	✓	X	X
In vivo	**BF^{+}**	✓	✓	✓	X	X*	✓

* Poor bone formation (≈5%)

Table 3. Phenotypes characterization of hBM-MSCs donors

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