Article

Megakaryocytes Enhance Mesenchymal Stromal Cells Proliferation and Inhibit Differentiation[†]

Running Head: Megakaryocytes control stromal cell fate

Arbi M. Emmakah[1], Hussain E. Arman[2], Marta B. Alvarez[2], Paul J. Childress[2], Joseph P. Bidwell[3], William S. Goebel[4], Tien-Min Gabriel Chu[1, 2, 5] , Melissa A. Kacena[2,3]*

[1]Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN 47908, USA
[2]Department of Orthopaedic Surgery, Indiana University School of Medicine, Indianapolis, IN 46202, USA
[3]Department of Anatomy and Cell Biology, Indiana University School of Medicine, Indianapolis IN 46202, USA
[4]Children's Clinical Research Center, Riley Hospital for Children, Indiana University Health, Indianapolis, IN 46202, USA
[5]Department of Biomedical and Applied Sciences, Indiana University School of Dentistry, Indianapolis, IN 46202, USA

Corresponding Author:
Melissa A. Kacena, Ph.D.,
Associate Professor of Orthopaedic Surgery
Indiana University School of Medicine,
Telephone: (317)-278-3482,
Fax: (317)-278-9568,
Email: mkacena@iupui.edu

This is the author's manuscript of the article published in final edited form as:

Emmakah, A. M., Arman, H. E., Alvarez, M. B., Childress, P. J., Bidwell, J. P., Goebel, W. S., Gabriel Chu, T.-M. and Kacena, M. A. (), Megakaryocytes Enhance Mesenchymal Stromal Cells Proliferation and Inhibit Differentiation. J. Cell. Biochem.. Accepted Author Manuscript. http://dx.doi.org/10.1002/jcb.26289

Abstract

Megakaryocytes (MKs) can induce proliferation of calvarial osteoblasts [Ciovacco et al., 2009], but this same phenomenon has not been reported for bone marrow stromal populations from long bones. Bone marrow contains several types of progenitor cells which can be induced to differentiate into multiple cell types. Herein, we examined mesenchymal stromal cell proliferation and osteoblastic differentiation when rabbit or mouse MK were cultured with *i)* rabbit bone marrow stromal cells, *ii)* rabbit dental pulp stromal cells, or *iii)* mouse bone marrow stromal cells. Our results demonstrated that rabbit and mouse stromal cells co-cultured with rabbit MK or mouse MK, have significant increases in proliferation on day 7 by 52%, 46%, and 24%, respectively, compared to cultures without MK. Conversely, alkaline phosphatase (ALP) activity was lower at various time points in these cells when cultures contain MK. Similarly, calcium deposition observed at day 14 rabbit bone marrow and dental pulp stromal cells and day 21 mouse bone marrow stromal cells was 63%, 69%, and 30% lower respectively, when co-cultured with MK. Gene expression studies reveal transcriptional changes broadly consistent with increased proliferation and decreased differentiation. Transcript levels of c-fos (associated with cell proliferation) trended higher after 3, 7, and 14 days in culture. Also, expression of alkaline phosphatase, osteonectin, osterix, and osteopontin, which are markers for osteoblast differentiation, showed MK-induced decreases in a cell type and time dependent manner. Taken together, these data suggest that MK play a role in stromal cell proliferation and differentiation, from multiple sites/locations in multiple species. This article is protected by copyright. All rights reserved

Keywords: Megakaryocytes; Dental Pulp Stromal Cells; Proliferation Differentiation

Introduction

Bone marrow contains many types of undifferentiated stem cells [e.g. mesenchymal and hematopoietic stem cells (MSC and HSC, respectively)] and differentiated lineage cells, [e.g. osteoblasts (OB), osteoclasts (OC), and MK] [Caplan, 2005; Mauney et al., 2005]. MSC are known to differentiate into several types of cells including cartilage-forming chondrocytes and bone-forming OB [Caplan, 1991; Caplan, 2005; Caplan and Bruder, 2001; Emmakah et al., 2017; Friedenstein et al., 1976; Mauney et al., 2005; Pittenger et al., 1999]. OB play an important role in forming new bone by producing osteoid, which contains mostly type I collagen and other types of proteins. Additionally, calcium, phosphate and other minerals are required to enhance skeletal stiffness [Caplan and Bruder, 2001; Raisz and Kream, 1983; Turner, 2002]. On the other hand, HSCs have the ability to differentiate into numerous mature hematopoietic bone marrow cells such as lymphocytes, plasma cells, eosinophils, bone-resorbing OC, and MK [Chan and Yoder, 2004; Morrison et al., 1995].

MK are polyploidy cells derived from MK/erythroid progenitors [Amano et al., 2005; Geddis, 2010]. Although MK comprise approximately 0.01% of total blood cells in bone marrow, these cells are responsible for generating blood platelets that produce a number of growth factors which contribute to hemostasis [Geddis, 2010; Kacena et al., 2012]. Interestingly, similar to OB and odontoblasts (dentin-forming cells), MK also express bone differentiation markers such as, osteocalcin [Ciovacco et al., 2010], osteonectin, osteopontin, bone morphogenetic protein 2 (BMP2), and bone sialoprotein [Kacena et al., 2012].

While the mechanisms have not yet been fully elucidated, growing evidence indicates that MK play important roles in skeletal homeostasis [Kacena et al., 2006b]. Indeed, a number of

models have demonstrated that mice with elevated numbers of MK have increased bone mass [Kacena et al., 2004]. Additionally, it has been reported that co-culturing MK with murine calvarial osteoblastic cells enhances proliferation by 3-6 fold compared to these cells cultured alone [Ciovacco et al., 2010]. Furthermore, it has been shown that MK inhibit OC development through secreted factors *in vitro* [Kacena et al., 2006b], Thus, it is thought that MK enhance bone mass by both inhibiting bone resorption via decreasing osteoclastogenesis and increasing OB proliferation, leading to a net increase in overall bone volume [Kacena et al., 2004].

Of note, the above studies were all completed in murine cells. Stromal cells (referred to here as OB) for the purposes of our work are defined as mesenchymal lineage cells from the bone marrow that are selected for adherent growth and the ability to differentiate into osteoblast-like cells in vitro. Herein, we are expanding our knowledge to understand cell-cell interactions by utilizing stromal cells and MK harvested from both mice and rabbits. Further, we demonstrate the location or the site from which stromal cells are harvested impacts their ability to respond to MK, as stromal cells were harvested from both the bone marrow as well as from dental pulp. Determining whether differences occur between species or sites of harvest will be important for assessing the overall utility of MK-mediated bone formation as a potential therapy for bone loss diseases or bone healing.

