EFFECT OF VITAMIN D PRETREATMENT OF HUMAN MESENCHYMAL STEM CELLS ON ECTOPIC BONE FORMATION

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Adult mesenchymal stem cells (MSCs) are used in contemporary strategies for tissue engineering. The MSC is able to form bone following implantation as undifferentiated cells adherent to hydroxyapatite (HA)/tricalcium phosphate (TCP) scaffolds. Previous investigators have demonstrated that human MSCs (hMSCs) can be differentiated to osteoblasts in vitro by the inclusion of vitamin D and ascorbic acid. The aim of this study was to compare the osteogenic potential of predifferentiated and undifferentiated bone marrow–derived, culture-expanded hMSCs adherent to synthetic HA/TCP (60%/40%) following subcutaneous engraftment in severe combined immunodeficiency (SCID) mice. During the final 3 days of culture, cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and antibiotics or media containing 25-mM calcium supplementation with vitamin D and ascorbic acid. Four weeks following implantation in SCID mice, scoring analysis of bone formation within the cubes revealed the absence of bone formation in unloaded cubes. Bone formation compared by a qualitative bone index was 7.23% for undifferentiated cells compared to 5.20% for differentiated cells. Minimal resorption was observed at this early time point. In this ectopic model, predifferentiation using a combination of vitamin D and ascorbic acid failed to increase subsequent bone formation by implanted cells. Following implantation of hMSCs adherent to an osteoconductive scaffold, host factors may contribute dominant osteoinductive signals or impose inhibitory signals to control the fate of the implanted cell. Predifferentiation strategies require confirmation in vivo.

INTRODUCTION

Predictable bone repair and regeneration is a fundamental aspect of contemporary dental implant therapy. Current methods for bone repair and augmentation include the use of autogenous bone grafting or guided bone regeneration using a wide range of alloplastic or allogeneic materials. New clinical approaches to bone regeneration

are of current interest and seek to reduce donor-site morbidity or to increase the efficacy of alloplastic materials. Testing of new materials during the early stages of development often involves rodent models of bone repair or induction. Critical-size calvaria and long-bone defect models are 2 common orthotopic models, while bone formation in subcutaneous pouches represents a common ectopic model for bone induction.

Tissue-engineering approaches to bone repair are of growing interest and can be evaluated in ectopic bone–repair models. Bone morphogenetic proteins (BMPs) were originally evaluated in this manner. Besides molecular approaches using BMPs, cellular approaches using adult human mesenchymal stem cells (hMSCs) are now reaching levels of clinical interest.[1,2] The hMSCs are multipotential cells capable of forming bone, cartilage, muscle, fat, and tendon. When placed onto hydroxyapatite (HA)/tricalcium phosphate (TCP) carriers in subcutaneous pouches of immunocompromised rodents, the bone marrow–derived mesenchymal stem cell (MSC) and adipose-derived MSC are able to direct ectopic bone formation in the absence of additional osteoinductive signals.[3–6] The amount of bone formed in the supporting scaffold is limited to less than half of the potential available space, even after 5 to 6 weeks of healing.[7]

Several factors may contribute to the control of bone formation by the scaffold-adherent MSC. Previous work has demonstrated a role for the scaffold in control of osteogenesis.[6,7] Additionally, the source of the MSC, including the age of the donor[8] or the tissue of origin, may also affect the extent of osteogenesis. The conditions of cell expansion vary in the literature and include the use of different culture-media supplements such as FGF2.[9] Culture conditions may affect the ultimate osteogenic potential of the scaffold-adherent MSC.

The ability to predictably enforce the osteoinductive program of the stem cell is one goal of bone-tissue engineering. The predifferentiation of cells by cell-culture supplements or BMPs could restrict differentiation along alternative pathways[10] and might offer an acceleration of the healing process. Current methods of osteoinduction of cultured hMSCs include culture of cells in the presence of dexamethasone, ascorbic acid, and β-glycerophosphate or in the presence of vitamin D. The aim of this study was to define the relative effectiveness of osteoblastic predifferentiation using vitamin D and calcium supplementation of culture hMSCs prior to implantation on scaffolds supporting ectopic bone formation.

