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Megakaryoctye-Bone Cell Interactions: The Role of Gap Junctions, Maturation, and Longevity

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MEGAKARYOCYTE-BONE CELL INTERACTIONS: THE ROLE OF GAP JUNCTIONS, MATURATION, AND LONGEVITY. Wendy A. Ciovacco, Carolyn G. Goldberg, Amanda F. Taylor, Justin M. Lemieux, Henry J. Donahue, Ying-Hua Cheng, Mark C. Horowitz, and Melissa A. Kacena. Department of Orthopaedic Surgery, Indiana University School of Medicine, Indianapolis, IN.

Research shows that megakaryocytes (MKs) can enhance bone volume by increasing osteoblast (OB) proliferation and inhibiting osteoclast (OC) formation. This cumulative work first explores the role of gap junction intercellular communication (GJIC) in MK-OB interactions, and secondly examines the effect of MK maturation state and MK number on skeletal homeostasis. In both studies, cell lineages were cultured as described below. In the gap junction (GJ) study, we used real-time PCR to test for MK expression of connexin 43 (Cx43), the predominant GJ protein found in bone cells. A dual-label parachute assay and FACS analysis assessed GJIC between MKs and OBs. Proliferation and differentiation assays of OBs cultured with and without MKs were performed. Here we demonstrate that: 1) MKs express Cx43, 2) MKs can functionally communicate with OBs via GJIC, 3) the addition of two distinct GJ uncouplers inhibits this communication, 4) inhibiting MK-mediated GJIC further enhances the ability of MKs to stimulate OB proliferation and, 5) blocking GJIC does not result in MK-induced reduction of OB differentiation. In the second study, increasing numbers of MKs were co-cultured with bone cells to see if increased MK number correlated with increased OB proliferation and decreased OC formation. In addition, MKs were separated using flow cytometry into 3 subpopulations based on maturation and effects on OB proliferation and OC formation were assessed. Finally, longevity studies on wild-type and mutant MKs were also conducted. In the second study we show that: 1) increased MK number corresponds with increased OB proliferation and decreased OC formation, 2) MK maturation stage does not alter the effect of MKs on bone cell lineages beyond the megakaryoblast stage, and, 3) GATA-1 deficient MKs survive longer than wild-type controls. Thus we demonstrate a novel interaction between two cell lineages only recently shown to be functionally connected, and make steps towards understanding how MKs exert their osteogenic effects.

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1. **INTRODUCTION**

While cells of the hematopoietic and mesenchymal lineage both originate in the bone marrow (BM) and are thus intimately related in space, only in the last few decades have functional links between the two lineages been sought. Hematopoietic and mesenchymal cells replicate in juxtaposition to each other, suggesting that local secretion of growth factors or cytokines by either lineage, or juxtacrine communication by cell contact, may have a direct or indirect effect on neighboring cells. In fact research now shows that these two systems originally studied in isolation are in fact functionally connected, with the skeletal system not just housing hematopoietic progenitors, but interacting with these cells in a variety of ways.

MKs are platelet progenitor cells which primarily reside in the BM, and their reciprocal relationship with bone cells is one of the burgeoning areas of research just described. Multiple studies have now provided ample in vivo and in vitro evidence showing that MKs affect the development and differentiation of both OCs and OBs. This cumulative work presents two independent studies that further the understanding of the MK-bone cell interaction. The first study explores GJIC between MKs and OBs, while the second looks at the effect of MK-maturation stage and increasing MK number on the ability of the lineage to affect skeletal homeostasis. The Methods, Results, and Discussion section are thus divided into two sections labeled GJIC, and MK Maturation and Longevity.

This Introduction begins with a general background discussion of MK-bone cell interactions, and then provides a brief, focused opening for both studies.

1. A. Background Information:

This opening review summarizes the current research on MK-bone cell interactions. It begins by describing several mouse models with dysregulated megakaryopoiesis and resultant skeletal pathology, then looks at MK-OC interactions in vitro, and finally at the in vitro effect of MKs on OBs.

1.A.1. In Vivo Evidence: Mouse Models

There are currently four known mouse models with dysregulated megakaryopoiesis and resultant significant increases in bone volume. Mice that overexpress thrombopoietin (TPO), the main MK growth factor, have an approximate 4-fold increase in BM MK number, and develop a concurrent osteosclerotic bone phenotype with increased bone mineral density (BMD) (1-5). Mice with a deficiency in the transcription factors GATA-1 or NF-E2, necessary for normal MK differentiation, develop marked increases in MK number with a concomitant reduction of platelet number, and a tremendous increase in trabecular bone (6-8). Most recently a novel mouse model of platelet-type von Willenbrand disease (Pt-vWD), with a platelet phenotype identical to the human form of the disease, showed a marked increase in splenic MK with splenomegaly, and a high bone mass phenotype with decreased serum measures of bone resorption (9).

1.A.1.a. TPO Overexpressing Mice

Given that TPO is the major MK growth factor, and is essential for lineage proliferation and differentiation, it is expected that a mouse model overexpressing TPO show dysregulated MK reproduction and maturation. Perhaps most surprising is the dramatic phenotype manifested in these mice, as a myelofibrotic syndrome with osteosclerosis develops by nine months of age in mice repeatedly injected with TPO or infected with a viral vector harboring the TPO gene (1-5).

Mice overexpressing TPO show a marked 4-fold increase in absolute MK number versus wild-type controls affecting all stages of differentiation (1, 2). Additionally, levels of transforming growth factor-β (TGF-β) and platelet-derived growth factor (PDGF) are elevated 2-fold and 5-fold respectively versus controls (1-5). TGF-β and PDGF are both growth factors expressed by MK with proven marked effects on bone cells (10, 11). This observed increase in MK expression of two growth factors, previously identified to stimulate OBs, raised the possibility that in this model MK secretion of TGF-β and PDGF resulted in the myelofibrotic,

osteosclerotic phenotype seen in TPO overexpressing mice. This hypothesis was supported by studies illustrating that in the absence of TGF-β, TPO overexpressing mice failed to exhibit the characteristic myelofibrotic, osteosclerotic syndrome. Irradiated, wild-type mice were engrafted with TGF-β-1-/- BM stem cells infected with a retrovirus encoding murine TPO protein to induce TPO overexpression, but no myelofibrosis or osteosclerosis developed. Furthermore, when irradiated, wild-type mice were repopulated with wild-type TPO overexpressing stem cells, the femurs now showed significant myelofibrosis and osteosclerosis 16-weeks post-transplantation (12), clear evidence that the MK secreted growth factor TFG-β plays a critical role in the final bone phenotype of TPO overexpressing mouse models.

Another study using transgenic mice constitutively expressing TPO showed predictably elevated numbers of MKs, with associated significant increases in plasma levels of both TGF-β-1 and osteoprotegerin (OPG) (13). OPG inhibits osteoclastogenesis and is expressed by MKs (14-19). This study implies that along with TGF-β, increased secretion of OPG by MKs contributes to the myelofibrotic, osteosclerotic phenotype of TPO overexpressing mice. Similarly, a study by Chagraoui et al. (20) implicates the upregulation of OPG and associated inhibition of osteoclastogenesis in the pathogenesis of osteosclerosis. Here, irradiated wild-type or OPG -/- mice were repopulated with either wild-type or OPG -/- BM stem cells infected with a retrovirus encoding murine TPO protein. While all mice showed subsequent increases in TGFβ-1 with associated myelofibrosis, only the wild-type recipients (engrafted with wild-type or OPG -/- stem cells) showed increased OPG plasma levels with associated osteosclerosis. As opposed to this bone dense phenotype, the OPG -/- recipients (engrafted with wild-type or OPG -/- stem cells) instead developed an osteroporotic phenotype (20). These results suggest that the OPG secreted by the transplanted BM stromal cells and OB caused the osteosclerosis seen in the wild-type hosts.

Yet another investigation studied the direct effects of TPO on OC formation in vitro and demonstrated a TPO dose-dependent decrease in OC number (21). This effect was most likely mediated by increased MK number in response to increased TPO stimulation. Thus it appears that TPO plays an indirect role in bone turnover by its proliferative effect on MK.

In summation, TPO overexpressing mice exhibit marked increases in MK number with simultaneous increases in BMD mediated by various MK secreted cytokines, specifically TFG-β and OPG.

1.A.1.b. GATA-1 and NF-E2 Deficient Mice

Pluripotential hematopoietic stem cells give rise to MKs through a stepwise differentiation process, with progression through each phase mediated by specific transcription factors, ultimately resulting in terminally differentiated MKs capable of releasing platelets. See Figure 1 below. The selective loss of any of the transcription factors regulating MK differentiation results in arrested development and accumulation of cells at the latest stage of maturation. Specifically, the loss of either GATA-1 or NF-E2 transcription factors produces dysregulated megakaryopoiesis, with GATA-1 knock-down and NF-E2 knock-out mice both displaying marked megakaryocytosis and a paradoxical thrombocytopenia (6, 7).

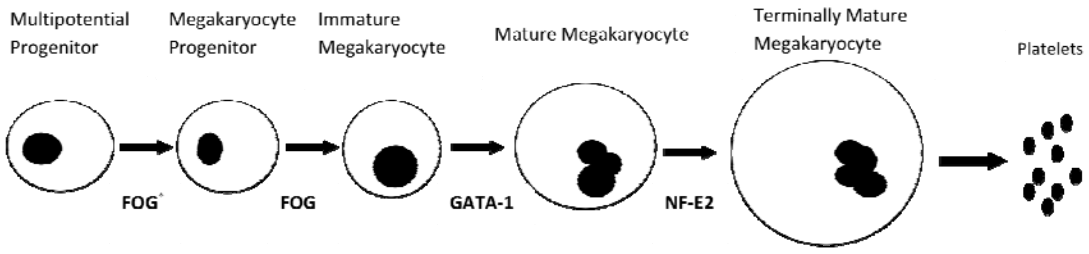

Figure 1: Stages of MK development and required transcription factors

GATA-1 transcription factor is one of the six members of the GATA family of zinc-finger transcription factors (GATA-1 through GATA-6 in vertebrates), with DNA binding activity in the C-terminal zinc finger of the single polypeptide chain. GATA-1, previously thought to be necessary exclusively for erythroid lineage development, is now known to play a critical role in MK differentiation. Its expression is restricted to the hematopoietic lineage almost entirely, with known expression by multi-potential hematopoietic progenitors, mast cells, and MKs (22). In GATA-1 knock-down models, MK number in the BM and spleen increases approximately 10-fold, while the peripheral platelet count is decreased to 15% of wild-type controls (23). We have demonstrated that GATA-1 deficient mice have a higher bone mass than controls, displaying more than a 3-fold increase in bone volume and bone formation detectable after approximately 4 months of age (8). At ages over a year, these animals ultimately develop a myelofibrotic phenotype (24). MKs from GATA-1 deficient mice are also less differentiated than wild-type MKs. Compared to normal controls, these MKs are morphologically smaller, they show evidence of retarded nuclear and cytoplasmic develoment, and GATA-1 deficient MKs express reduced levels of mRNA encoding markers of cellular maturity (23).

