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# THREE-DIMENSIONAL COLLAGEN GEL CULTURE PROMOTES OSTEOBLASTIC PHENOTYPE IN BONE MARROW DERIVED CELLS

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## INDEXING WORDS

collagen; three-dimensional culture; bone marrow

## SYNOPSIS

When calvarial cells or osteogenic cell lines were cultured in type I collagen gel, calcification was observed early and diffusely compared to monolayer culture. Bone marrow derived cells were cultured in three-dimensional type I collagen gel to investigate whether the cells can differentiate into osteogenic cells. In terms of efficient induction of osteogenic differentiation, we compared collagen gel culture to type I collagen coated dish culture and collagen-free plastic dish culture by morphology, alkaline phosphatase activity, and mRNA expression for type I collagen and osteopontin. Bone marrow derived primary cells formed colonies consisting of fibroblastic cells positively expressing alkaline phosphatase activity. Mineral deposition was observed in both primary and the 3rd passaged cells cultured in collagen gel, whereas the 3rd passaged cells on plastic dishes failed to be mineralized. Cells in collagen gel showed higher alkaline phosphatase activity than those in the other two methods suggesting that three-dimensional collagen network stimulated osteoblastic differentiation effectively. The expression level for type I collagen mRNA of the cells in collagen gel was three times higher in the 3rd passaged cells, and was slightly decreased in primary cells compared to the other two methods. The osteopontin mRNA expression of the cells in collagen gel was four times higher in the 3rd passaged cell culture but lower in primary cell cultures. These results suggested that collagen gel culture might be a useful environment for osteogenic induction of passaged cells derived from bone marrow.

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## INTRODUCTION

Collagen is not only a major component of extracellular matrix in vivo, it also acts as an important substrate for cell culture in vitro influencing cell proliferation, migration and differentiation.[2,3,10,13,18] Several studies have indicated that type I collagen induces osteogenic differentiation, leading to expression of the osteogenic phenotype and matrix mineralization.[4,14,15] When the multipotential mesenchymal clonal cell line (ROB-C26) was cultured on type I collagen coated plate, the promotion of osteoblastic phenotype was characterized by increase in mRNA expression of type I collagen, alkaline phosphatase and osteopontin.[19] Moreover, collagen has been shown to play an important role in signal transduction, affecting receptors for calcitonin and parathyroid hormone in UMR 106-66 osteoblast-like cells[8] and collagen-$\alpha 2\beta 1$ integrin interaction in murine osteoblastic cells.[24]

Three-dimensional type I collagen gel culture has been tested using various kinds of cell types. Chondrocytes cultured long term in collagen gel maintain morphology and phenotype without dedifferentiation.[9] Fibroblastic colonies positive for alkaline phosphatase staining were formed when bone marrow cells were cultured in collagen gels, although those fibroblasts in monolayer culture differentiated into adipocytic lineage.[16] Thus collagen gel culture systems may allow cells the expression, maintenance of phenotype and differentiation.

A few three-dimensional type I collagen gel cultures have also been reported for osteogenic cells. Rat calvarial cells cultured in collagen gel showed increased alkaline phosphatase activity and mineralization when compared to conventional monolayer culture.[25] Rat calvarial cells and MC3T3-E1 osteogenic cells, when cultured in collagen gel, are known to show calcification and differentiation.[7,21] This calcification was observed in earlier periods than in the monolayer culture which served as their control. Calcification in the monolayer culture was only observed in the nodule where cells grew multilayered, compared to diffuse calcification in the collagen gel.

Collagen gel may have potential as a filling material for large bone defects commonly seen following trauma or tumor resection. Sweeney et al. demonstrated total repair of critical sized bone defects through intramembranous ossification with type I collagen gel.[22] It is generally accepted that bone marrow stromal cells have potential to differentiate into osteoblastic cells and to form mineralized bone-like nodules in the presence of ascorbic acid and $\beta$-glycerophosphate in vitro.[11,12] The objectives of this study were to investigate whether bone marrow derived cells cultured in collagen gel can differentiate into osteogenic cells and to establish a method of delivering osteogenic cells to bone defects using bone marrow derived cells. In terms of efficient induction of osteogenic differentiation, we compared three-dimensional collagen gel culture system to a collagen coated dish or a collagen-free plastic dish monolayer culture.