MATERIALS AND METHODS

Matrix preparation

Synthetic HA/TCP (60%/40%) ceramic was cut with a diamond blade on a rotary instrument into 3 mm × 3 mm cubes, ultrasonically cleaned of particulate debris, and sterilized by autoclave.

Cell preparation

The treatment of hMSCs with vitamin D in Dulbecco's modified Eagle's medium (DMEM) with osteogenic supplements was evaluated to confirm osteoinduction. The hMSCs were provided in cryopreservation vials (5–10 × 10^6) by Osiris Therapeutics Inc (Baltimore, Md) as passage 2 or passage 3 cells. At confluence, cells were passaged using 0.05% Trypsin/EDTA (Life Technologies, Rockville, Md) and plated into 24-well culture dishes at an initial density of 25 000 cells per well. Cells were cultured for 0 to 14 days in DMEM (Life Technologies) supplemented with 10% fetal calf serum (FCS), 100 U penicillin/mL, 100 μg streptomycin/mL, 0.2 mM ascorbic acid, 10 mM β-glycerophosphate, and 10 μM 1α,25dihydroxy-cholecalciferol (Sigma Chemical Company, St Louis, Mo). The comparison of vitamin D– and dexamethasone-induced differentiation revealed no differences for cells grown in DMEM (data not shown). Cell layers were either fixed or harvested after 1, 3, 7, or 14 days in culture. Cell layers were stained for mineral accumulation by the von Kossa method, and harvested cells were lysed for RNA isolation and reverse transcription polymerase chain reaction analysis of osteocalcin, bone sialoprotein (BSP), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression (Figure 1).[11]