NF-E2 transcription factor is a heterodimeric nuclear protein comprised of two polypeptide chains, both belonging to the basic leucine zipper family of transcription factors. The p18 subunit is ubiquitously expressed (25, 26), while the expression of hematopoietic-specific 45-kDa subunit is restricted to erythroid precursors, MKs, mast cells, and multipotential progenitors, similar to GATA-1. Like the GATA-1 knock-down model, MK number in the BM and spleen of adult mice lacking p45 NF-E2 is increased, though not as profoundly (2-5 fold), and a severe thrombocytopenia develops due to maturational arrest of MK development, with essentially no detectable platelets in the peripheral blood (less than 5% of control levels) (6). Interestingly, we have shown that NF-E2 deficient mice also develop a high bone mass phenotype, with up to a 5-fold increase in bone volume and bone formation parameters (8, 27). NF-E2 deficient mice respond to exogenous TPO with marked proliferation, but there is no detectable increase in

platelet production in vivo; and although MK number is markedly elevated, TPO levels are normal in NF-E2 deficient mice (6, 28).

Mice deficient in GATA-1 and NF-E2 transcription factors necessary for proper MK differentiation have strikingly similar phenotypes characterized by marked megakaryocytosis, thrombocytopenia, and significantly increased BMD.

1.A.1.c Murine Model of Pt-vWD

Suva et al. (9) recently developed a mouse model of Pt-vWD by creation of a transgenic cassette containing the human Pt-vWD point mutation (G233V) capable of expression in a murine colony. This gain of function mutation affects the GP-Ibα subunit of the platelet glycoprotein Ib-IX receptor complex which normally binds von Willebrand factor and promotes platelet adhesion during vascular damage. A limited number of mutations such as G233V are known that alter the glycoprotein receptor complex configuration while still allowing interaction with soluble von Willebrand factor, and this is the pathogenesis behind Pt-vWD (9).

The Pt-vWD mutation causes a phenotype in mice that mirrors the human disorder, with platelet dysfunction and resultant impaired hemostasis. The G233V mutant mice have a modest thrombocytopenia, with platelet counts reduced by 20%, and significantly increased bleeding time versus wild-type mice expressing the normal human GP-Ibα subunit. Mutant mice were also found to have spleens that were 2.5 times as large as wild-type controls, leading investigators to histological evaluation and discovery of a tissue-specific megakaryocytosis with marked increase in splenic MK number. The observation of MK dense spleens prompted an evaluation of the mouse BM to determine if MK dysregulation was affecting skeletal homeostasis. While there was no increase in the number of MKs in the BM of the mutant mice, histological examination revealed a high bone mass phenotype detectable as early as 2 months, and bone mass

progressively increased with age. Mutant bones also exhibited increased biomechanical strength versus wild-type controls (9).

Further BM analysis revealed a decrease in OC number, with no matched decrease in OBs, suggesting the high bone mass phenotype was attributable to decreased OC number and resultant decreased bone breakdown. Additionally, ex vivo cultures further demonstrated that BM cultures from the Pt-vDW mice had a significant decrease in the number of tartrate resistant acid phosphatase (TRAP)-positive OC-like cells versus wild-type controls (TRAP is a biomarker of OC number). Ex vivo cultures failed to show a difference in OB number or differentiation versus wild-type controls (9).

The investigators attempted to identify expression of the GP Ib-IX receptor complex on OC to determine if the Pt-vWD mutation was having a direct effect on OC proliferation, but transcript profiling and immunofluoresence failed to find GP-Ib-IX expression by OCs. This supports prior evidence that the receptor complex is expressed exclusively on platelets as an MK lineage specific gene product (9).

The Pt-vWD murine model displays a phenotype similar to the human disease it was designed to mimic. In addition to disrupted hemostasis, these mice have increases in splenic MK number, increased BMD, and a decreased number of OCs. Although the exact mechanism by which this tissue-specific megakaryocytosis contributes to decreased OC population and decreased osteoclastogenesis remains to be elucidated, this study further confirms the specific links between MK function, platelet development, and OC proliferation and differentiation (9).

Taken collectively, the four mouse models detailed above illustrate the complex role of MK in regulating skeletal mass, and show that disruptions in various points of MK differentiation and development consistently lead to an osteosclerotic phenotype.

1.A.2. MK-OC Interactions

MKs have the ability to directly and indirectly affect osteoclastogenesis, as evidenced by recent studies. The direct effects of MKs on OC differentiation are complex because MKs express proteins that both enhance and inhibit osteoclastogenesis. RANKL is critical for OC development, and while it is thought that OBs and OB precursors account for the majority of RANKL found within the BM, MKs have also been shown to express RANKL (14, 19, 29, 30). However MKs can also inhibit osteoclastogenesis as studies have demonstrated that MK express or secrete several factors known to downregulate OC terminal differentiation, specifically OPG (an antagonist of RANK signaling), interleukin (IL)-10, IL-13, TGF-β and granulocyte/macrophage colony-stimulating factor (GM-CSF) (11, 14-18, 31-34). Thus, MKs have the potential to significantly affect OC number through their expression of factors that both promote and retard osteoclastogenesis. Of importance, our group as well as others, have demonstrated that when OC progenitors are cultured with MKs or in MK-conditioned medium, in vitro OC development is significantly inhibited by up to 10-fold (21, 31, 35). We also demonstrated that OPG expression alone is not responsible for this inhibition, as MKs derived from OPG deficient mice also inhibit OC formation in vitro (31). As a result, we are currently working to isolate and identify the MK-secreted OC inhibitory factor using biochemical separation techniques including HPLC. We have identified a single fraction with strong inhibitory activity containing less than 30 proteins. Interestingly, none of the major factors known to inhibit osteoclastogenesis, namely OPG, IL-4, IL-10, IL-12, IL-13, IL-18, interferon gamma (IFN-γ), TGFβ, GM-CSF, OC inhibitory lectin (OCIL), calcitonin, amylin, and calcitonin gene-related peptide, are present in the isolated fraction (31). Therefore while direct, in vitro evidence of MK-induced inhibition of OC development exists, the factor(s) or mechanism responsible remains unknown.

MK can also indirectly influence osteoclastogenesis. MKs increase OB and fibroblast proliferation by direct cell-to-cell contact (8, 36-40). OBs and fibroblasts are cells with known effects on osteoclastogenesis, including the expression of RANKL and OPG (41-48). In addition to increasing OB proliferation, MKs increase OB expression of OPG when co-cultured (14). As described previously, OPG inhibits osteoclastogenesis, so a potential indirect path for MK inhibition of OC formation exists.

In conclusion most direct and indirect evidence suggests that MK act to inhibit the differentiation of cells of the OC lineage. MK express numerous proteins known to inhibit OC formation, co-culturing MK with OC progenitors results in significant inhibition of OC formation, and MK indirectly increase OPG secretion by both OBs and fibroblasts. However, under certain physiological circumstances MK expression of RANKL may be an important stimulator of osteoclastogenesis, particularly during inflammatory responses such as rheumatoid arthritis (49).

1.A.3. MK-OB Interactions

Studies have shown that MKs affect OB development by the secretion of bone matrix proteins and growth factors, and by directly increasing OB proliferation.

MKs or their platelet products secrete multiple bone matrix proteins, namely: osteocalcin, osteonectin, bone sialoprotein, and osteopontin (50-54). MKs also secrete multiple growth factors crucial for bone remodeling, including: TGFβ-1, PDGF, VEGF, and bone morphogenetic protein (BMP)-2, -4, and -6 (10, 11, 55). Therefore MKs could impact bone formation and bone remodeling, especially in the setting of elevated local concentrations.

Our in vitro evidence demonstrates that MKs enhance OB proliferation 3-6 fold by a direct cell-to-cell contact mechanism (8). Additionally, co-cultures of MKs with BM stromal cells also enhances osteoblastogenesis, again by a mechanism requiring direct cell-to-cell contact

(36). Although these data demonstrate that MKs mediate OB proliferation by a juxtacrine signaling mechanism, the exact method remains to be identified.

1.B. GJIC and MK Maturation and Longevity

As the above review shows, it is now imminently clear that the hematopoietic and bone lineages are connected not just by proximity, but by functionality, and that MK induced effects on OB and OC are multiple and complex. Several mouse models demonstrate that alterations and increases in cells of the MK lineage can lead to increases in bone volume in vivo; and, in vitro data shows a primarily inhibitory effect of MKs on OCs, and substantial pleiotropic effects on OBs. However, the exact mechanisms of inhibition and induction need to be further elucidated.

1.B.1. GJIC

Our previous studies showed that not only does MK conditioned medium fail to enhance OB proliferation (8), but that when separated from OBs by a 0.1μm membrane in a transwell co-culture system, MKs again do not increase OB proliferation (8) . These data suggest that MK secreted factors such as cytokines or growth factors are not responsible for the induction in OB proliferation, but rather MKs stimulate OB proliferation through some sort of direct cell-cell contact, or juxtacrine, mechanism. There are 3 basic juxtacrine mechanisms: 1) a protein on one cell binds its receptor on another cell, 2) a receptor on one cell binds to its ligand on the extracellular matrix secreted by another cell and, 3) cytoplasm from one cell is directly to the cytoplasm of a second cell. GJIC is an example of the latter mechanism. Because GJIC can also be blocked with 0.4 μm membranes (unpublished observation, H.J.D. and A.F.T.) and because Cx43, the predominant GJ protein expressed by bone cells, has been immunolocalized in MKs (56), GJIC could also be responsible for the MK-induced enhancement in OB proliferation. Therefore, in this study we wanted to determine whether MKs are capable of communicating with

OBs through GJs, and whether MK-mediated GJIC is responsible for the MK-induced enhancement in OB proliferation.