## MATERIALS AND METHODS

*Isolation and culture of bone marrow cells*

Bone marrow cells were harvested from the femurs of adult mice as previously reported.[6] Femurs were aseptically excised, cleaned of soft tissue and the metaphyseal ends were cut off. The marrow was flushed out from the medullary canal with serum free $\alpha$MEM. Single cell suspension

was obtained by graded passages with different sized needles. After washing with phosphate buffered saline (PBS), cells were resuspended with αMEM supplemented with 10% fetal calf serum (FCS) and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin). For the primary cell cultures, bone marrow cells obtained from one femur were plated on two 35mm collagen free plastic dishes or embedded in two collagen gels and incubated in a fully humidified atmosphere of 5% $CO_2$ in air at 37°C. For the passaged cell culture, cells were harvested with trypsin/EDTA before mineralization and plated on collagen free plastic dishes at a density of $2.0x10^4/cm^2$ in culture medium. Primary and passaged cell cultures had 50 μg/ml of ascorbic acid and 10 mM β-glycerophosphate added on day 2. Culture medium was changed every two days.

*Collagen gel culture*

Purified acid soluble type I collagen from calf skin (Sigma) was dissolved in 1 mM acetic acid at 4°C to make a final concentration of 0.3% (w/v). This collagen solution was sterilized with ultraviolet light on ice for 2 hours. Initial gel preparation involved 1 volume of 10x αMEM, 1 volume of FCS and 8 volumes of cooled type I collagen solution (0.3%) mixed on ice. Six volumes of cell suspension were mixed with 10 volumes of collagen mixture. This mixture was plated and incubated at 37°C for 15 minutes. After gelation, the gels were overlaid with culture medium.

*Collagen coated dish*

Dishes were coated with type I collagen solution (10 μg/ml) for 2 hours at 37°C and washed with PBS before plating cells.

*Histological examination*

For the histological examination, 50 μg/ml of ascorbic acid and 10 mM of β-glycerophosphate were added to the culture medium. Gels were scraped off with a spatula, transferred on to the glass slides and dried. Gels were then fixed in 10% neutral buffered formalin and stained with Alizarin red-S. Alkaline phosphatase was stained with a commercially available alkaline phosphatase staining kit (Sigma: 86-R). The gels were fixed with citrate-acetone-formaldehyde solution and incubated with reagent for 15 minutes in the dark.

*Alkaline phosphatase activity*

For the measurement of alkaline phosphatase activity, first passaged cells obtained by trypsinization were plated on plastic dishes and collagen coated dishes or embedded into collagen gel at density of $3.0x10^5$/35mm dish. In order to eliminate the effect of ascorbic acid or β-glycerophosphate on alkaline phosphatase activity, neither of them were added to the culture medium. After 6 days culture, cells were washed well with PBS and homogenized in 50 mM Tris/HCl buffer (pH 7.6) containing 0.5% Triton X-100.[24] The supernatant was mixed with substrate (7.6 mM p-nitrophenyl-phosphate in 5 mM $MgCl_2$ in 0.75 mM 2-amino-2-methyl-l-propanol pH10.3 ), and incubated for 10 minutes at 37°C. The absorbance at 405 nm was compared against a standard curve obtained with p-nitrophenol. Noncollagenous protein concentrations were determined using Bio-Rad protein assay (Bio-Rad). This assay reagent does

not react with type I collagen (data not shown).

*Preparation of cDNA insert for probe*

Bone marrow cells were cultured for one week in αMEM supplemented with 10% FCS, antibiotics, 50 μg/ml of ascorbic acid and 10 mM of β-glycerophosphate. Total RNA was isolated using TRIzol (Gibco) as per manufacturer's instruction and reversetranscribed to cDNA. Reverstranscribed cDNA was PCR-amplified with primers: 5'-GGTGCCCCCGGTCTTCAG-3' and 5'-AGGGCCAGGGGGTCCAGCATTTC-3' for α1(I) procollagen, 5'-CCAACGGCCGA-GGTGATAG-3' and 5'-CAGGCTGGCTTTGGAACTTG-3' for osteopontin. Expected product lengths were 528bp and 332bp respectively. The PCR products were subcloned into pCR 2.1-TOPO vector (Invitrogen). The cDNA inserts were excised by PCR with T7 and M13 reverse primers, purified by gel extraction kit (Qiagen), and then used as probes for Northern blotting. The probe for GAPDH was kindly provided by Dr. Robert J Alpern (University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.).[1]