Materials and Methods

Osteoblastic cell Isolation and Culture

All protocols for animal use were approved by the Institutional Animal Care and Use Committee at the Indiana University School of Dentistry or the Indiana University School of Medicine. For all studies cells were generated from C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) or from New Zealand White Rabbits (Charles River Laboratories, Wilmington MA). Briefly, to isolate rabbit DPSCs, the oral cavity was washed several times with 70% ethanol, followed with 100% povidone – iodine, 70% ethanol, and then swabbed with 1X DPBS, (Corning Cellgro®, Manassas, VA). Dental pulp tissues were carefully removed and immediately transferred into Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum or FBS (HyClone®, Logan, Utah, USA), 1% penicillin-streptomycin-glutamate (HyClone®, Logan, Utah, USA), and 1 µg/mL amphotericin (Life Technologies, Grand Island, NY, USA). The extracted pulp tissues were washed three times by vortexing and centrifuging at $500 \times g$ for 5 min at room temperature. The pulp was then placed into a fresh tube containing 5mL of a 0.27% enzymatic solution type II collagenase and thermolysin (Worthington, Lakewood, NJ, USA), was incubated in a 37 C° shaking water bath (70 rpm) for 15-20 minutes, and was transferred into a new tube containing 5 mL of fresh enzymes. This process was repeated three times. After each digestion, the extracted enzyme solutions were immediately neutralized with an equal volume of culture medium containing 10% FBS. The digested pulp was then centrifuged at $500 \times g$ for 5 min to yield a cell pellet, which was re-suspended and plated in a 15 cm^2 tissue culture dish.

BMSCs were isolated based on previously described method by Li *el at* [Li et al., 2009].

Briefly, the rabbit tibiae and femora were harvested, transferred into 1X DPBS, and placed on ice. In a tissue culture hood, the epiphyses of the bones were removed, and an 18-gauge needle with a 10 mL syringe was used to flush the marrow cavity with culture medium. The resultant bone marrow suspension was centrifuged and washed with medium twice and re-suspended in fresh culture medium. The cells were divided into two groups, 50% of the bone marrow suspension was reserved for BMSC isolation and 50% was reserved for differentiation with MK. Harvested bone marrow cells were plated in a 15 cm tissue culture dish. Unless otherwise stated, all rabbit stromal cell cultures were maintained at 37°C in a humidified incubator with 5% CO_2, in culture medium comprised of DMEM with10% FBS, 1% penicillin-streptomycin-glutamate, 1 µg/mL amphotericin. The medium was changed every 3 days and passaged by detachment with trypsin when the cultures became approximately 80-90% confluent. Passage 1–3 cells were used for experiments in this study.

Immortalized mouse C57BL/6 BMSC, used in this study, were previously characterized by Alvarez et al. [Alvarez et al., 2012]. Mouse BMSC were cultured in alpha-MEM with10% FBS, 1% penicillin-streptomycin-glutamate, 1 µg/mL amphotericin. The medium was changed every 3 days. The cells were passaged by detachment with trypsin when the cultures became approximately 80-90% confluent.

MK were isolated from the fetal livers of E13-E15 C57BL/6 mice as previously described [Ciovacco et al., 2010; Kacena et al., 2006b]. MK were also isolated from the rabbit bone marrow suspensions. To increase MK expansion and differentiation, single cell suspensions were plated in DMEM medium with 100 ng/mL recombinant human thrombopoietin, (rhTPO) (PeproTech, Rocky Hill, NJ, USA). Cultures were maintained for five days at which time MK were separated and collected from other cells by using a one-step albumin gradient as previously described

[Ciovacco et al., 2010]. Briefly, the bottom layer of the gradient comprised 3 mL of 3% Bovine Serum Albumin Fatty Acid Free (BSA) (Millipore, Kankakee, IL, USA), the middle layer 3 mL of 1.5% BSA solution, and the top layer 3 mL of MK cell suspension. The gravity gradient remained at room temperature for approximately 30-40 minutes at which time the MK were collected from the bottom of the tube and the number of isolated MK was determined.

Cell Proliferative Activity

Cell proliferation was assessed by the metabolic activity of the cells by using an AlamarBlue assay. Briefly, rabbit stromal cells were cultured in 24 well plates at a seeding density of 1.5×10^4 cells/mL. Rabbit stromal cells were cultured either alone (positive control group) or co-cultured with rabbit MK at 1:1 ratio. Additionally, rabbit MK were cultured alone at seeding density of 1.5×10^4 cells/mL to serve as a negative control group. Immortalized mouse BMSC were cultured at 1×10^3 cells/mL following the same design of the rabbit stromal cell groups. At the time of assessment, culture medium was removed and cells were washed three times with 1X PBS, pH 7.4, 37 C°. Next, 10% AlamarBlue (Thermofisher Scientific, Waltham, MA, USA) solution was added to each well (500 µL). Rabbit stromal cells were incubated for 3 hours and mouse BMSC were incubated for 50 minutes. Finally, 150 µl aliquots of AlamarBlue solution were analyzed in triplicate on a microplate reader (Epoch, BioTek, Winooski, VT, USA) for absorbance at wavelengths of 570 and 600 nm.

Alkaline Phosphatase (ALP) Activity

Stromal cells were quantified for ALP activity after 1, 3, 7, and 14 days in culture. At each designated time-point, cells were washed three times with 1X PBS. Subsequent, lysis buffer was prepared in 10 mL double deionized water containing 0.1% triton X (Sigma-Aldrich®, St. Louis, MO, USA), and 0.1% Protease Inhibitor, Protease Inhibitor Cocktail Kit (Thermofisher Scientific, Rockford, IL, USA), and then 300 µL lysis buffer was added to each sample. Samples were collected and frozen at -80˚C. Once all samples were collected, samples were then thawed at room temperature and placed on ice. Lysed cell suspensions were centrifuged at 10,000 rpm for one minute at 4˚C. 10 µL of the supernatant (in triplicate) from each sample was assayed for ALP activity assay (Sensolyte ® pNPP Alkaline Phosphatase Assay kit, Sigma-Aldrich®, St. Louis, MO, USA) and for total protein detection, Pierce BCA Protein Assay Kit (BCA) (Thermo Scientific, Rockford, IL, USA) according to the manufactures' protocols. Finally, ALP activity was normalized to total protein concentration for each sample.

Quantification of Calcium Deposition

Quantitative analysis of Alizarin Red staining was carried out at days 7, 14, and 21. Briefly, stromal cells were washed three times with 1X PBS, pH 7.4. Cells were then fixed with cold 70% ethanol and placed at -20˚C for 1 hour. Plates were warmed to room temperature for an hour. 70% ethanol was removed from samples and washed four times with 1X PBS, pH 7.4. The cells were stained at room temperature with Alizarin Red Staining reagent 40 mM, 4.2 pH (Sigma-Aldrich®, St. Louis, MO, USA), by adding 1 mL/well for 10 minutes, with gentle shaking in a dark room. Alizarin Red staining reagent was removed and the plate was washed with double deionized water

four times, and then washed once more with 1X PBS for 15 minutes. Subsequently, 1X PBS was removed and then 500 µL of 1% Cetylpyridinium Chloride (CPC) (Sigma-Aldrich®, St. Louis, MO, USA) was added for 15 minutes with gentle shaking to extract calcium from each sample. 150 µL from each sample (triplicates) was immediately read at 562 nm wavelength.

Extraction of mRNA and Real-Time PCR

Total RNA was isolated from cultured cells for the analysis of stromal cell proliferation and osteoblastic differentiation markers. At days 7, 14, and 21, cells were vigorously washed three times with 1X PBS, pH 7.4 to remove MK and then treated with 500 µL cell lysis buffer from E.Z.N.A.® Total RNA Kit I (OMEGA bio-teck, Norcross, GA, USA). Cells were transferred into in 1.5 mL Eppendorf microcentrifuge tube, and homogenized using a 26-gauge needle with a 1 mL syringe. RNA was isolated according to the manufacturer's protocol. Isolated RNA was then reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Indianapolis, IN, USA). Quantitative PCR (Applied Biosystems, Foster City, CA) was performed using SYBR Master Mix (Life Technologies, Grand Island, NY, USA) and analyzed for several genes (Table 1). A calibration curve was performed and all oligonucleotides were tested to ensure specificity and sensitivity. For each sample, arbitrary units obtained using the standard curve and the expression of GAPDH was used to normalize the amount of the investigated transcript.