1.B.2. MK Maturation and Longevity

As detailed above, there are several well-studied mouse models with dysregulated megakaryopoiesis and resultant high bone mass phenotypes. Until now, most theories attempting to explain the high bone mass phenotype in the aforementioned mouse models focused on the increase in MK number, with the logical assumption that if MKs favor bone deposition, more MKs equates to a higher bone mass. Here we test this hypothesis, but also probe further and examine MKs cultured from GATA-1 deficient mice to see if there is an inherent quality about the mutant cell lineage itself, such as stage of maturation or increased viability, that favors net bone formation more than wild-type MKs.

2. STATEMENT OF PURPOSE

Hypothesis: 1) That MKs can communicate with OBs by gap junction intercellular communication and that this communication influences OB proliferation; 2) the state of MK maturation affects bone homeostasis, with immature MKs favoring bone deposition more than well-differentiated states; 3) GATA-1 deficient MKs live longer than wild-type counterparts, contributing to increased bone deposition

Aims:

1) Determine if MKs express Cx43
2) Determine if MKs and OBs communicate by GJIC
3) Determine the effect of MK-mediated GJIC on OBs
4) Determine if MKs sorted by maturation state have different effects on the OB and OC lineages
5) Determine if increasing MK number in co-culture with bone cells further increases OB proliferation and or decreases OC development
6) Determine if there is a difference in longevity between wild-type MKs and GATA-1 deficient MKs

3. METHODS

Please note that an * next to the name of a procedure indicates that this was not performed by the student.

3.A. GJIC

3.A.1. Preparation of Fetal Liver Derived MKs:

Murine MKs were prepared as previously described (8, 31). In brief, fetuses were dissected from pregnant mice at E13-15. The livers were removed and single cell suspensions made by forcing cells thru sequentially smaller gauge needles (18G, 20G, 23G). Cells were washed 2x with DMEM + 10% FCS and then seeded (5 fetal livers/100 mm dish) in 100 ml culture dishes, in DMEM + 10% FCS + 1% murine TPO (5). After 3-5 days, when the cells become confluent, MK were obtained by separating them from the lymphocytes and other cells using a one-step albumin gradient to obtain a 95% pure MK population (57). The bottom layer was 3% albumin in PBS (Bovine Albumin, protease free, fatty acid poor, Serologicals Proteins Inc., Kankakee, IL), the middle layer was 1.5% albumin in PBS, and the top layer was media containing the cells to be separated. All of the cells sedimented through the layers at 1g for approximately 40 minutes at room temperature. The MK fraction was collected from the bottom of the tube.

3.A.2. Preparation of neonatal calvarial cells (OB):

Murine calvarial cells were prepared as previously described (58,59). Briefly, calvaria from mice less than 48 hours old were pre-treated with EDTA in PBS for 30 min. The calvaria were then subjected to sequential collagenase digestions. Cells were collected following incubation in collagenase. Fractions 3-5 were used as the starting population. These cells were > 95% OB or OB precursors by a variety of criteria (58, 60, 61). Freshly prepared OBs were used for all studies.

3.A.3. MEG-01 Cell line:

MEG-01 cells (ATCC, Rockville, MD) are a human megakaryoblastic cell line which was established from the BM of a patient with chronic myelogenous leukemia. MEG-01 cells express the MK marker platelet glycoprotein (GP) IIB/IIIa on their cell surface and possess no markers for B or T lymphocytes or for myeloid cells (62). MEG-01 cells were grown in RPMI 1640 supplemented with 10% FCS. MEG-01 cells were collected from maintenance culture, washed, and used in the parachute assay as described below (MEG-01 cells as well as primary MKs were utilized).

3.A.4. RNA Extraction and Real-Time PCR:

Cells were washed 2-4 times with PBS prior to RNA isolation. RNA was isolated from the cells using trizol (Invitrogen Corporation, Carlsbad, CA) or a NucleoSpin II RNA Purification kit (BD Biosciences, San Jose, CA) incorporating an on-column DNase treatment to remove contaminating genomic DNA. For real-time PCR, cDNA was prepared from 5μg of total RNA using Sprint PowerScript Reverse Transcriptase (BD Biosciences) and oligo$(dT)_{12-18}$ primers. The cDNA was purified using an Amicon YM30 filter device (Millipore, Danvers, MA). Alternatively, cDNA was prepared from 2μg of total RNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA). Quantitative real-time PCR was performed on a Cepheid Smart Cycler or on a 7500 Fast Real-Time PCR System (Applied Biosystems) using Platinum Taq polymerase (Invitrogen Corporation) and Sybr Green I (Invitrogen Corporation) incorporation or using the Power Sybr Green PCR Master Mix (Applied Biosystems), respectively.

The quantitative comparison between samples was calculated using comparative C_T. The data are normalized by subtracting the difference of the threshold cycles (C_T) between the gene of interest (e.g. Cx43) and the C_T of the housekeeping gene glyceraldehyde 3-phosphate

dehydrogenase (GAPDH). This value is defined as the C_T. To compare the relative expression of Cx43 in two types of cells (e.g. primary C57BL/6 MKs vs. primary C57BL/6 OBs) the C_T must be calculated. C_T for this example is the absolute value of the difference between the C_T for the MK and the OB or C_T MK - C_T OB. The relative difference (fold increase or decrease) in expression is calculated as 2^{-CT}. The following primer sequences were used:

Cx43 forward primer:	5' CCTTTGACTTCAGCCTCCAA
Cx43 reverse primer:	5' CATGTCTGGGCACCTCTCTT
Osteocalcin forward primer:	5' AAGCAGGAGGGCAATAAGGT
Osteocalcin reverse primer:	5' TTTGTAGGCGGTCTTCAAGC
Alkaline phosphatase forward primer:	5' GCTGATCATTCCCACGTTTT
Alkaline phosphatase reverse primer:	5' CTGGGCCTGGTAGTTGTTGT
Type I collagen forward primer:	5' CAGGGAAGCCTCTTTCTCCT
Type I collagen reverse primer:	5' ACGTCCTGGTGAAGTTGGTC
GAPDH forward primer:	5' CGTGGGGCTGCCCAGAACAT
GAPDH reverse primer:	5' TCTCCAGGCGGCACGTCAGA

3.A.5. Dual-Label Parachute Technique and FACS Analysis:

To assess GJIC in co-cultures containing MKs (or MEG-01 cells) and OBs, we utilized a dual-label parachute technique which has previously been described (63). Our protocol was adapted from Ziambaras et al (64). In brief, donor cells (MKs) were simultaneously labeled with 10 μM calcein-AM (Invitrogen Corporation) and 10 μM 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen Corporation) for 25 minutes at 37C. Cells were washed once in HBSS and once in PBS prior to dropping onto receiving cells (OBs were ~90-95% confluent). Cultures were incubated for 75 minutes at 37C. It should be noted that the number of donor cells (MKs) was titrated in initial experiments from 2,000-200,000 cells/well. This titration demonstrated that 20,000 cells/well was in the dynamic range and was

used for subsequent parachute assays.

Prior to conducting FACS studies, the parachute assay was examined microscopically to confirm transfer of dye from MKs to OBs. Visual inspection confirmed the viability of both the donor and receptor cells and demonstrated that the dye-transfer was specific. The double labeling of the MKs with DiI is a secondary confirmation that the donor cells are viable and that dye is not permeating from the cells. As a result of these initial demonstrations (data not shown) we then proceeded to conduct FACS analysis.

For FACS analysis studies, cells were trypsinized to release OBs (during washes most of the MKs were removed, remaining MKs were gated out) and cells were fixed in 4% paraformaldehyde, resuspended in FACS buffer, and FACS analyzed on a Facstar Plus Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ). Because the calcein is able to permeate gap junctions, any OBs which fluoresce green have had calcein transferred to them through GJs. Because DiI can not permeate, cells which fluoresce red are the originally labeled donor cells (MKs). In all FACS studies strict threshold controls were employed, according to the method previously published (65), to insure that only specific dye transfer was detected. First, although MKs are nonadherent and were washed from the OBs prior to FACS, as a precaution we did have a donor control (positive control) containing double labeled MKs. We set the thresholds so that any DiI (red) labeled cells were excluded. Second, OBs cultured alone served as our negative control to which we applied a second threshold to exclude 99.5% of unlabeled OBs. Cells that fell in to the gate set between these 2 thresholds were deemed to be calcein positive OBs that had communicated with MKs.

In some experiments, cells were treated with increasing concentrations of 18α-glycyrrhetinic acid (αGA, Sigma, St. Louis, MO) or oleamide (Sigma) to assess the ability of a GJ uncoupler to inhibit MK-OB GJIC. Here, αGA or oleamide was added to the receiving OBs 30 minutes prior to dropping donor cells (MKs also pretreated with inhibitors) into cultures.

3.A.6 Proliferation Analysis:

To examine the proliferative capacity of OBs (2500 OBs/well, optimal, pretested) cultured with and without MKs (5000 MKs/well, optimal, pretested) that were exposed to αGA (50 μM, optimal, pretested) or vehicle control (DMSO), cells were seeded in triplicate into 96-well tissue culture plates and incubated for up to 6 days at 37C in α-MEM supplemented with 2% FCS. In all of the proliferation studies, as in the parachute assay studies, both MKs and OBs were pre-incubated with inhibitors (or vehicle control) for 30-60 minutes prior to seeding. As has been described by Davidson et al (66), our preliminary studies demonstrated that the inhibitory response of αGA was reduced in 10% FCS as compared to 2% FCS (data not shown); therefore, for these studies 2% FCS was utilized. Proliferation was measured every 2-3 days by the incorporation of ^{3}H-thymidine (1 μCi/well; 5-8 Ci/mmol) added during the last 16 hours of culture (67). To assess OB proliferation alone, MK were removed from wells (4 washes) prior to measuring incorporation of ^{3}H-thymidine (8).