*Northern blotting*

The expression levels of α1(I) procollagen and osteopontin mRNA in the 3rd passaged cell were detected by Northern blotting. The same culture conditions that were described in the alkaline phosphatase activity measurement was used in this experiment. Ten μg of total RNA was size-fractionated on 1% agarose/formaldehyde gels and transferred to positively charged nylon membrane (Hybond-N+, Amersham). Membranes were prehybridized for 30 minutes and hybridized for 1 hour at 65°C in Rapid-hyb buffer (Amersham) containing $^{32}$P-labeled probes at a concentration of $10^6$ cpm/ml. Hybridized membranes were washed twice with 2x SSC, 0.1% SDS at room temperature and twice with 0.1x SSC, 0.1% SDS at 65°C for 15 minutes each. Washed membranes were then exposed to X-OMAT films (Kodak) with an intensifying screen at -80°C. Radioactive intensities of α1(I) procollagen, osteopontin and GAPDH mRNA were analyzed by NIH image software. The levels of each mRNA expression were indicated by the relative intensities to GAPDH mRNA.

## RESULTS

*Morphology and histology*

Primary cell culture: Immediately after gelation, cells embedded in collagen gel showed a round shape and were uniformly dispersed in the three-dimensional arrangement. A lot of small fibroblastic colonies were formed within a week and a few round cell colonies that seemed to differentiate into heamatopoietic cells were also observed. Cells in the fibroblastic colony were arranged radially and tended to form thin cytoplasm processes extending to neighboring cells in order to link each other (Fig. 1). Most of these cells stained positive for alkaline phosphatase. By the middle of the second week colonies became mineralized as shown by Alizarin red-S staining (Fig.2). Mineralization in plastic dish culture was seen at one week (data not shown).

Third passaged cell culture: Trypsinized cells were embedded into the gel at a density of

$2.0x10^5/cm^3$. Several hours after gelation, all cells extended cytoplasmic processes similar to fibroblasts. As days passed cell density was increased and gel volume was decreased by gel contraction as had been described previously.[7,20,26] Toward the end of two weeks, the three-dimensional uniform arrangement was partially destroyed and replaced with a lot of thick column-like formations (Fig.3). Mineral deposition then occurred in these column-like formations as confirmed by Alizarin red-S staining. In contrast, passaged cells on plastic dishes did not form column-like structure and did not stain with Alizarin red-S indicating that no mineral deposition occurred in plastic dish cultures (data not shown).

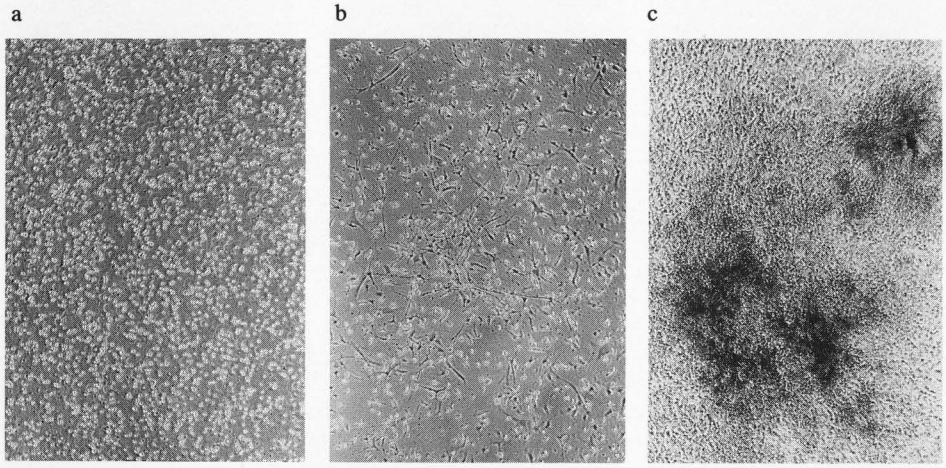


Fig. 1. Morphology of bone marrow derived primary cells cultured in type I collagen gel. Phase contrast photomicrograph. (a) Three-dimensional cell arrangement immediately after embedding. (b) Fibroblastic colony on day 7 of culture. (c) Dense nodule formation on day 14 of culture.