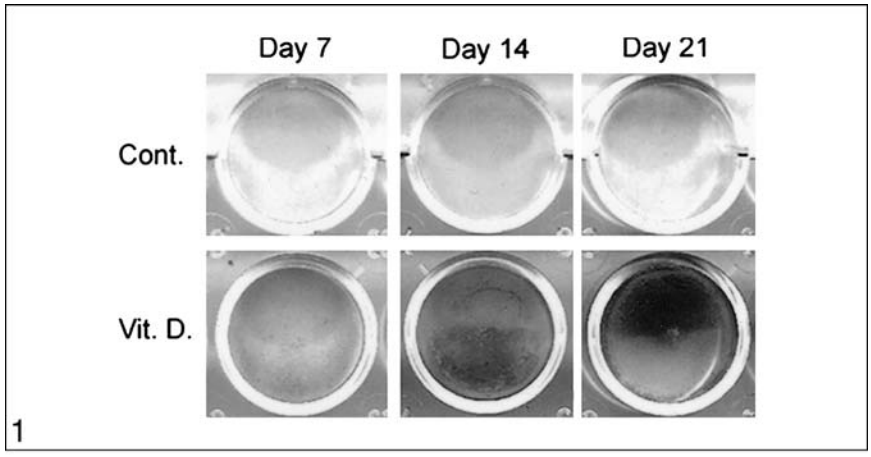
hMSC preparation and implantation

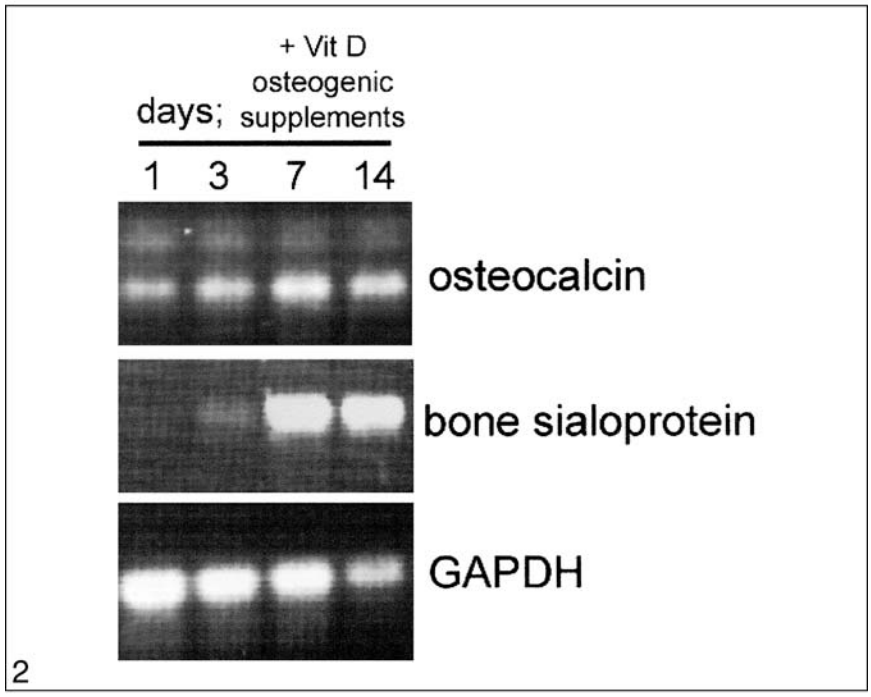
Undifferentiated, culture-expanded hMSCs from a single donor were obtained (Osiris Therapeutics Inc, Baltimore, Md). The hMSCs were expanded in DMEM containing 10% FCS and penicillin/streptomycin. All cubes were submerged and vacuumed in 100 μg/mL bovine
plasma–derived Fibronectin (bFN; catalog #GEB, Lot #124021, RD Inc, Minneapolis, Minn). The bFN-coated cubes were rinsed with phosphate-buffered saline (PBS) and then loaded with hMSCs by incubation for 3.5 hours in 1 × 10^7 hMSC/mL. Eight cubes were loaded with vitamin

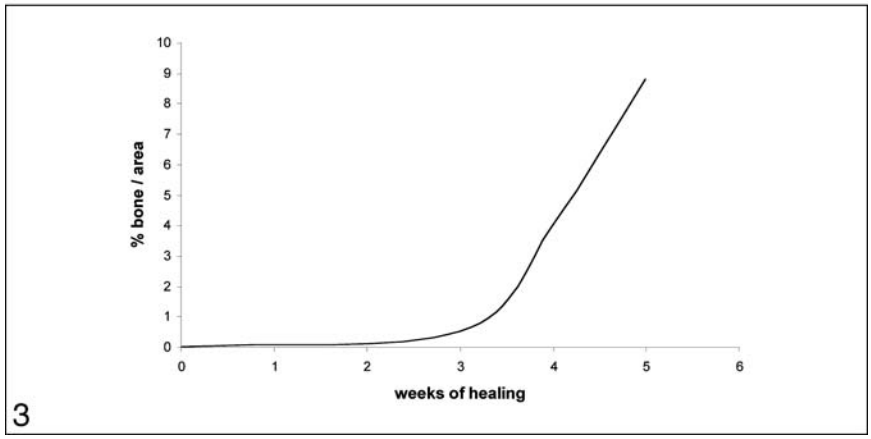
D/calcium–pretreated hMSCs, 8 cubes were loaded under vacuum with hMSCs grown in unsupplemented growth medium, and 4 additional cubes were similarly treated in cell-free DMEM.

Ectopic osteogenesis assay

The University of North Carolina-Chapel Hill Animal Care and Use Committee approved Protocol 02-117.0, which was used in this study and observes the National Institutes of Health guidelines for the care and use of laboratory animals. Samples were implanted in subcutaneous pouches on the dorsum of severe combined immunodeficiency (SCID) mice (n = 4) in an ectopic cube assay.[12–14] Samples (6 per mouse) were randomly implanted among mice and implantation sites, and incisions were closed with tissue staples. After 4 weeks, the mice were sacrificed and samples were retrieved. The matrices/formed tissues were immediately placed in 10% paraformaldehyde for 3 hours and rinsed. Preparation