3.A.7. Differentiation Studies *:

To examine the differentiation of OBs (20,000 OBs/ml, optimal, pretested) cultured with and without MKs (25,000 MKs/ml, optimal, pretested) that were exposed to αGA (40-50 μM, optimal, pretested) or vehicle control (DMSO), cells were seeded in triplicate into either 6-well or 24-well tissue culture plates and incubated for up to 14 days at 37C in α-MEM supplemented with 2% FCS and 50 μg/ml ascorbic acid on day 0. It should be noted that cells were only fed only 1X/week (day 7) and at this time point fresh MKs were added as non-adherent MKs were removed with feeding. On the day 7 feed, fresh ascorbic acid and αGA/vehicle were added to cultures and they were additionally supplemented with 5mM β-glycerophosphate to induce mineralization. Differentiation experiments ended on day 14 when mineralized nodules were evident in control OB cultures.

To assess the effects of MKs on OB differentiation, gene expression levels of alkaline phosphatase, type I collagen, and osteocalcin were measured in OBs using real-time PCR. In addition, functional assays for alkaline phosphatase enzyme activity and mineralization were also performed. With regard to real-time PCR, studies were conducted as outline above with the following alterations. Cultures were washed with PBS 4x to remove MKs and these real-time PCR experiments were performed using the Applied Biosystems platform.

3.A.8. Alkaline Phosphatase Activity*:

Alkaline phosphatase activity was determined by the colorimetric conversion of *p*-nitrophenol phosphate to *p*-nitrophenol (Sigma) and normalized to total protein (BCA, Pierce, Rockford, IL) (68). Briefly, cells were washed 2x with PBS, subsequently lysed with 0.1% (vol/vol) Triton X-100 supplemented with a cocktail of broad-range protease inhibitors (Pierce), subjected to two freeze-thaw cycles, and cleared via centrifugation. Lysates were incubated with 3 mg/ml *p*-nitrophenol phosphate in an alkaline buffer (pH 8.0) (Sigma) for 30 min at 37°C, the reaction was stopped by the addition of 20 mM NaOH and read at 405 nM (GENios Plus, Tecan, San Jose, CA). The enzymatic activity of alkaline phosphatase was determined by comparison with known *p*-nitrophenol standards (Sigma).

3.A.9. Quantative Analysis of Calcium Deposition *:

Calcium deposition was assessed by eluting Alizarin Red S from cell monolayers as previously described by Stanford et al (69). Briefly, monolayers were washed 2x with PBS, subsequently fixed in ice cold 70% (v/v) ethanol for 1 hr, and then washed 2x with water. Monolayers were stained with 40mM Alizarin Red S (pH 4.2) for 10 min (room temperature, shaking), unbound alizarin red was removed by washing with water (5x) and with PBS (1x for 15 minutes, room temperature, shaking). Bound Alizarin Red was eluted by incubating monolayers with 1% (v/v) cetylpyridinium chloride in 10mM sodium phosphate (pH 7.0) for 15 min (room

temperature, shaking). From aliquots, absorbance was measured at 562 nm (GENios Plus, Tecan), and Alizarin Red concentrations were calculated from measured standards (Ca/mol of dye in solution).

3.A.10. Statistical Analysis:

Two-way factorial analyses of variances were used to compare groups, with MKs and αGA being the independent variables. In the event of a significant interaction, pair-wise Bonferroni comparisons were made to explore individual group differences while controlling for the elevated family-wise error associated with performing multiple comparisons. All analyses were performed with the Statistical Package for Social Sciences (SPSS 6.1.1; Norusis/SPSS Inc., Chicago, IL) software and were two tailed with a level of significance set at 0.05.

3.B. MK Maturation and Longevity

3.B.1.Mice:

For these studies GATA-1 deficient and C57BL/6 mice were used. Generation and breeding of mutant mice with selective loss of GATA-1 was described previously (7, 70). In brief, a DNAse I-hypersensitive region (HS) was identified upstream of the GATA-1 promoter and was subsequently knocked-out by insertion of a neomycin-resistant cassette. This resulted in mice with reduced levels of GATA-1 mRNA and protein (3-5 fold reduction in protein), a functional knock-down (7, 70) GATA-1 deficient mice are maintained on the C57BL/6 background.

3.B.2. Preparation of Fetal Liver Derived MKs:

Murine MKs were prepared as previously described in the above section (8, 31).

3.B.3. Preparation of MK CM

To generate MK CM, $1X10^6$ MKs/ml were cultured in α-media containing no serum for 3 days. After 3 days CM was collected, sterile filtered, and stored at -80° C until use. For the studies here CM was used at 3%, 10%, and 30% (vol:vol).

3.B.4. Preparation of neonatal calvarial cells (OB):

Murine calvarial cells were prepared as previously described in the above section. (58-61). Freshly prepared OBs were used for all studies.

3.B.5. Proliferation Analysis:

The proliferative capacity of OBs was examined as previously described in the above section (66, 67, 8)

3.B.6. In Vitro OC-like Cell Formation Models *:

OC-like cells were generated by three previously described methods (71-74). First, co-cultures containing $2x10^6$ BM cells/ml and 20,000 primary calvarial OB/ml were grown in α-MEM supplemented with 10% FCS and 10^{-8} M $1,25(OH)_2D_3$. The media was changed every other day for 6-8 days. Second, $2x10^6$ BM cells/ml were cultured in α-MEM supplemented with 10% FCS and 30 ng/ml of recombinant murine M-CSF (Research Diagnostics Inc., Flanders, NJ) and 50 ng/ml of recombinant human RANKL (Research Diagnostics Inc.). Media was changed every third day for 6-9 days (until OC were visible). Third, a new OC generation model, using a Pax5-/- spleen cell line (SCL) as the source of OC precursors, was used as has been previously described (73). In brief, the Pax5-/- SCL is highly enriched in OC precursors and when cultured with M-CSF and RANKL, OC develop in a shorter time (3-4 days) than C57BL/6 BM cell cultures (6-8 days). As detailed by Horowitz et al. (73) the Pax5-/- SCL is 97% $CD11b^+$ (Mac-1),

96% CD16/32$^+$ (FcγR), and >90% CD115$^+$ (c-fms) as demonstrated by FACS analysis. In addition, the Pax5-/- SCL does not express CD19, CD45R (B220), CD117 (c-kit), Ly-6A/E, Ly-6C, Ly-6G (Gr-1), NK1.1, or TCRαβ (TCR). These data indicate that the Pax5-/- SCL expresses a monocyte/macrophage-like phenotype with no T or B cells present (73). For experiments using the Pax5-/- SCL, 100,000 cells/ml were cultured with M-CSF and RANKL (as above) and if necessary the media was changed on the third day. Cells were usually fixed at day 4 or 5, stained, and counted as described below.

3.B.7. Flow Cytometry *:

Fetal liver cells were removed from dishes prior to BSA gradient separation as described above and washed with PBS containing 2% FCS. Staining was performed in PBS with 2% serum. Anti-CD41, CD61, CD49b, and CD49d, were purchased from PharMingen. Anti- CD42d was purchased from RDI. Light scatter and fluorescence of individual cells was measured by a Facstar Plus flow cytometer, and cells were sorted base on their antigen expression as described here. Cells were sorted into 3 separate subpopulations (in order of increasing maturity): megakaryoblast, MK immature, and MK mature.

Megakaryoblasts are CD61$^+$ CD41$^-$cells (75, 76). Anti-mouse CD41 and CD61 antibodies were obtained from PharMingen.

Immature MKs are CD41$^+$ CD49d$^+$ cell (77). Anti-mouse CD49d were also obtained from PharMingen.

Mature MKs are CD41$^+$ CD49b$^+$ (77) or CD41$^+$ CD42d$^+$ (77) cells. Anti-mouse CD49b and CD42d antibodies were purchased from PharMingen and Research Diagnostics, Inc (RDI, Flanders, NJ), respectively.

These 3 subpopulations of cells were then analyzed for their ability to induce OB proliferation or inhibit OC formation as described above.

3.B.8. Longevity Studies *:

C57BL/6 and GATA-1 deficient MKs (40,000 cells/ml) were cultured in the presence or absence of TPO (100 ng/ml) and with or without OBs (20,000 cells/ml). In other words, there were 4 groups examined for each type of MK: 1) MK alone; 2) MK+TPO; 3) MK+OB; and 4) MK+OB+TPO. Cells were cultured in 24-well culture plates and the media used in these studies was α-MEM supplemented with 10% FCS. As MKs can be removed with normal feedings, media was replenished 1/week (if counts were performed, feeding followed counting). Specifically, for the first feeding, an additional 1 ml of medium was added to cultures. On the second feeding 1 ml of medium was carefully removed from the top on the well using a pipet and an additional 1 ml of fresh medium was added to the cultures. All subsequent feedings were done as described for the second feeding. Fresh TPO was added to the cultures indicated (100 ng). Number of viable MKs present in these cultures was recorded.

3.B.9. Statistics

Unless otherwise stated, all data are presented as the Mean ± 1 SD. Student's t-test was used to determine significant differences, with $p<0.05$ (Systat 6.0 for Microsoft Windows, SPSS Inc., Chicago). Experiments are always repeated, in some cases multiple times. Within individual experiments, data points are based on a minimum of triplicate samples.

4. RESULTS

4.A. GJIC

4.A.1. MKs Express Cx43:

To determine whether MKs express Cx43, real-time PCR analysis was completed. As described above, the comparative C_T method was used to compare Cx43 expression in different types of cells. GAPDH mRNA served as an internal control for the Cx43 mRNA. mRNA was extracted separately from 3 different cultures of fetal liver derived MKs and primary calvarial OBs (both from C57BL/6 mice), and the real-time PCR was performed in duplicate. The results shown below in Figure 2 revealed the presence of mRNA for Cx43 in C57BL/6 MKs, although the expression of Cx43 in the MKs was less than that observed in C57BL/6 OB specimens.