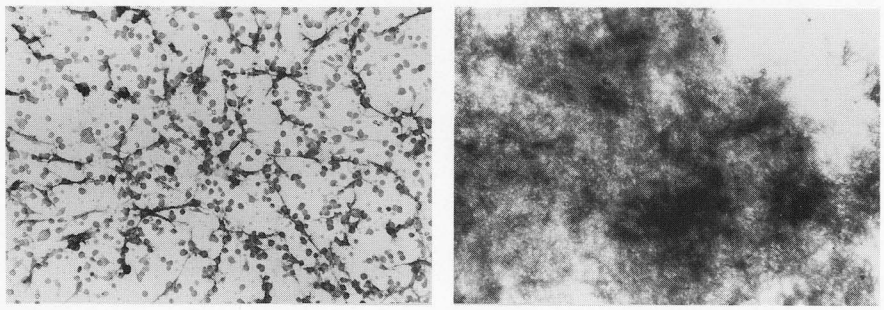

Fig. 2. Histology of bone marrow derived primary cells embedded in type I collagen gel. (a) Fibroblastic colony on day 7 of culture (alkaline phosphatase staining). (b) Mineralized nodule on day 14 of culture (Alizarin red S staining).

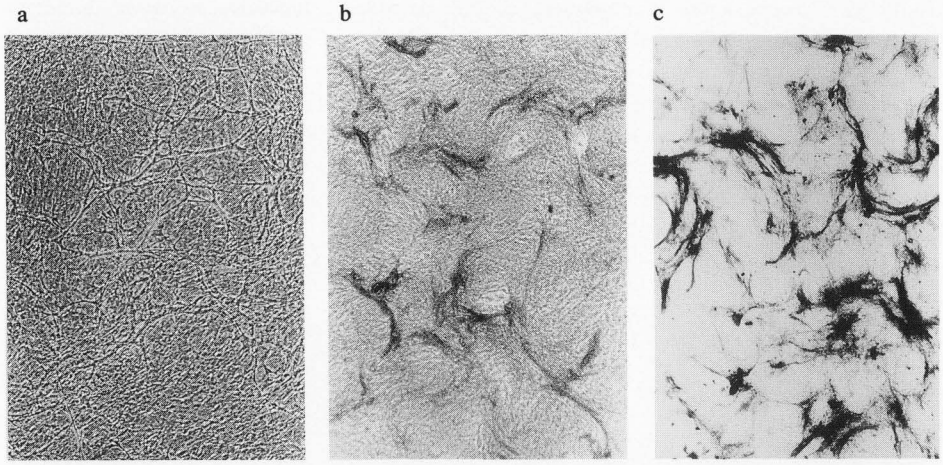


Fig.3. Morphology and Histology of the 3rd passaged cells cultured in type I collagen gel. (a) Confluent culture on day 7 of culture. (b) Column like structure formation on day 14 of culture. (c) Mineral deposition on day 17 of culture (Alizarin red-S staining).

*Alkaline phosphatase activity*

Alkaline phosphatase activity which is one of the earliest markers of osteoblast phenotype was compared within the three culture methods. Activity in the cells grown on the collagen coated dishes was 2 fold higher than that of conventional plastic dish (Fig.4). However cells embedded in collagen gel showed significantly higher activity than those of the plastic dish and the collagen coated dish ($P < 0.01$, ANOVA test).

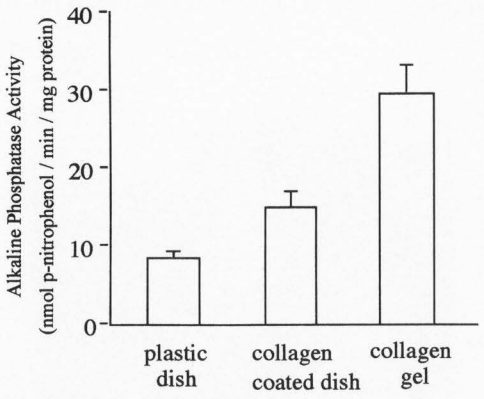


Fig.4. Alkaline phosphatase activity in bone marrow derived 1st passaged cell after 6 days of culture. Data represent the mean of triplicate cultures.