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FIGURES 1–3. FIGURE 1. von Kossa staining of human mesenchymal stem cells cultured for the 14-day period in the presence or absence of vitamin D revealed the development of a mineralizing matrix in the vitamin D–treated cultures only. FIGURE 2. Vitamin D induction of cultured human mesenchymal stem cell osteoblastic differentiation. Reverse transcription polymerase chain reaction analysis of osteocalcin, bone sialoprotein (BSP), and glyceraldehyde-3-phosphate dehydrogenase (as shown) was performed for RNAs isolated from cultures grown for 1, 3, 7, and 14 days. Note the induction of BSP beginning at Day 7. The expression of osteocalcin at these early time points is due to vitamin D supplementation. FIGURE 3. Time-dependent accumulation of bone in ectopically implanted mesenchymal stem cell–loaded hydroxyapatite/tricalcium phosphate cubes. These data represent composite information obtained from 3-, 4-, and 6-week healing performed using multiple donors.

for conventional bright-field microscopy included decalcification with 10% formic acid. Cubes were embedded in paraffin, and 5-μm sections were prepared and stained using hematoxylin and eosin. Four sections per group were digitally captured using an Olympus DX41 microscope (Olympus America Inc, Melville, NY) and a Sony DX390 camera (Sony Electronics Inc, Park Ridge, NJ) using Bioquant software (BIOQUANT Image Analysis Corp, Nashville, Tenn). The percentage of tissue within the field that was identified as bone was calculated by scoring.

RESULTS

Vitamin D induction of cultured hMSC osteoblastic differentiation during a relatively short-term, 14–21-day culture period in media supplemented with osteogenic supplements and 10-mM vitamin D resulted in osteoblastic differentiation, as demonstrated by culture layer von Kossa staining for mineralization (Figure 1) and osteoblast-specific gene expression (Figure 2). In particular, BSP expression was elevated after only 3 days in culture and remained high at 14 days. The onset of positive von Kossa staining was consistent with the observed BSP expression. The evaluation of osteocalcin served to demonstrate the effectiveness of vitamin D treatment, as this vitamin D–responsive gene was induced within the first hours of treatment and remained high throughout the culture period.

In this study, a 4-week healing period was chosen for the evaluation of ectopic bone formation. The development of histologically evident bone was initially observed at 3 weeks following implantation of hMSC-loaded HA/TCP constructs and, by 6 weeks, 8% to 10% of the evaluated area was filled with bone (Figure 3). All mice survived the implantation procedure for the 4-week period without signs of infection or dorsal cutaneous lesion formation.

Histologic analysis of the ectopically formed tissue revealed the absence of bone in all of the implanted cell-free constructs. In the hMSC-loaded and vitamin D/calcium-pretreated hMSC-loaded samples, there was an absence of inflammation within the tissues formed in the scaffold pores and surrounding the constructs. Cartilage and muscle tissue in the scaffold pores were not observed in any of the tissue sections (Figures 4 and 5). Bone formation was observed in all hMSC-loaded samples. The formed bone tissue was associated with well-vascularized connective tissue. There was little evidence of active osteoclastic activity occurring at the surface of the matrices at this 4-week time point (Figure 6).

Bone formation differed between the vitamin D–treated, hMSC-loaded scaffolds and the untreated hMSC-loaded scaffolds. More bone formation was qualitatively revealed for the untreated hMSCs when the number of bone-filled pores was counted. No pores contained bone in any of the cell-free samples. When scored according to the method of Hanada et al,[13] the average bone score for untreated hMSCs and vitamin D–treated, hMSC-loaded scaffolds were 7.23 and 5.20, respectively. The histomorphometric quantification of bone/area forming in untreated hMSC-loaded cubes was 4.50% ± 2.5 and 3.15% ± 1 for the vitamin D–treated, hMSC-loaded cubes. These values were not significantly different.