Statistics Analysis

Unless otherwise indicated, all data are expressed as the mean ± SD. Generated data was compared using the Student t-test. Significant differences between each group were determined at $p^* < 0.05$ and $p^{**} < 0.01$. All experiments were repeated between 2 to 6 times using cells generated from different animals at each time point. All assays were completed in triplicate.

Results

Proliferative Activity of Stromal Cells

We have found that co-culture of bone marrow and dental pulp stromal cells with MK increases proliferation. Previously, we have shown that proliferation of primary mouse calvarial OB and BMSC increased up to 6 fold when co-cultured with MK [Cheng et al., 2015; Ciovacco et al., 2010; Ciovacco et al., 2009; Kacena et al., 2004; Melissa A. Kacena, March 2012]. In this study, we sought to further examine the effects of MK on stromal cell proliferation and differentiation by examining cells from two species (rabbit and mouse) and from different locations (bone marrow vs. dental pulp). Throughout we will refer to rabbit bone marrow stromal cells as rBMSC, rabbit dental pulp stromal cells as rDPSC, and mouse immortalized bone marrow stromal cells as mBMSC. Corresponding populations cultured in the presence of MK will be rBMMK, rDPMK, and mBMMK, respectively. MK were generated from bone marrow isolated from rabbit femur and tibia. Figures 1A-C- shows the proliferative activity of stromal cells ±MK (n = 4 /group/study) at days 1, 3, 5, and 7. MK cultured alone are included for comparison. By day 3 and continuing till day 7, we observed increased proliferative activity over time in all rDPMK and mBMMK groups compared to cultures without MK (Figure 1A & 1C). In the rabbit bone marrow cells the rBMMK cells had significantly higher proliferative activity compared to rBMSC at day 7 only. However, a similar trend was observed at day 3 and day 5, but these increases did

not reach statistical significance (Figure 1B). Taken together, in general, MK stimulate proliferation of all tested rabbit and mouse stromal cells.

Alkaline Phosphatase (ALP) Activity of Stromal Cells

Next, we investigated the effects of MK on differentiation by co-culturing MK with stromal cells from rabbits or mice, and examined ALP activity (n = 4/group/study). As illustrated in Figure 2, overall, starting at day 3, stromal cells cultured with MK have significantly reduced ALP activity compared to stromal cells cultured alone. In rabbit bone marrow cells, the rBMMK group had less ALP activity than rBMSC group at day 3, 7, and 10. This difference was lost by day 14 (Figure 2A). Similar results were observed in the rabbit dental pulp cells, however the rDPMK group and rDPSC group were not different at day 3, but were significantly different on days 7, 10 and 14 (Figure 2B). The mouse bone marrow cells experienced the most robust MK-dependent decrease in ALP activity, with mBMMK having lower values than mBMSC at all time points after day 1 (Figure 2C). We did not find any significant differences in ALP activity of stromal cells at day 1 due to co-culture with MK. In summary, we found a strong trend for deceased alkaline phosphatase activity in the presence of MK in all stromal populations tested.

Quantification of Calcium Deposition

Next, we measured calcium deposition, as a marker for differentiation in our rabbit and mouse cultures. The results agree with the ALP data above suggesting MK-induced delay in osteoblastic differentiation. Endpoint measures were made at day 1, 7 and 14 post seeding in rabbit cells, and day 7, 14, and 21 post-seeding in mouse cells. The mouse cells were cultured for a longer time frame based on previously published data demonstrating a longer time to mineral deposition [Alvarez et al., 2012]. Figure 3A shows that rBMMK calcium deposition was lower than rBMSC

at day 7 and 14. Consistent with this, rDPMK cells had lower calcium deposition than rDPSC at these time points as well. Interestingly, calcium deposition was significantly higher in the rDPMK cultures as compared to the rDPSC group at day 1 after seeding cells, suggesting that co-culture with MK reversed this difference (Figure 3B). Much like the rabbit dental pulp cells, mouse bone marrow stromal cells showed a significant increase in calcium deposition at the earliest time point, and lower deposition at the latter 2 time points (Figure 3C) when co-cultured with MK. This increase at the earliest time point, followed decreases for the rDPMK and mBMMK groups remains an unexplained observation and will require more detailed study. Despite this however, the data thus far suggests MK-induced proliferation and delay in differentiation is a conserved phenomenon in mice and rabbits, and is a common feature of bone marrow and dental pulp stromal cells. Additionally, these data are consistent with previous co-culture experiments in which co-culture with MK resulted in a significant reduction in mouse calvarial OB mineralization [Ciovacco et al., 2009].

Effect of Megakaryocytes (MK)on Stromal Cells Gene Expression

To evaluate if MK-induced phenotypic changes are also reflected at the level of gene transcription, we used quantitative real-time PCR to measure levels *c-fos* (known to be positively associated with proliferation) and 6 genes associated with osteoblastic differentiation in our culture system at days 3, 7, and 14. These osteoblast differentiation genes were *Runx2* (Runt-related transcription factor 2), *Alk Phos* (alkaline phosphatase), *Col1A1* (collagen, type I, alpha 1), *ON* (osteonectin, or Sparc), *Osx* (osterix, or sp7), and *Opn* (osteopontin, or Spp1).

As expected based on the observed increased proliferation, *c-Fos* expression was

significantly upregulated in the rBMM and rDPMK groups on day 3 (Figure 4A & 5A). By day 7 of co-culture, c-fos expression was not affected by MK in both rabbit-derived populations (Figure 4B & 5B). By day 14, the rDPMK cells again showed a significant upregulation of c-fos compared to controls (Figure 5C). In mBMMK cultures there was a consistent trend for higher c-fos expression compared to mBMSC at all time points, but this difference never reached statistical significance (Figure 6A-C). This is potentially because these immortalized cells were selected and clonally expanded based on proliferative potential, which may differentially affect gene expression compared to rabbit primary cells [Alvarez et al., 2012]. Consistent with this, we noted a similar expression pattern for *c-fos* in MC3T3-E1 cells, a spontaneously immortalized murine cell line (data not shown).

Runx2 is required to establish the genetic program of osteoblastic differentiation, and surprisingly its expression did not change in rBMMK cells across all time-points analyzed compared to controls (Figure 4A-C). In contrast, rDPMK cells experienced a significant decrease in Runx2 expression compared to rDPSC at all time-points (Figure 5A-C). In mBMMK, average expression of Runx2 was not different at any time point (Figure 6A-C) similar to that seen in the rabbit cells of the bone marrow.

Alp is a secreted enzyme in bone and can be used as an early marker for osteoblastic differentiation. On day 3, rBMMK and rDPMK were not different from rBMSC and rDPSC, respectively (Figures 4 & 5). However, by day 7 in co-culture with MK, *Alp* transcript levels of rDPMK had declined significantly from controls (Figure 5). At day 14, the rBMMK were equivalent to the rBMSC with respect to *Alp* expression, but the differences persisted in the rDPMK group compared to the rDPSC group (Figures 4 & 5). The mBMMK cells had a stable pattern of reduced *Alp* expression at all time-points compared to the mBMSC cultures (Figures 4-

6).