*Expression of mRNA*

Osteopontin is also an osteoblastic marker which is known as a marker for mature osteoblasts. In order to investigate the potential of collagen gel culture for the osteoblastic differentiation of bone

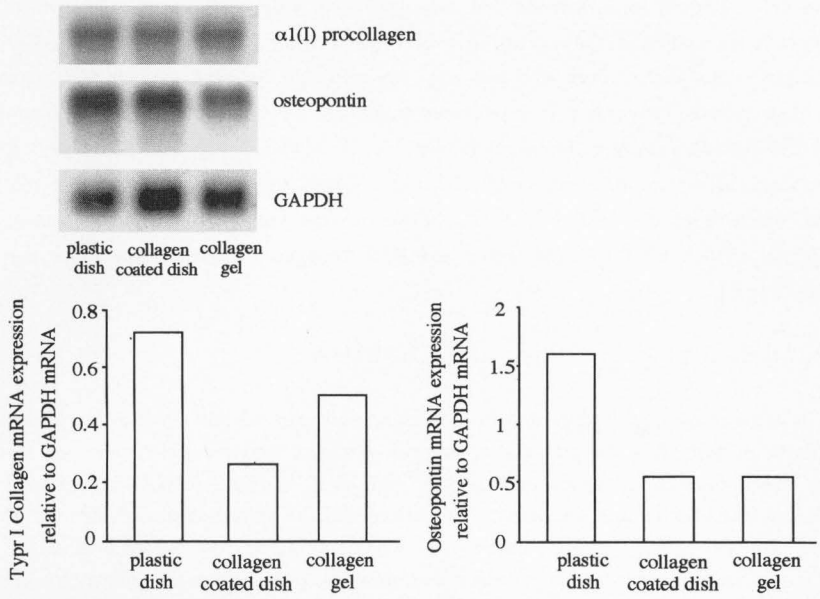


Fig. 5. Northern blot analysis of α1(I) procollagen, osteopontin and GAPDH mRNA in bone marrow derived primary cells.

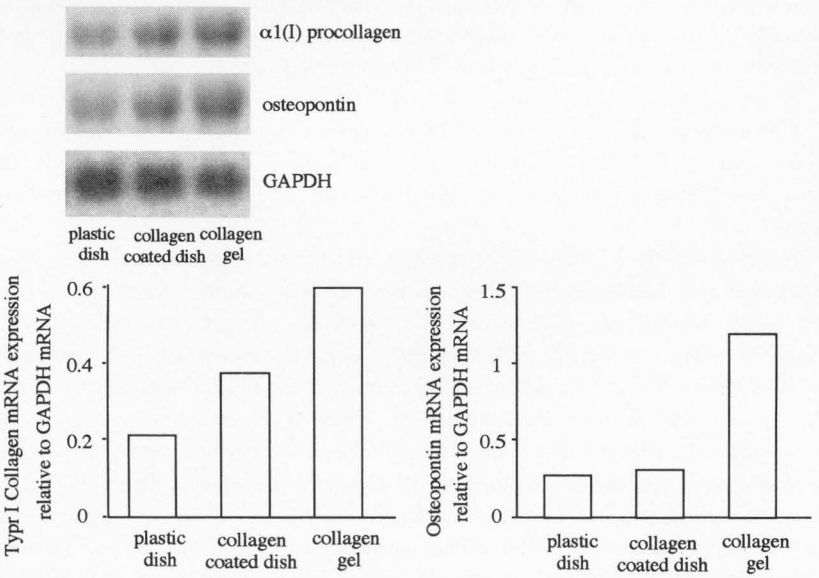


Fig. 6. Northern blot analysis of α1(I) procollagen, osteopontin and GAPDH mRNA in bone marrow derived the 3rd passaged cells.

marrow cells, primary cells and the 3rd passaged cells were used for this experiment. When primary cells were cultured, the expression level of type I collagen mRNA was slightly decreased in the collagen coated dish culture, and was not changed in the collagen gel culture compare to the plastic dish culture. Expression levels of osteopontin mRNA were higher in plastic or collagen coated dish cultures than in collagen gel culture (Fig.5). Type I collagen mRNA expression in the 3rd passaged cells was three times higher in the collagen gel cultures than that of plastic dish cultures while twice the control level of expression was seen in the collagen coated culture. Osteopontin mRNA level was four times higher in collagen gel than in the other two culture methods (Fig.6).