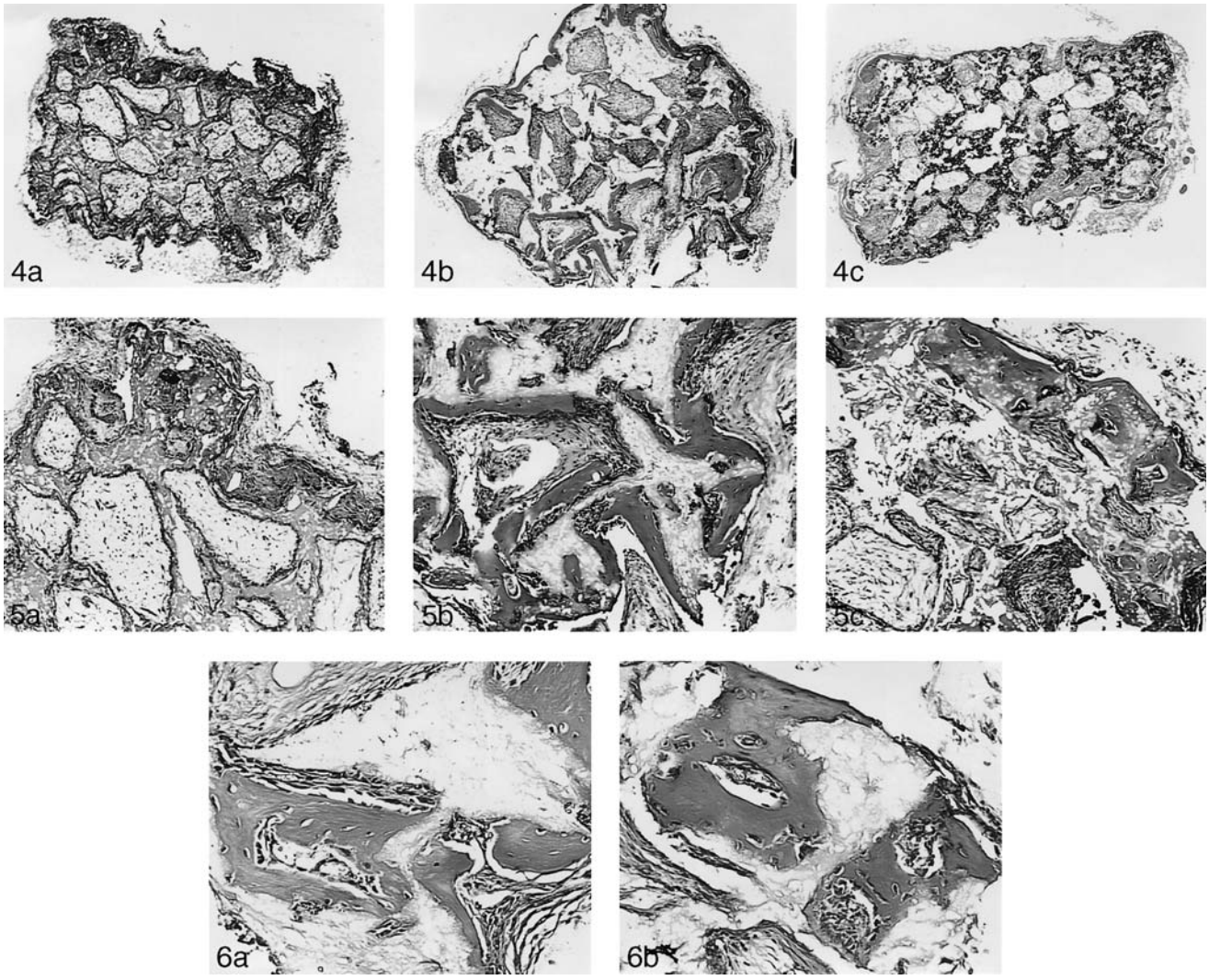
DISCUSSION

In this study, the ectopic bone–formation model was used to examine the effect of vitamin D pretreatment of culture-expanded hMSCs on the subsequent behavior following hMSC implantation adherent to HA/TCP scaffolds. When compared to untreated, culture-expanded hMSCs, vitamin D–treated hMSCs failed to produce more bone following implantation on HA/TCP scaffolds.

Vitamin D treatment of hMSCs has previously been shown to support or induce osteoblastic differentiation.[15] We hypothesized that vitamin D treatment of cultured hMSCs prior to implantation would initiate osteoblastic differentiation in culture and, thereby, accelerate in vivo bone formation. When compared to cells grown without vitamin D supplementation, this treatment resulted in greater alkaline phosphatase expression and more mineralization of the cell-culture matrix.[16] The in vitro differentiation of hMSCs cultured with vitamin D–supplemented media occurred rapidly in this study.