MK did not cause a significant change of type I collagen (Col1A1) expression in rBMMK cultures compared to rBMSC cultures at any time point (Figure 4A-C). In stark contrast, co-culture with MK resulted in a significant reduction in expression in the rDPMK group at all time points (Figures 5A-C). Collagen transcript levels were consistently higher in the mBMMK group compared to the mBMSC group across all time points, though these expression differences failed to reach statistical significance (Figure 6A-C).

Expression of the other OB differentiation associated genes *ON*, *Osx*, and *Opn* were significantly upregulated (or nearly so, p=0.055 for Osx) in rBMMK at day 3 (Figure 4A). This trend reversed by day 7, however, not all differences reached significance. *Osx* was the only gene to show a statistically significant difference (Figure 4B). By day 14, *Osx* and *Opn* were both significantly downregulated, with *ON* borderline at p=0.05 (Figure 4C). In the rDPMK population, these genes were not different from the rDPSC populations at day 3 (Figure 5A), but were all significantly downregulated by day 7 (Figure 5B). These genes all returned to control levels by day 14, except for *Opn* which maintained a downregulated state in rDPMK compared to controls (Figure 6C). Expression of *Osx* and *Opn* in the mouse cells was not significantly different throughout the experimental time frame, except for *ON* which was consistently downregulated by MK in co-culture (Figures 6A-C).

To summarize the gene expression studies, MK-induced changes were cell type and time point dependent. For example, expression of *Alp* tracked very closely with the activity of this enzyme seen in Figure 2. However, *c-fos* expression only tracked closely with the AlamarBlue assay data with 3 days of co-culture. Also, co-culture with MK generally down regulated

expression ~~gene expression~~ of genes associated with osteoblastic differentiation at the later time points analyzed in rabbit cells.

Discussion

Clinical use of mesenchymal lineage cells is an ongoing area of translational research. These cells show promise to help regenerate numerous skeletal tissues including both long bones and craniofacial bones. The source of mesenchymal cells differs by anatomical location which may impact findings. On the other hand, ex vivo expansion of these cells may be required to obtain sufficient cell numbers for utilization, which also requires investigation into their proliferative and maturation profiles ex vivo. In some applications, ex vivo expansion/proliferation will be critical, while simultaneously retaining the ability to differentiate into bone formation OB. Here we examine the ability of MK to increase proliferation while inhibiting differentiation of 3 types of stromal cells generated from two animal species. The latter also improves translational possibility as it demonstrates these properties are not restricted to one animal type and are more likely to be successful in humans.

Previously, we have reported *in vitro* studies that show the influence of MK on bone cells such as mouse calvarial OB and OC [Cheng et al., 2015; Ciovacco et al., 2010; Ciovacco et al., 2009]. To better characterize this influence, here we examined the effect of MK on proliferation and differentiation of stromal cells from rabbit bone marrow and dental pulp, and in an immortalized line of mouse BMSC. To accomplish this we co-cultured the three stromal cell populations in the presence or absence of MK and analyzed proliferation, enzyme activity, mineral deposition, and gene expression. We hypothesized that MK-induced increases in proliferation and decreases in differentiation would be seen in the cell types tested. Similar to our previously

reported co-culture studies, with calvarial OB, we found that MK increased cell proliferative activity in rabbit BMSC and DPSC as well as mouse BMSC (Figure 1).

Although the mechanism by which MK enhance cellular proliferative activity has not been completely described, we have previously demonstrated that direct cell-cell contact is required for this increase [Ciovacco et al., 2009; Miao et al., 2004], and that this mechanism is partially mediated by integrin β1 signaling [Melissa A. Kacena, 2006]. Of note our previous studies demonstrated that MK increase the progression of primary mouse OB through the G1/S checkpoint of the cell cycle [Cheng et al., 2015]. Consistent with this, our data shows an upregulation of c-fos, an immediate early transcription factor associated with proliferation [Pai and Bird, 1994], by MK at day 3 in all three stromal cell cultures (Figure 4). Thus, we now show that stromal cells generated from different anatomical locations, two different species, and even an immortalized cell line (which was established in part based on its proliferative capacity) are all impacted by MK stimulation.

Consistent with previous reports [Alge et al., 2010] rabbit BMSC have a reduced proliferation and differentiation capacity compared to rabbit DPSC. For example, the ALP activity values of rabbit DPSC was consistently higher compared to rabbit BMSC at days 10 and 14 in Figures 2A and 2B. Although, the molecular mechanisms behind the differential response between the two anatomical sites is not clear, it may be due to their unique roles, dental pulp stromal cells being responsible for the production of dentin, whereas bone marrow stromal cells are responsible for the production of bone. That said, future investigations into these differences might yield a novel response pathway present in dental pulp cells not seen in bone marrow cells, which could be targets for therapeutic intervention.

In general, cells proliferation and differentiation have an inverse relationship [Ruijtenberg

and van den Heuvel, 2016]. Since proliferation indices were markedly increased by MK, another goal of this study was to examine the effects of MK on stromal cell differentiation. Specifically, to investigate the ability of MK to increase or to decrease differentiation of stromal cells. Thus, we employed two functional assays of differentiation: ALP activity (an essential early marker for OB differentiation) and calcium deposition (demonstrating a late stage of OB differentiation, a measure of mineralization). Generally, we found that co-culture with MK decreased ALP activity (and mRNA expression of ALP) in all cell types examined (Figures 2, 4, 5, and 6). Similarly, MK decreased calcium deposition in the later time-points, again in all cell types (Figure 3). Ciovacco et al showed similar results when the authors cultured mouse calvarial OB with MK [Ciovacco et al., 2009]. However, here we observed at very early time-points that calcium deposition was significantly higher for stromal cells cultured with MK. Thus our data appears to suggest that MK induced proliferation occurs at the expense of differentiation which is consistent with general observations that cellular proliferation often occurs at the cost of differentiation [Ruijtenberg and van den Heuvel, 2016], and is a conserved mechanism.

Next, we examined mRNA expression of genes associated with OB differentiation (Figures 4-6). As mentioned above and would be expected based on ALP activity assays, on the whole, ALP mRNA expression was reduced in all 3 stromal populations co-cultured with MK. On days 7 and 14 mRNA expression levels of Col1, ON, and OPN were reduced in both rabbit BMSC and DPSC cultures containing MK as compared to those cultured without MK (Figures 5 and 6). This trend, however, was not observed in the immortalized mouse BMSC cultures, especially for Col1, which is the opposite of the rabbit findings reported here and our previous reports using primary mouse calvarial OB cultures [Ciovacco et al., 2009]. A possible explanation for this apparent discrepancy may be the fact that the mouse BMSC used in this study are an immortalized cell line

that was genetically modified to proliferate while maintaining its ability to differentiate into multiple cell types. Thus, the effects of immortalizing these cells may be responsible for their differential collagen expression.