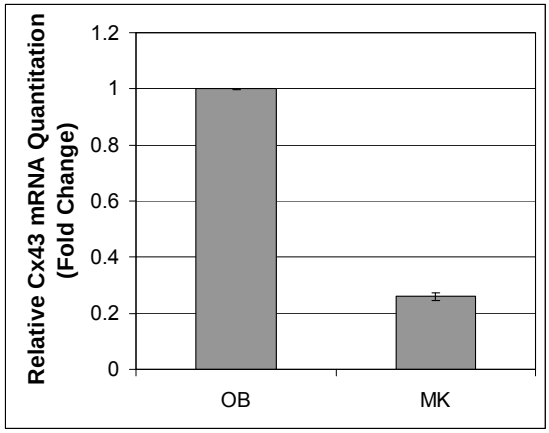

Figure 2. Real- time quantitative PCR analyses show the expression of Cx43 in C57BL/6 MK and OB (n=3). Results are reported as fold change or relative quantitation of target mRNA expression (2^{-Ct} method), normalized to an endogenous control (GAPDH) and relative to a calibrator (OB RNA sample). Error bars represent the standard error of the mean associated with the ΔΔCt value.

4.A.2. MKs Form Functional GJs which can be used to Communicate to OBs:

To determine whether MKs communicate with OBs via GJIC, MKs and OBs were set-up in the "parachute assay", and FACS analysis was performed. FACS analysis was used to show the transfer of calcein to OBs (Figure 3A), demonstrating that MK-OB GJIC occurs. It should be noted that these experiments were also conducted by replacing MKs with the MEG-01 cell line

and similar data were obtained (data not shown).

4.A.3. αGA and Oleamide Significantly Inhibit MK-OB GJIC:

Because our data showed that MK-OB GJIC occurs, we wanted to confirm that MK-OB GJIC was specific. This was accomplished by using two GJ uncoupling agents, αGA and oleamide (66, 78). αGA or oleamide were titrated into OB cultures (added to OBs 30 minutes prior to dropping MKs onto OBs) and then the cultures were analyzed by FACS analysis as above. Figure 3B shows that GJIC remained unchanged with 10 μM of αGA. However, with 20 - 50 μM of αGA, MK-OB GJIC was reduced (Figures 3C-3E), and with 100 μM of αGA, significant inhibition of MK-OB GJIC was observed (Figure 3F). We also performed these experiments with MEG-01 cells replacing MKs and similar results were observed (data not shown). In studies where we utilized a different GJ uncoupler we found that both 75 and 90 μM of oleamide resulted in a significant reduction in GJIC, 25% and 30% respectively (data not shown).

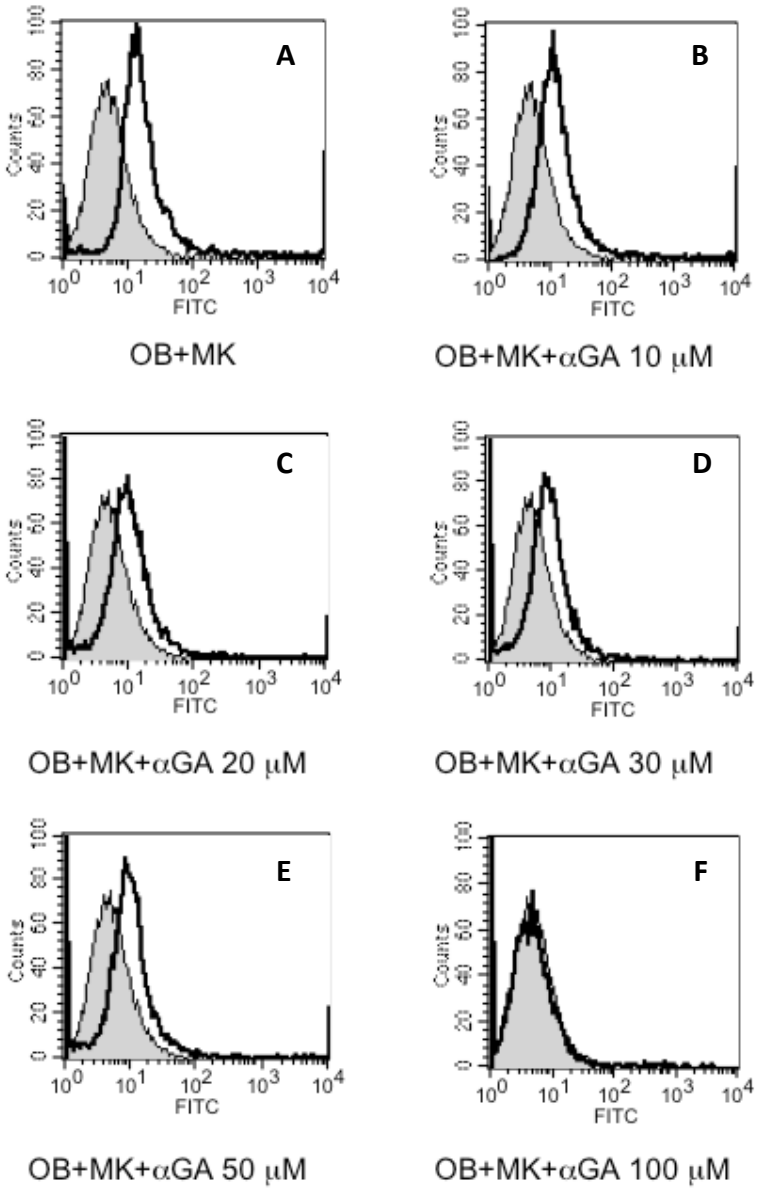

Figure 3. Histograms from FACS analysis of OBs after dual-labeled MKs were dropped onto OBs in parachute assay and GJ were allowed to form. 3A shows the percentage of OBs in which calcein-Am was transferred from MKs. 3B-2F shows the percentage of OBs in which calcein-AM was transferred from MKs when cultures were treated with various concentrations of αGA (10-100 μM). The filled profile is that of the negative control samples. The open profile is that of the experimental cells. In the experimental cell population there are many cells which do not uptake dye (overlap with the negative control population) and those cells shifted to the right of the negative control population are the cells which have taken up the dye.

4.A.4. Inhibiting MK-OB GJIC Enhances OB Proliferation In Vitro:

Next we wanted to determine whether MK-mediated GJIC affected OB proliferation. OBs were cultured alone, or were co-cultured with MK and grown for 3 days (similar data were obtained when cultures were grown for 4, 5, or 6 days, data not shown). To examine whether GJIC altered OB proliferation, some wells were treated with 50 μM of αGA (OBs alone and OBs co-cultured with MKs). N=9 for each of the 4 groups. As we have previously reported and as is shown below in Figure 4, addition of MKs to OB cultures significantly enhances OB proliferation by greater than 50% over a 3 day period of time (8). Next, we show that OB proliferation was significantly reduced when OBs were cultured with αGA in the absence of MKs. There was approximately a 50% reduction in OB proliferation when OB were cultured with αGA. This suggests that OBs communicate with each other via GJIC to increase their own proliferation, and that αGA inhibits this communication, leading to a reduction in proliferation. Importantly we show that when GJIC between MKs and OBs is inhibited by treating co-cultures with αGA, OB proliferation is enhanced even further than when MKs alone are added to OBs (compared to the appropriate control, OB treated with αGA, there was approximately a 250% increase in OB proliferation). Although MK-mediated proliferation was increased only a modest 14% in cultures containing αGA as compared to those without αGA, the difference was found to be significant ($p=0.048$). These data suggest that under normal conditions GJIC between MKs and OBs decreases the degree by which MKs enhance OB proliferation, and that blocking GJIC with an inhibitor such as αGA allows MKs to exert signals for proliferation unopposed by the inhibitory GJ signals.

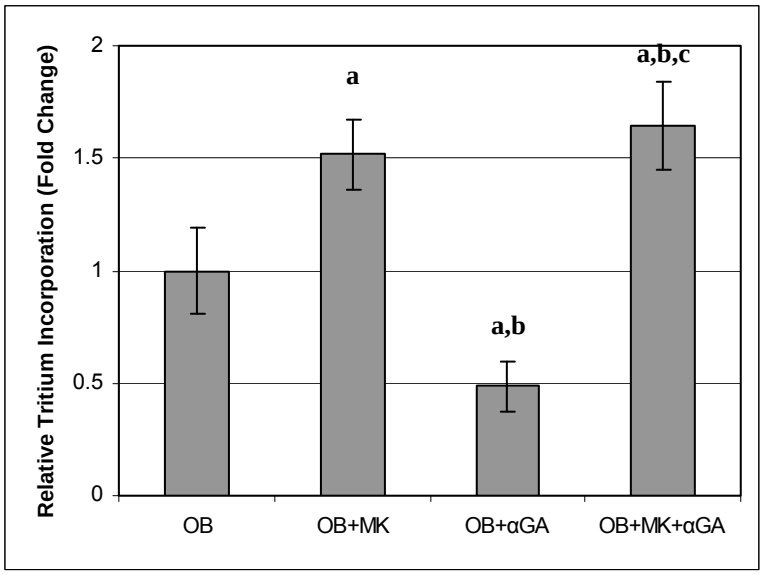

Figure 4. Proliferation of OBs was assessed by tritium incorporation. Proliferation of OBs alone in the following culture conditions was assessed: OB, OB+MK, OB+αGA, and OB+MK+αGA (n=9). MKs were removed via washing prior to analysis and MK cultures alone served as a background control (<200 counts were observed, data not shown). Error bars represent standard deviations associated with the mean tritium level. All pair-wise comparisons between groups were statistically significantly different from each other ($p<0.05$). a=significant difference from OB, b=significant difference from OB+MK, c=significant difference from OB+αGA.

4.A.5. Effects of MKs and GJIC on OB Differentiation In Vitro:

To further examine the effects of MKs on OB differentiation and the role of GJIC in mediating these effects we cultured 4 groups of cells: OB, OB+MK, OB+αGA, and OB+MK+αGA, and examined the following OB differentiation markers: type I collagen, osteocalcin, and alkaline phosphatase mRNA expression, alkaline phosphatase activity, and calcium deposition as a marker for mineralization. For all differentiation studies n=8-9. It should be noted that the OB and OB+MK groups also contained DMSO (vehicle control).