The concept of differentiating hMSCs prior to implantation or predifferentiation is not restricted to the use of vitamin D. Vitamin D pretreatment of cultured hMSCs results in a population of mature osteoblasts that synthesized more matrix,[16] demonstrating that this agent stimulates their osteogenic differentiation. Herbertson and Aubin[17] demonstrated that dexamethasone significantly increased the number of alkaline phosphatase–positive colonies; in addition, they verified the reduction of hematopoietic cells expressing leukocyte common antigen.

Other reagents can be used for the osteoblastic differentiation of hMSCs. For example, dexameth-



FIGURES 4–6. FIGURE 4. General histomorphology of ectopic-tissue formation 4 weeks following implantation. (a) Cell-free scaffold. (b) Undifferentiated, cell-loaded scaffold. (c) Vitamin-D/calcium-treated, cell-loaded scaffold (original magnification ×1.25). FIGURE 5. Identification of osseous tissue in the ectopically formed tissue 4 weeks after implantation. (a) Cell-free scaffold. (b) Undifferentiated, cell-loaded scaffold. (c) Vitamin-D/calcium-treated, cell-loaded scaffold (original magnification ×4). FIGURE 6. High-power evaluation of bone formed by (a) undifferentiated, cell-loaded scaffold and (b) vitamin D/calcium-treated, cell-loaded scaffold.

asone and ascorbic acid are used to induce hMSC osteogenesis in culture and support hMSC osteogenesis in vivo.[18] Other predifferentiation schemes have been used. Treatment of cultured MSCs with inducing factors such as FGF2, BMP2, BMP6, GDF5, and BMP7 have all been shown to increase in vitro differentiation.[17–20] The BMPs have been included in stem cell–containing tissue-engineering constructs, and they increased bone formation.[21] However, this is a chronic treatment in vivo that would require substantial BMP quantities and differ from a predifferentiation approach. Short-term pretreatment or long-term treatment with BMPs also supports greater in vivo differentiation of implanted MSCs.[9,16,21,22] It remains to be determined if this differentiation scheme can enhance hMSC osteogenesis in vivo.

Recent studies using vitamin D in culture of osteoblastic and MSCs suggest that unitary or linear commitment of hMSCs has not been achieved. Both osteogenic and adipogenic traits are revealed by hMSCs following treatment with vitamin D.[22] It is possible that a common lineage is utilized during the initial differentiation of these cells in culture.[23]

As a naturally occurring osteotrophic hormone, the use of vitamin D may have merit in creating a simple tissue-

engineering construct. The hMSC differentiation along the osteoblastic lineage using the BMP family of osteoinductive factors is effective. Pretreatment of hMSCs with different BMPs, and the fabrication of tissue engineering-constructs containing BMPs in combination with stem cells, have resulted in greater bone formation when BMPs or BMP-encoding viral vectors were included in stem cell-containing constructs than when stem cell constructs were used alone. The complexity of a BMP or viral construct containing hMSCs may face significant challenges in the translation of such strategies to the clinical environment.

A simple predifferentiation scheme that is mediated by culture conditions may be more directly translated. However, the effectiveness of predifferentiation has been questioned in other recent studies. The use of MSCs adherent to an extracellular matrix within a titanium mesh scaffold was effective in healing critical-size defects in the rat.[25] The predifferentiation of the cell-loaded constructs did not increase the amount of bone formed. In fact, constructs that were cultured for only 1 day performed better than the 3- to 16-day cultured samples.

This preliminary study utilized cells from a single donor. One potential explanation for the absence of vitamin D responsiveness in the in vivo assay is donor variability; yet, these cells were differentiated in culture. Ongoing investigation of the in vitro effects of vitamin D predifferentiation as performed for this study may indicate potential factors that further limit greater osteogenesis following implantation.

In conclusion, this report reiterates the capacity of culture expanded hMSCs to form bone at ectopic sites when implanted adherent to an HA/TCP scaffold. The short-term predifferentiation-using media supplementation with vitamin D did not accelerate or increase the amount of bone formed within the scaffolds. Continued development of culture conditions that promote ex vivo expansion and preserve the multipotentiality of the adult stem cell cultures remain an important focus of investigation in the translation of this promising approach to bone repair.

NOTE

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