In conclusion, we have shown that MK play a critical role in stromal cells proliferation and differentiation. Our results suggest that long term (21-day) co-culture with MK enhance proliferation and inhibit differentiation of stromal cells into OBs. Importantly, we show this effect is seen in 2 species and in cells from 2 distinct anatomical locations. Although the role of integrins, pyk2, Mdm2, and several other signaling proteins have been described as being involved in this process, much remains to be understood [Cheng et al., 2015; Kacena et al., 2012; Kacena et al., 2006a; Kacena et al., 2004]. That said, a potentially important observation from this work is that co-culture with MK increases proliferation of stromal cells with OB differentiation potential. This observation may have important implications for ex-vivo expansion of mesenchymal lineage cells which maintain their osteogenic potential. Additionally, previous studies have demonstrated increased numbers of MK *in vivo*, leads to increased bone mass – apparently at odds with the *in vitro* observation that MK decrease osteoblast differentiation. An interesting potential explanation for this could be that *in vivo* direct cell contact with MK lead to increased proliferation of osteoblast precursors. As this pool expands and cell contact with MK are severed, these cells are able to continue down the osteoblast differentiation pathway. The observation that MK co-culture did not change *Runx2* in rBMMK or mBMMK cells is consistent with this explanation. While much work is needed to further identify the specific mechanisms that control MK-mediated stromal cell proliferation and differentiation, these studies provide justification for continuing to explore these mechanisms.

Acknowledgments

Figure Legends

Figure 1. Proliferative activity of stromal cells co-cultured with or without MK. (A) Rabbit MK, rBMSC, and rBMMK; (B) Rabbit MK, rDPSC, and rDPMK; and (C) Mouse MK, mBMSC, and mBMMK. Proliferative activity was assessed on days 1, 3, 5, and 7. Error bars reflect the standard deviation of the mean. Significant differences between groups are indicated by the error bars: *$p<0.05$ and **$p<0.01$.

Figure 2. ALP activity of rabbit and mouse stromal cells cultured with or without MKs. (A) rBMSC cultured with or without MK; (B) rDPSC cultured with or without MK; and (C) mBMSC cultured with or without MK. ALP activity was assessed at five different time-points (days, 1, 3, 7, 10, and 14). Enzymatic ALP activity was calculated per min and normalized per mg of protein. Error bars reflect the standard deviation of the mean. Significant differences between groups are indicated by the error bars: *$p<0.05$ and **$p<0.01$.

Figure 3. Calcium deposition in stromal cells co-cultured with or without MKs. (A) rBMSC cultured with or without MK; (B) rDPSC cultured with or without MK; and (C) mBMSC cultured with or without MK. Rabbit bone marrow stromal cells were assessed on days 1, 7, and 14 while mouse stromal cells where assessed on days 7, 17, and 21. Error bars reflect the standard deviation of the mean. Significant differences between groups are indicated by the error bars: *$p<0.05$ and **$p<0.01$.

Figure 4. mRNA expression of several genes known to be important regulators of OB proliferation and differentiation in rBMSC cultured with or without MK. Cultures were examined on days 3 (A), 7 (B), or 14 (C). Error bars reflect the standard deviation of the mean. Significant differences between groups are indicated by the error bars: *$p<0.05$ and **$p<0.01$.

Figure 5. mRNA expression of several genes known to be important regulators of OB proliferation and differentiation in rDPSC cultured with or without MK. Cultures were examined on days 3 (A), 7 (B), or 14 (C). Error bars reflect the standard deviation of the mean. Significant differences between groups are indicated by the error bars: *$p<0.05$ and **$p<0.01$.

Figure 6. mRNA expression of several genes known to be important regulators of OB proliferation and differentiation in mBMSC cultured with or without MK. Cultures were examined on days 3 (A), 7 (B), or 14 (C). Error bars reflect the standard deviation of the mean. Significant differences between groups are indicated by the error bars: *$p<0.05$ and **$p<0.01$.

References

Alge DL, Zhou D, Adams LL, Wyss BK, Shadday MD, Woods EJ, Gabriel Chu TM, Goebel WS. 2010. Donor-matched comparison of dental pulp stem cells and bone marrow-derived mesenchymal stem cells in a rat model. J Tissue Eng Regen Med 4:73-81.

Alvarez MB, Childress P, Philip BK, Gerard-O'Riley R, Hanlon M, Herbert BS, Robling AG, Pavalko FM, Bidwell JP. 2012. Immortalization and characterization of osteoblast cell lines generated from wild-type and Nmp4-null mouse bone marrow stromal cells using murine telomerase reverse transcriptase (mTERT). J Cell Physiol 227:1873-82.

Amano H, Hackett NR, Rafii S, Crystal RG. 2005. Thrombopoietin gene transfer-mediated enhancement of angiogenic responses to acute ischemia. Circ Res 97:337-45.

Caplan AI. 1991. Mesenchymal stem cells. J Orthop Res 9:641-50.

Caplan AI. 2005. Review: mesenchymal stem cells: cell-based reconstructive therapy in orthopedics. Tissue Eng 11:1198-211.

Caplan AI, Bruder SP. 2001. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. Trends Mol Med 7:259-64.

Chan RJ, Yoder MC. 2004. The multiple facets of hematopoietic stem cells. Curr Neurovasc Res 1:197-206.

Cheng YH, Streicher DA, Waning DL, Chitteti BR, Gerard-O'Riley R, Horowitz MC, Bidwell JP, Pavalko FM, Srour EF, Mayo LD, Kacena MA. 2015. Signaling pathways involved in megakaryocyte-mediated proliferation of osteoblast lineage cells. J Cell Physiol 230:578-86.

Ciovacco WA, Cheng YH, Horowitz MC, Kacena MA. 2010. Immature and mature megakaryocytes enhance osteoblast proliferation and inhibit osteoclast formation. J Cell Biochem 109:774-81.

Ciovacco WA, Goldberg CG, Taylor AF, Lemieux JM, Horowitz MC, Donahue HJ, Kacena MA. 2009. The role of gap junctions in megakaryocyte-mediated osteoblast proliferation and differentiation. Bone 44:80-6.

Emmakah AM, Arman H, Bragg J, Greene T, Alvarez M, Childress P, Goebel WS, Kacena M, Lin CC, Chu TM. 2017. A Fast-Degrading Thiol-Acrylate based Hydrogel for Cranial Regeneration. Biomed Mater.

Friedenstein AJ, Gorskaja JF, Kulagina NN. 1976. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. Exp Hematol 4:267-74.

Geddis AE. 2010. Megakaryopoiesis. Semin Hematol 47:212-9.

Kacena MA, Eleniste PP, Cheng YH, Huang S, Shivanna M, Meijome TE, Mayo LD, Bruzzaniti A. 2012. Megakaryocytes regulate expression of Pyk2 isoforms and caspase-mediated cleavage of actin in osteoblasts. J Biol Chem 287:17257-68.

Kacena MA, Gundberg CM, Horowitz MC. 2006a. A reciprocal regulatory interaction between megakaryocytes, bone cells, and hematopoietic stem cells. Bone 39:978-84.

Kacena MA, Nelson T, Clough ME, Lee SK, Lorenzo JA, Gundberg CM, Horowitz MC. 2006b. Megakaryocyte-mediated inhibition of osteoclast development. Bone 39:991-9.

Kacena MA, Shivdasani RA, Wilson K, Xi Y, Troiano N, Nazarian A, Gundberg CM, Bouxsein ML, Lorenzo JA, Horowitz MC. 2004. Megakaryocyte-osteoblast interaction revealed in mice deficient in transcription factors GATA-1 and NF-E2. J Bone Miner Res 19:652-60.