As illustrated below in Figure 5, a greater than 50% reduction in type I collagen, osteocalcin, and alkaline phosphatase gene expression was observed in OB cells when MKs were cultured with them (in mineralizing culture medium) for 14 days. Also shown in Figure 4 was the even greater reduction in expression of all 3 genes when cells were cultured with αGA. This suggests that GJIC is critical for proper expression of several OB genes. Interestingly, in contrast

to the proliferation data, when MKs were added to OB cultures containing αGA, expression of the genes examined was unaltered in OBs.

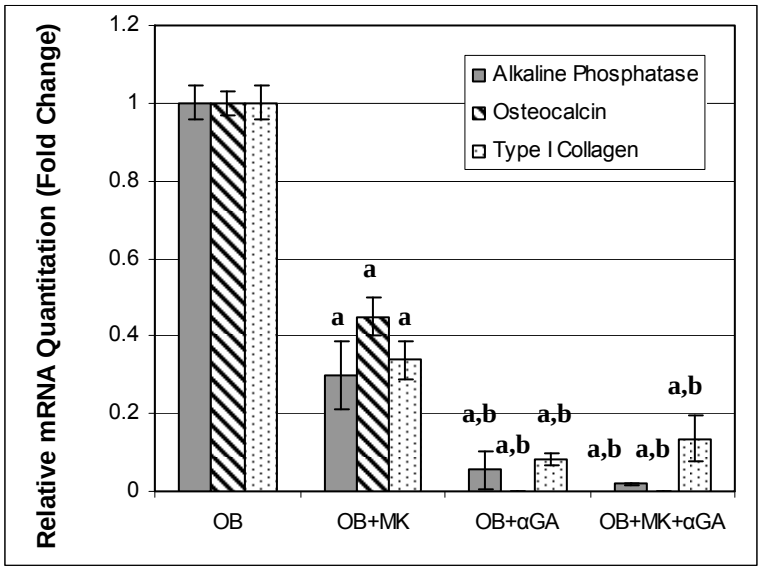

Figure 5. Real- time quantitative PCR analyses show the osteoblastic expression of alkaline phosphatase, osteocalcin, and type I collagen in OB, OB+MK, OB+αGA, and OB+MK+αGA cultures (n=8-9). MKs were removed via washing prior isolation of OB RNA. Results are reported as fold change or relative quantitation of target mRNA expression (2^{-Ct} method), normalized to an endogenous control (GAPDH) and relative to a calibrator (OB RNA sample). Error bars represent the standard deviation associated with the mean ΔΔCt value. With regard to mRNA expression of a single gene of interest, no significant difference was detected when comparing OB+αGA vs. OB+MK+αGA. All other pair-wise comparisons between groups (single gene of interest) were significantly different from each other ($p<0.05$). a=significant difference from OB (gene of interest only), b=significant difference from OB+MK (gene of interest only).

In addition to mRNA expression of several important OB genes, functional data was collected with regard to alkaline phosphatase activity and mineralization. As seen in Figure 6 below, alkaline phosphatase activity mirrored gene expression data. Specifically, OB alkaline phosphatase activity was significantly reduced in OBs co-cultured with MKs. When OBs were cultured with αGA, alkaline phosphatase activity was further reduced. Like gene expression, when MKs were added to OB cultures with αGA, alkaline phosphatase activity was unaltered.

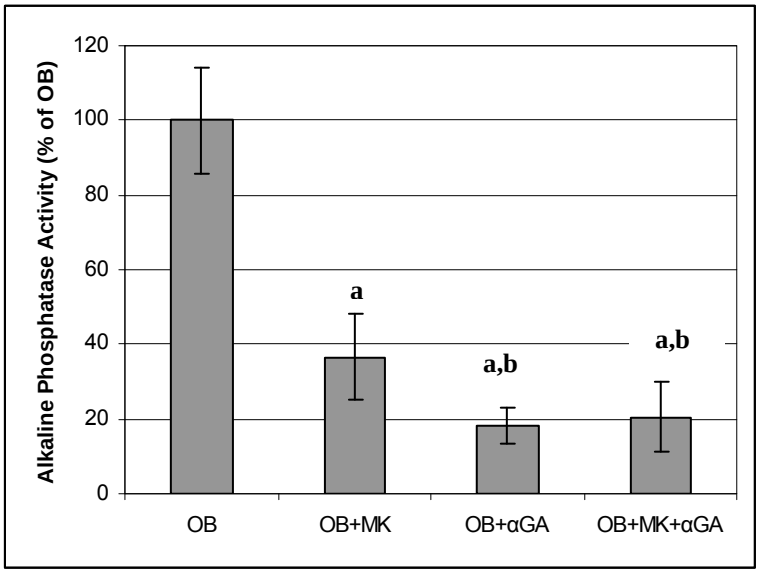

Figure 6. Alkaline phosphatase activity in OB cells from OB, OB+MK, OB+αGA, and OB+MK+αGA cultures (n=8-9). MKs were removed via washing prior to alkaline phosphatase determinations. Results are reported as a percentage of OB control cultures (OB). Error bars represent the standard deviation associated with the mean. With regard to alkaline phosphatase activity, no significant difference was detected when comparing OB+αGA vs. OB+MK+αGA. All other pair-wise comparisons between groups were significantly different from each other ($p<0.05$). a=significant difference from OB, b=significant difference from OB+MK.

Finally, bound calcium was assessed as a functional measure of mineralization. As with the other measures of OB differentiation, OBs co-cultured with MKs exhibited reduced levels of calcium deposition (Figure 7). When OBs were cultured with αGA calcium deposition was further reduced. However, unlike the other measures of differentiation, when MKs were added to OB cultures containing αGA, calcium deposition significantly increased. This latter trend is similar to what was observed in OB proliferation studies.

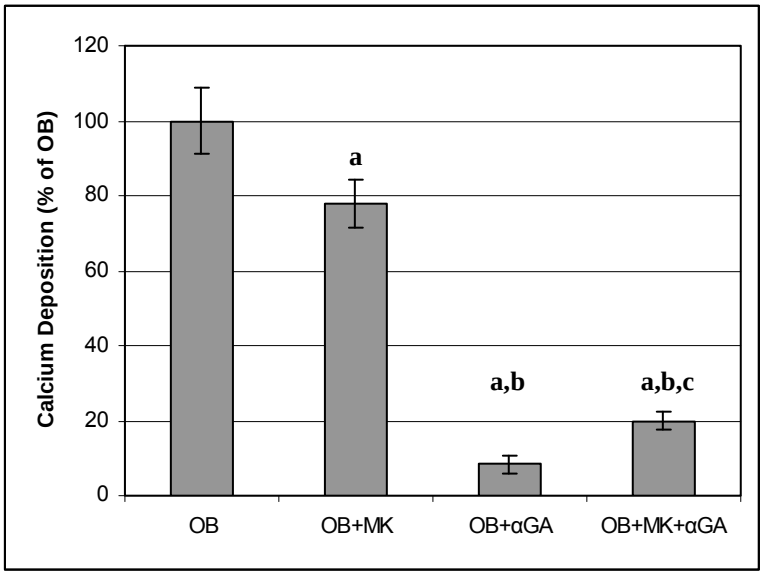

Figure 7. Calcium deposition in OB cells from OB, OB+MK, OB+αGA, and OB+MK+αGA cultures (n=8-9). MKs were removed via washing prior to calcium determinations. Results are reported as a percentage of OB control cultures (OB). Error bars represent the standard deviation associated with the mean. All pair-wise comparisons between groups were statistically significantly different from each other ($p<0.05$). a=significant difference from OB, b=significant difference from OB+MK, c=significant difference from OB+αGA.

4.B. MK Maturation and Longevity

4.B.1. Effect on MK Number on OB Proliferation

To determine if MK number affected the degree of OB proliferation we cultured increasing numbers of C57BL/6 and GATA-1 deficient MKs (0, 2500 and 5000) with 2500 OBs, and OB proliferation was determined by relative tritium incorporation as outlined above (n=9). The results at day 3 are shown in Figure 8 below. When OBs were co-cultured with 2500 MKs, derived from either C57BL/6 or GATA-1 deficient mice, OB proliferation was similarly elevated by 92% and 91%, respectively. When OBs were co-cultured with 5000 MKs, derived from either C57BL/6 or GATA-1 deficient mice, OB proliferation was enhanced by 127% and 171%, respectively. It should be noted that OB proliferation was significantly increased when co-cultured with either C57BL/6 or GATA-1 deficient MKs (as compared to OBs cultured alone) at both concentrations. Importantly, although it appears that OB proliferation is further elevated

when co-cultured with 5000 GATA-1 deficient MKs as compared to 5000 C57BL/6 MKs, no significant difference was detected (p=0.11). Thus, these results show that as MK number increases, OB proliferation increases regardless of whether C57BL/6 or GATA-1 deficient MKs are used.

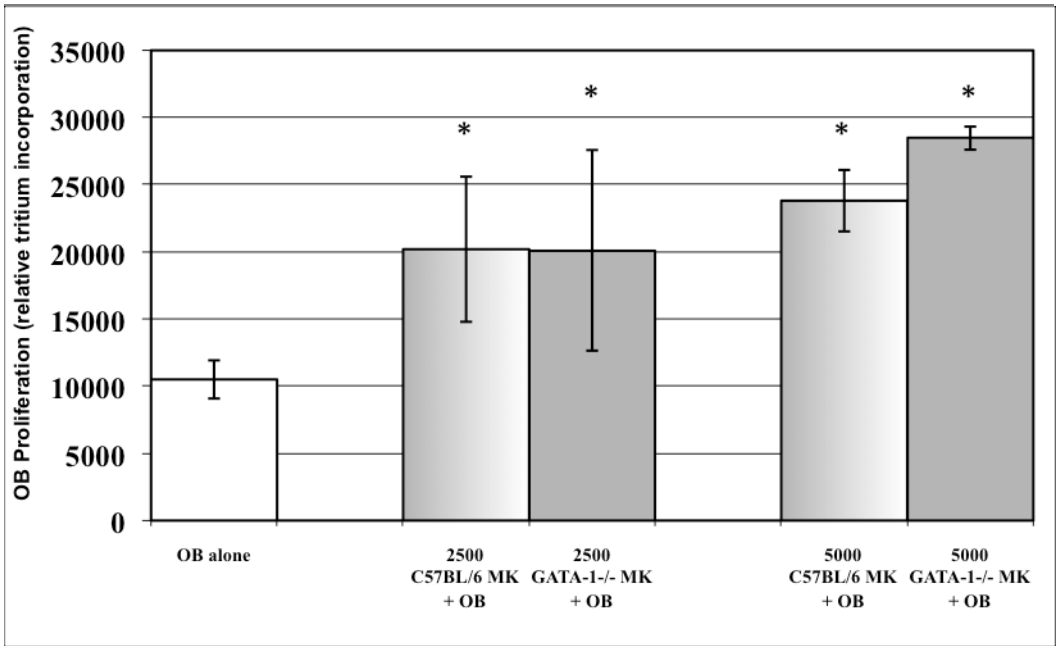

Figure 8. Affect of MK number on OB proliferation. Increasing numbers of GATA-1 deficient and C57BL/6 MKs similarly enhanced OB proliferation. A significant, greater than 90% increase in OB proliferation was observed when OBs were cultured with either 2500 C57BL/6 or GATA-1 deficient MKs. Culturing OBs with 5000 MKs further enhanced OB proliferation. * Denotes a significant difference in proliferation compared to the OB alone control group, as assessed by the Student's t-test, where $p<0.05$.