## DISCUSSION

In terms of osteogenic differentiation, bone marrow derived cells are reported to develop the osteoblastic phenotype in the presence of ascorbic acid and β-glycerophosphate.[11,12] One of the known effects of ascorbic acid on osteoblastic cells is to increase mRNA expression of type I procollagen, osteopontin and osteocalcin. Activity of alkaline phosphatase was also reported to be augmented in osteoblastic cells by supplement with ascorbic acid in culture medium.[5,27] Thus, ascorbic acid seems to be effective in inducing or maintaining the osteoblastic phenotype in vitro.

This study found that the 3rd passaged bone marrow derived cells on standard plastic dishes failed to form column-like structures and to be stained with Alizarin red-S (evidence of mineralization) in spite of supplementing with the ascorbic acid and β-glycerophosphate. On the other hand, primary or even the 3rd passaged bone-marrow derived cells appeared to differentiate into osteoblast like cells positively expressing alkaline phosphatase and forming mineralized nodules, when the cells were grown in three-dimensional type I collagen gel.

Cell replication in gel culture conditions has been reported to be suppressed,[9,17] which may explain why the mineralization of primary cells cultured in collagen gel was delayed in comparison with those in monolayer plastic dish culture in this study. Thus, bone marrow derived cells would seem to have a potential for differentiating into osteoblastic lineage depending on the environmental conditions.

The reason why type I collagen can stimulate osteoblastic differentiation is still elusive though it was reported that osteoblastic cell lines and calvarial cells cultured on collagen coated dishes showed higher alkaline phosphatase activity than those cultured on plastic dishes.[2,10,13,19] Interference of collagen binding to α2β1 integrin by integrin antibody or DGEA peptide decreased alkaline phosphatase activity, suggesting that interaction of exogenous collagen and an integrin is required for osteoblastic differentiation.[23] Also inhibition of endogenous collagen synthesis decreases an alkaline phosphatase activity. It is possible that not only the exogenous collagen but also endogenously synthesized collagen may play an important role in the osteoblastic differentiation through collagen-integrin interaction.

We tested three different types of culture conditions in order to seek the preferable condition for osteoblastic differentiation of bone marrow derived cells as seen by the alkaline phosphatase activities. Though collagen coated plate increased alkaline phosphatase activity as previously reported, cells cultured in three-dimensional collagen gel showed significantly higher enzyme

activity than other two culture conditions. Therefore our results suggest that three-dimensional collagen gel culture may be suitable for the differentiation of both primary and especially passaged bone marrow derived cells to osteoblastic lineage.

Lynch reported that when the calvarial cells were plated on collagen coated dishes, type I collagen mRNA expression was reduced up to 75% compared to that of cells grown on non-coated plastic dishes. On the other hand, Shi showed increased expression of type I collagen mRNA in ROB-C26 cells when cultured on collagen coating.[10,19] Shi also reported that osteopontin mRNA level was elevated when ROB-C26 cells were cultured on collagen coated plate.[19] In order to obtain significant amounts of cells for bone grafting, bone marrow derived cells may have to be cultured for long periods. These passaged cells are subject to be dedifferentiated to non-osteogenic lineage. In our study, the type I collagen mRNA expression level in the 3rd passaged cells embedded in collagen gel was three times higher than that in cells grown on non-coated plastic dishes, whereas it was not elevated in primary cells. The osteopontin mRNA expression of the 3rd passaged cells in the collagen gel culture was four times higher than monolayer culture conditions. Primary cells in the collagen gel culture expressed less osteopontin mRNA than monolayer culture conditions. These results of Northern blotting suggest that collagen gel promotes the expression of type I collagen and osteopontin mRNA in multi-passaged bone marrow derived cells. As type I collagen is the major extracellular matrix component of bone, collagen gel culture may be suitable for bone matrix formation.

In conclusion, collagen gel appeared to be a viable environment for expanding bone marrow derived cells by ex vivo culture and may well be a useful vehicle for delivering these cells to fill critical bone defects.

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