Li Y, Chen S, Yuan J, Yang Y, Li J, Ma J, Wu X, Freund M, Pollok K, Hanenberg H, Goebel WS, Yang FC. 2009. Mesenchymal stem/progenitor cells promote the reconstitution of exogenous hematopoietic stem cells in Fancg-/- mice in vivo. Blood 113:2342-51.

Mauney JR, Volloch V, Kaplan DL. 2005. Role of adult mesenchymal stem cells in bone tissue engineering applications: current status and future prospects. Tissue Eng 11:787-802.

Melissa A. Kacena PPE, Ying-Hua Cheng, Su Huang, Mahesh Shivanna, Tomas E. Meijome, Lindsey D. Mayo, and Angela Bruzzaniti. March 2012. Megakaryocytes Regulate Expression of Pyk2 Isoforms and Caspase-mediated Cleavage of Actin in Osteoblasts. J Biol Chem 287(21):17257-17268.

Melissa A. Kacena TN, Mary E. Clough, Sun-Kyeong Lee, Joseph A. Lorenzo, Caren M. Gundberg, Mark C. Horowitz. 2006. Megakaryocyte-mediated inhibition of osteoclast development. BONE 39:991-999.

Miao D, Murant S, Scutt N, Genever P, Scutt A. 2004. Megakaryocyte-bone marrow stromal cell aggregates demonstrate increased colony formation and alkaline phosphatase expression in vitro. Tissue Eng 10:807-17.

Morrison SJ, Uchida N, Weissman IL. 1995. The biology of hematopoietic stem cells. Annual review of cell and developmental biology 11:35-71.

Pai SR, Bird RC. 1994. c-fos expression is required during all phases of the cell cycle during exponential cell proliferation. Anticancer Res 14:985-94.

Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. 1999. Multilineage Potential of Adult Human Mesenchymal Stem Cells. Science 284:143-147.

Raisz LG, Kream BE. 1983. Regulation of bone formation. N Engl J Med 309:29-35.

Ruijtenberg S, van den Heuvel S. 2016. Coordinating cell proliferation and differentiation: Antagonism between cell cycle regulators and cell type-specific gene expression. Cell Cycle 15:196-212.

Turner CH. 2002. Biomechanics of bone: determinants of skeletal fragility and bone quality. Osteoporos Int 13:97-104.

Table 1. Mouse and rabbit primers used for RT-PCR

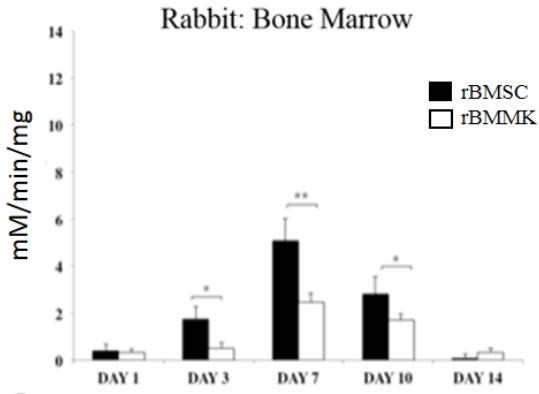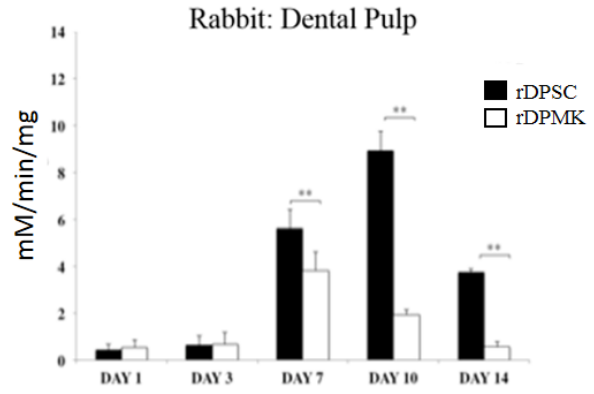| Gene	Species	Forward	Reverse
ALPL	Mouse	5'-GCTGATCATTCCCACGTTTT-3'	5'-CTGGGCCTGGTAGTTGTTGT-3'
	Rabbit	5'-AACCCTTCACTGCCATCCTG-3'	5'-GTTGTGAGCGTAGTCCACCA-3'
c-FOS	Mouse	5'-ACTTCTTGTTTCCGGC-3'	5'-AGCTTCAGGGTAGGTG-3'
	Rabbit	5'-GTAGACTAGGGAGGACGCGA-3'	5'-ACACACTCCATGCGTTTTGC-3'
RUNX2	Mouse	5'-CGACAGTCCCAACTTCTT-3'	5'-CGGTAACCACAGTCCCAT-3'
	Rabbit	5'-GTAGAGCCACACACATGGATAA-3'	5'-CCCTGATCTACTGCTTGGTAAG-3'
COL1a1	Mouse	5'-ACGTCCTGGTGAAGTTGGTC'	5'-CAGGGAAGCCTCTTTCTCCT-3'
	Rabbit	5'-CATAAAGGGTCACCGTGGCT-3'	5'-GCCGTTGAGTCCATCTTTGC-3'
SPARC	Mouse	5'-ATCCAGAGCTGTGGCACACA-3'	5'-GGAAAGAAACGCCCGAAGA-3'
	Rabbit	5'-TGGATGGTTTGTTGTTCTGC-3'	5'-AGGTGGTGATGGGGTTACAA-3'
SP7	Mouse	5'-CCCTTCTCAAGCACCAATGG-3'	5'-AGGGTGGGTAGTCATTTGCATAG-3'
	Rabbit	5'-TTCGGATGAGCTGGAACGTC-3'	5'-TTTGCTCAGGTGGTCACTCC-3'
SPP1	Mouse	5'-ACTCCAATCGTCCCTACAGTCG-3'	5'-TGAGGTCCTCATCTGTGGCAT-3'
	Rabbit	5'-AACAAGAGACCCTCCCGAGT-3'	5'-TCGGCATCGTCGGATTCATT-3'
GAPDH	Mouse	5'-CGTGGGGCTGCCCAGAACAT-3'	5'-TCTCCAGGCGGCACGTCAGA-3'
	Rabbit	5'-AGTATGATTCCACCCACGGC-3'	5'-GATGGCCTTCCCGTTGATGA-3'