4.B.2. Effect on MK Number on OC Inhibition

To determine if MK number affected the degree of OC inhibition we cultured 100,000 OC progenitor cells/ml with increasing concentrations of C57BL/6 and GATA-1 deficient MK CM (0%, 3%, 10%, and 30%, vol:vol), and mature multi-nucleated OC number was determined (n=9). The results are shown in Figure 9 below. Of note, the OC data presented here utilize the Pax5-/- spleen cell line model system as OCs develop in the shortest time with this system and for the flow cytometry, we required a model system where OCs were generated in the shortest

amount of time (fewer media changes mean fewer sorted MKs required to generated enough CM). We did however demonstrate that in all the OC generation models described, GATA-1 deficient and C57BL/6 MK CM similarly inhibited OC formation (data not shown and (31)). When Pax5-/- OC progenitors were cultured with 3% C57BL/6 MK CM or 3% GATA-1 deficient MK CM, there was a 66% and 80% reduction, respectively, in OC formation as compared to OC progenitors cultured without CM. Like with OB proliferation, no significant difference was detected between the ability of 3% GATA-1 deficient MK CM and 3% C57BL/6 CM to inhibit OC formation ($p=0.14$). With 30% MK CM no OCs were detectable in cultures containing C57BL/6 or GATA-1 CM. These results show that just as with OB proliferation, MK number plays a critical role in the inhibition of OC formation.

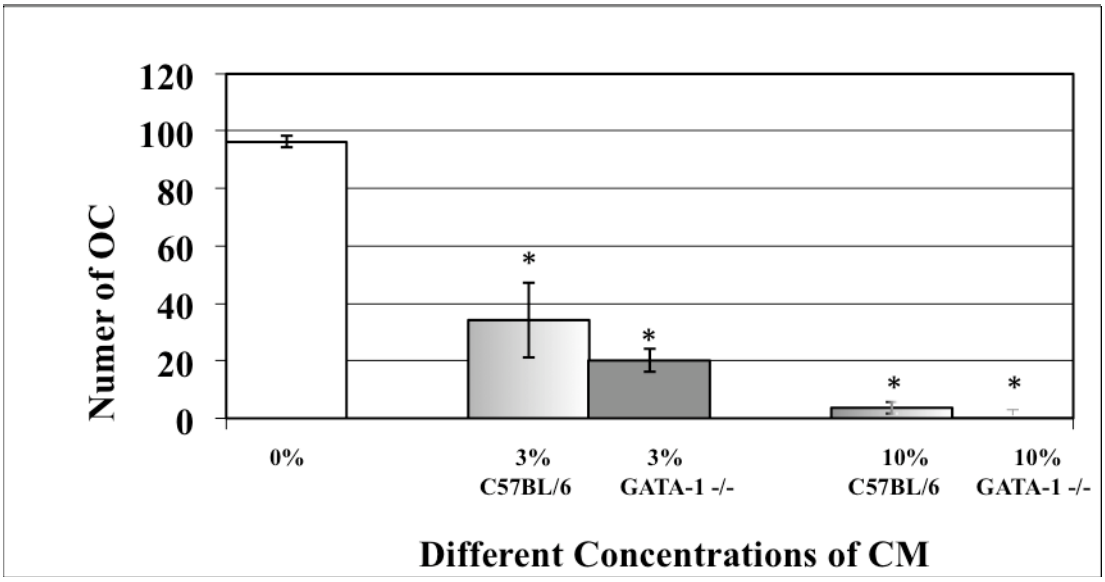

Figure 9. Affect of MK CM concentration on osteoclastogenesis. Increasing concentrations of GATA-1 deficient and C57BL/6 MK CM similarly inhibited OC development. A significant, greater than 65% reduction in the number of OCs formed was observed with just 3% CM. With 30% CM from either C57BL/6 or GATA-1 deficient MKs, complete inhibition in OC formation was seen. * Denotes a significant difference in OC number compared to control groups cultured without CM (0%), as assessed by the Student's t-test, where $p<0.05$.

4.B.3 Effect on MK Maturation Stage on OB Proliferation

To determine whether the stage of MK maturation influenced OB proliferation, C57BL/6 MKs were sorted into 3 subpopulations (megakaryoblasts, immature MKs and mature MKs) based on antibody binding to cell surface markers characteristic of each subpopulation (flow cytometry). 2500 MKs from each individual population, as well as 2500 MKs from a mixed MK population (~90-95% pure, BSA separated MK population harvested from fetal livers as outlined above (40)), were added to cultures containing 2500 OBs, and OB proliferation was determined (n=6). The results are shown in Figure 10 below. Like before, we examined OB proliferation on day 3. Interestingly, the megakaryoblasts had no significant effect on OB proliferation. However, the BSA separated MK population, the immature MK population, and the mature MK population all had a similar effect on OB proliferation (no significant differences were detected between these groups). Importantly these results show that while megarkaryoblasts have no

effect on OB proliferation, immature and mature MKs have virtually the same proliferative effect on OBs.

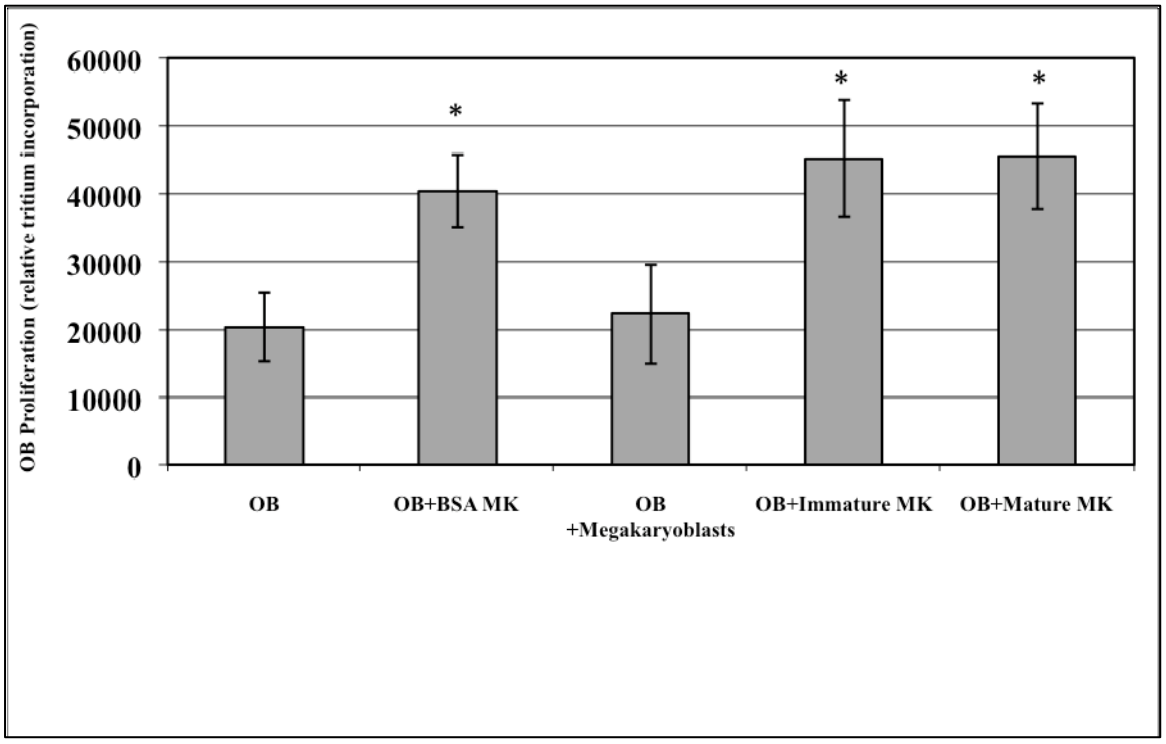

Figure 10: Affect of MK maturation stage on OB proliferation. 2500 BSA separated C57BL/6 MKs or flow cytometry separated immature and mature MKs all similarly enhanced OB proliferation, while 2500 megarkaryoblasts did not alter OB proliferation. * Denotes a significant difference in proliferation compared to the OB control group, as assessed by the Student's t-test, where $p<0.05$.

4.B.4. Effect of MK Maturation Stage on OC Inhibition

To determine whether the stage of MK maturation influenced OC formation, we again sorted C57BL/6 MKs into 3 subpopulations (megakaryoblasts, immature MKs and mature MKs). These individual subpopulations, as well as the BSA separated MKs, were cultured for 3 days ($1x10^6$ cells/ml), their CM was collected, sterile filtered, and 3% CM (vol:vol) was added to OC generating cultures as before (n=6). The results are shown below in Figure 11. To better compare to our previous data (Figure 9) all OC results were normalized (control cultures were set to 100). Interestingly, similar to the OB proliferation data, 3% megakaryoblast CM did not alter OC formation. Also like OB proliferation data, 3% CM from BSA separated C57BL/6 MKs,

immature MKs, and mature MKs all similarly inhibited OC formation by 63%, 69%, and 65% respectively (no significant differences were detected between these groups). Thus, these results show that similar to the OB data, megakaryoblasts have no effect on OC development, but immature and mature MKs have virtually the same inhibitory effect on OC development.