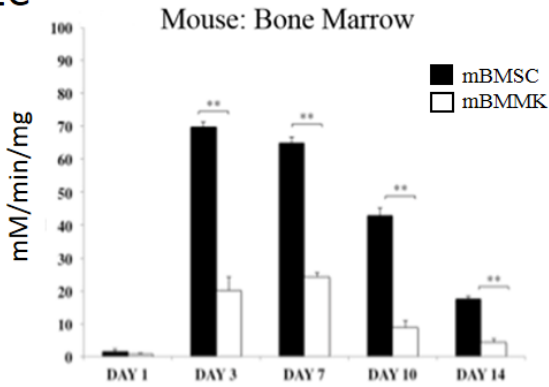# Cell Proliferation
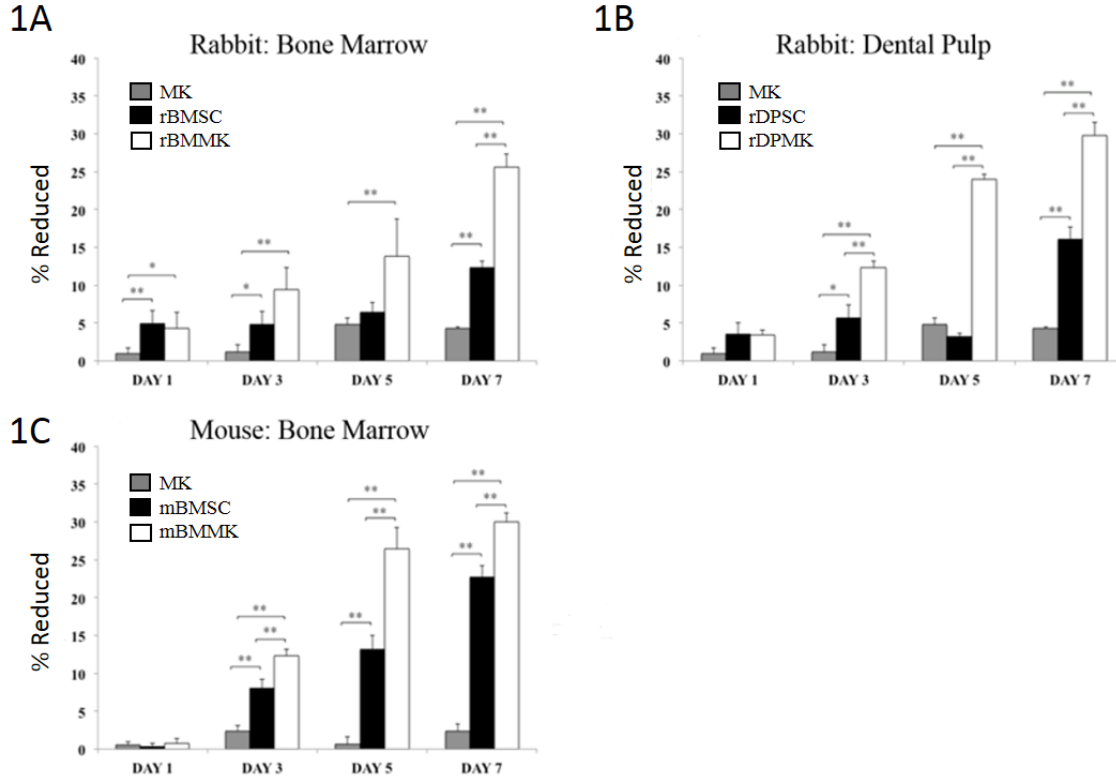
Figure 1

Alkaline Phosphatase Activity

2A

Rabbit: Bone Marrow

■ rBMSC
☐ rBMMK

2B

Rabbit: Dental Pulp

■ rDPSC
☐ rDPMK

2C

Mouse: Bone Marrow

■ mBMSC
☐ mBMMK

Figure 2

Mineralization

3A

Rabbit: Bone Marrow

■ rBMSC
□ rBMMK

3B

Rabbit: Dental Pulp

■ rDPSC
□ rDPMK

3C

Mouse: Bone Marrow

■ mBMSC
□ mBMMK
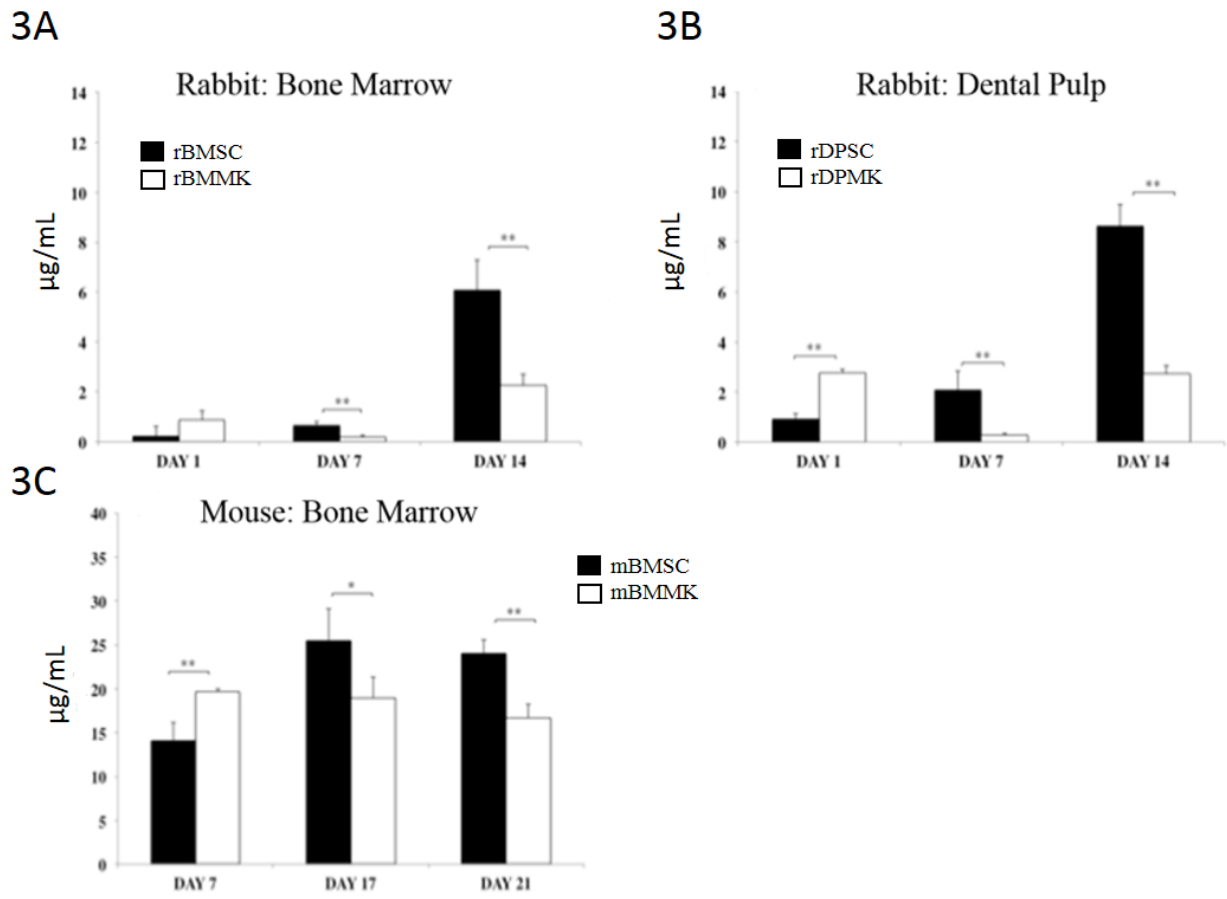
Figure 3

Rabbit: Bone Marrow
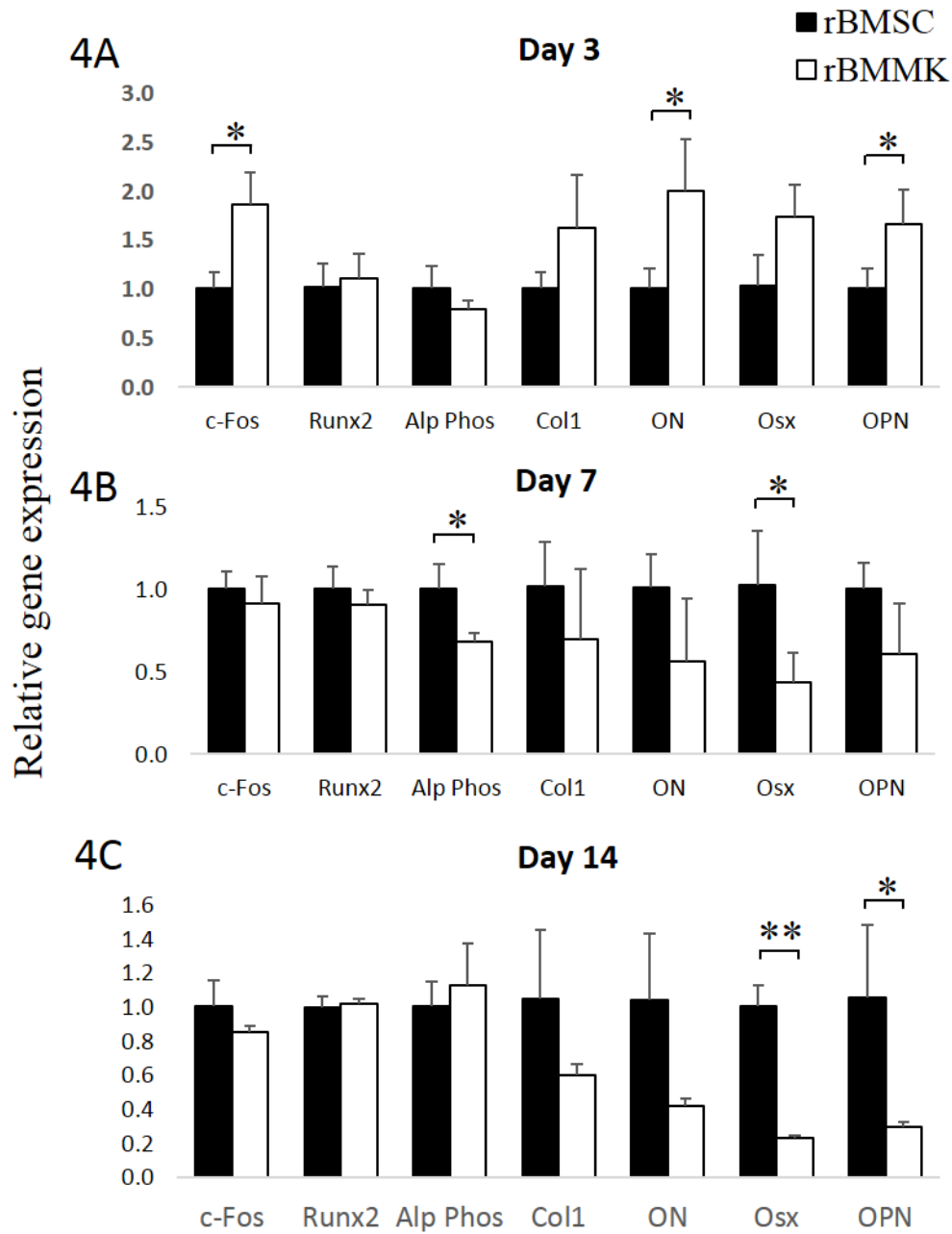
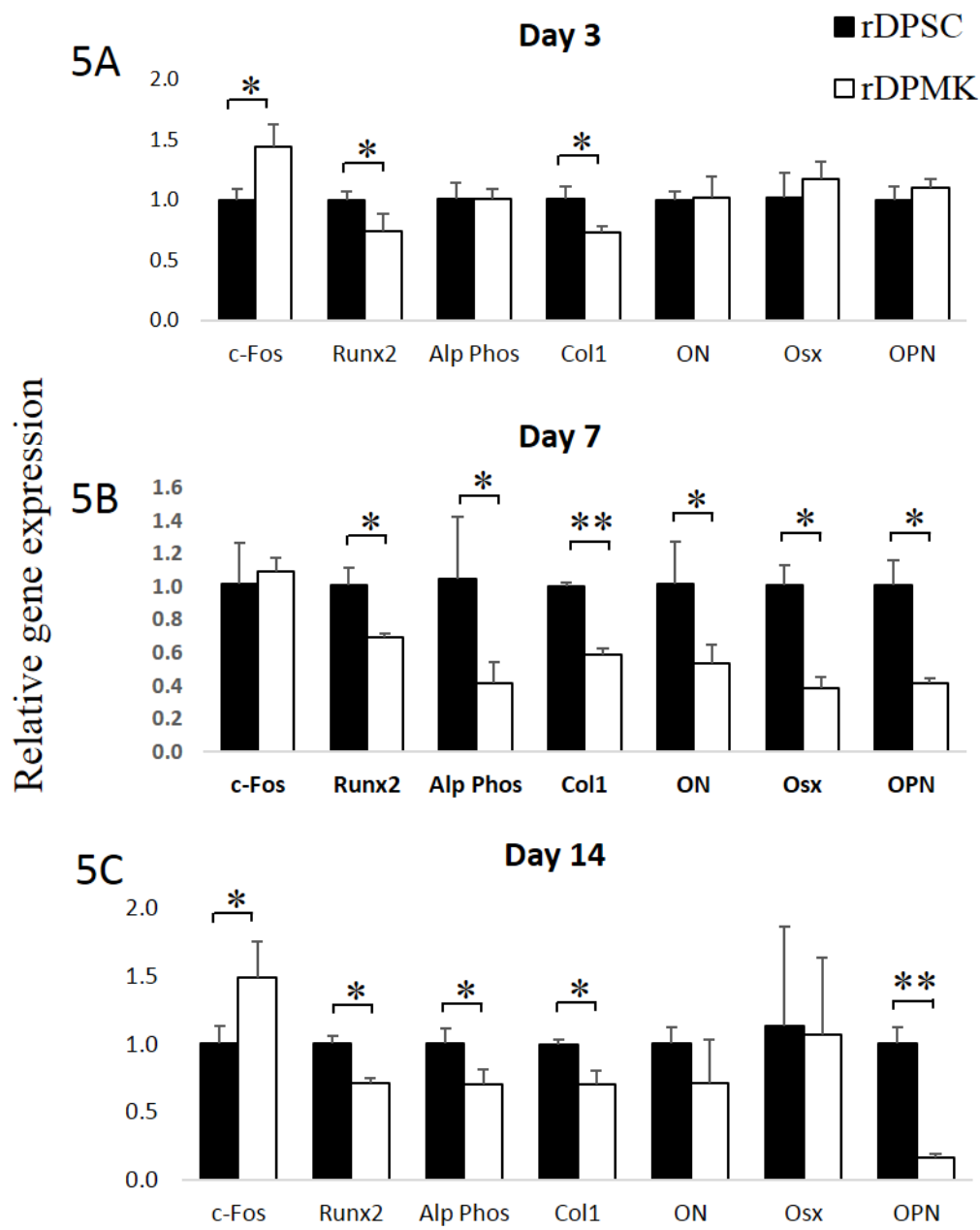Figure 4

Rabbit: Dental Pulp

Day 3

5A

■ rDPSC
□ rDPMK

Day 7

5B

Day 14

5C

Figure 5

Mouse: Bone Marrow

6A

6B

6C

Figure 6