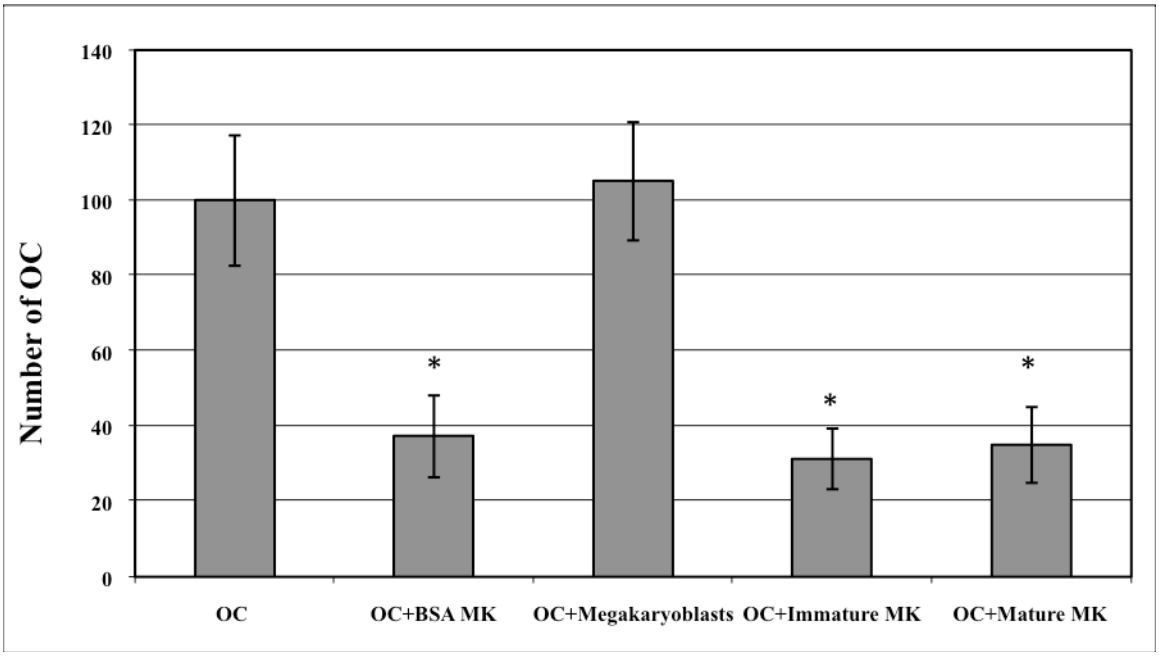

Figure 11. Affect of MK maturation stage on OC inhibition. 3% C57BL/6 MK CM from BSA separated or flow cytometry separated immature and mature MKs similarly inhibited OC development, while 3% CM from megakaryoblasts did not inhibit OC development. * Denotes a significant difference in OC number compared to OC control group, as assessed by the Student's t-test, where $p<0.05$.

4.B.5. Distribution of MK Subpopulations in C57BL/6 and GATA-1 Deficient Fetal Livers

C57BL/6 and GATA-1 deficient fetal liver cultures stimulated with TPO appear similar in their relative distribution of megakaryoblasts and immature MK if they are collected on day 3, but on day 4 the distribution is starting to change with an increase in mature MK in C57BL/6 cultures. Our flow cytometry data indicated day 4 fetal liver C57BL/6 cultures (total cells prior to BSA gradient separation to enrich for MKs) contained 1.83% of megakaryoblasts, 3.22% of immature MKs, and 4.95% of mature MKs, while GATA-1 deficient cultures contained 3.41% of megakaryoblasts, 4.58% of immature MKs, and 2.01% of mature MKs (based on equivalent cell

numbers). As would be expected, this data indicates that the C57BL/6 MKs are more differentiated than GATA-1 MKs.

4.B.6. MK Longevity:

To formally quantify if GATA-1 deficient MKs outlive their C57BL/6 counterparts we cultured C57BL/6 or GATA-1 deficient MKs alone, with TPO, with OBs, or with TPO and OBs. Viable MK number was recorded until no viable MKs were detected (n=4). The data from this study is presented in Figure 12 below. Virtually all of the C57BL/6 and GATA-1 deficient MKs cultured alone died by day 9 in culture (data not shown). The cultures of MKs with TPO added fared only slightly better, perhaps extending their lifespan by 2+ days (data not shown). Thus without the addition of OBs, viable MKs from both populations were not detected by approximately day 9. Interestingly, when MKs were co-cultured with OBs, MK survival was increased, but by day 15 virtually no viable MKs were detected (data not shown). However, the addition of both TPO and OBs significantly prolonged the lifespan of both C57BL/6 and GATA-1 deficient MKs. On day 15 when viable MKs were not detected in the other cultures, viable C57BL/6 MKs and GATA-1 deficient MKs were seen in culture containing both TPO and OBs. Both MK lineages peaked at day 20 with C57BL/6 MKs averaging 169±55 and GATA-1 deficient cultures averaging 450±100. After that, C57BL/6 MK number precipitously declined and by day 34 no viable MKs were detected. However, while the number of viable GATA-1 deficient MKs also declined, they significantly outlived their wild-type counterparts. It was not until day 69 that no viable GATA-1 deficient MKs were detected. It is important to remember that in these studies we cultured cells in OB media, not in MK media, which may negatively impact MK survival. That being said, overall these data show that the combination of TPO and OBs enhances MK survival, and that GATA-1 deficient MKs survive longer than C57BL/6 MKs when co-cultured with TPO and OBs.

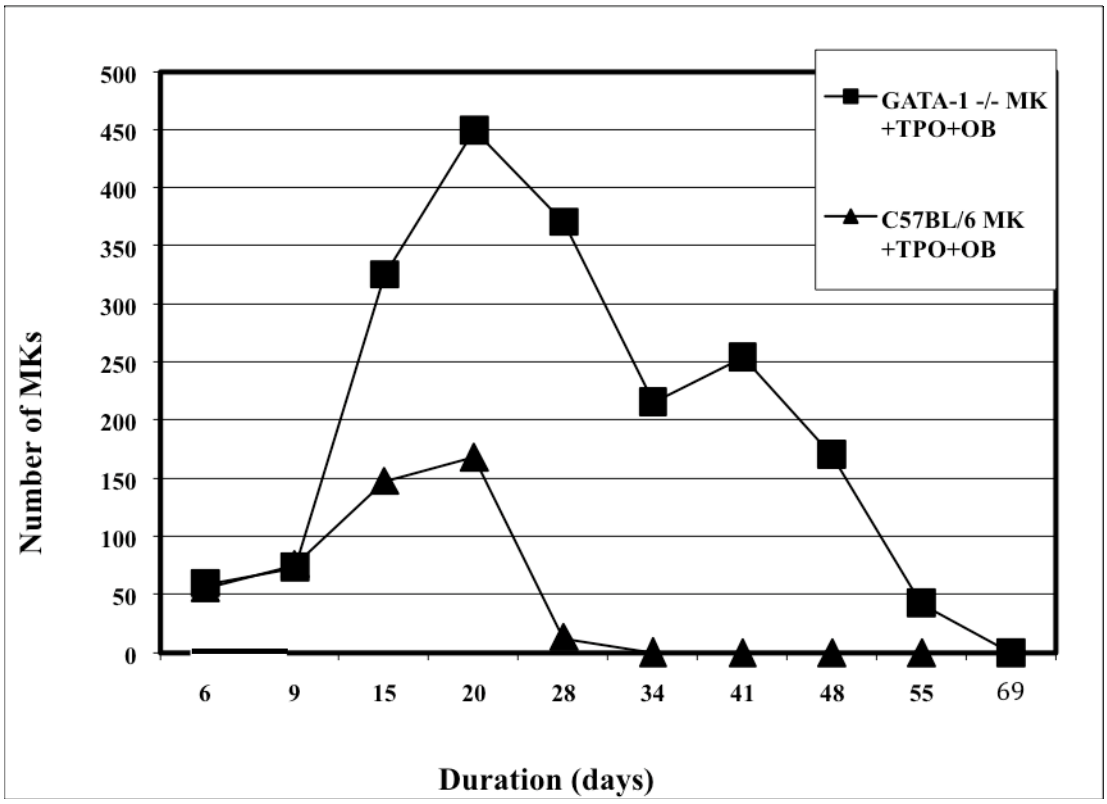

Figure 12. Viable MK number over time. C57BL/6 and GATA-1 deficient MKs were cultured alone, with TPO, with OBs, or with TPO and OBs, and viable cell number recorded. The combination of OBs and TPO increased MK survival. GATA-1 deficient MKs survived for longer than did C57BL/6 MK

5. DISCUSSION

5.A. GJIC

GJs are pores or channels that span the cellular membrane and allow molecules less than 1kD to pass from one cell to another, providing bilateral communication between the cells' cytoplasm. GJs are formed by two hexameric hemichannels, or connexons, composed of six protein subunits, termed connexins (Cxs). There are more than 20 identified mammalian Cxs (79), each with a different molecular permeability and a unique capability to interact with specific other connexins (80). Different tissue types express different Cxs, with Cx43 being the predominant GJ protein expressed by bone cells. OBs, OCs and osteocytes have all been shown to express Cx43, and Cx45 and Cx46 have also been detected (81). Importantly, one study also found MK expression of Cx43 during immunostaining of human and mouse BM biopsies. (56). This suggests that MKs may be able to communicate through GJs. Our previous studies have shown that MKs can enhance OB proliferation by up to 6-fold in vitro, that MK secreted growth factors or cytokines are not responsible for the enhancement in OB proliferation, and that the proliferative influence is blocked when the lineages are separated by a 0.1 μm membrane. Since MKs stained positively for Cx43, and because GJIC can be blocked by 0.4 μm transwell membranes (unpublished observation, H.J.D and A.F.T.), we investigated whether MKs can communicate with OBs through GJs, and whether MK-mediated GJIC was responsible for the MK-induced enhancement in OB proliferation we previously reported (8).

In this study we demonstrated that MKs expressed Cx43 (Figure 2), that functional GJs formed between MKs and OBs (Figure 3), and that GJIC between MKs and OBs could be significantly inhibited with exposure to a GJ uncoupler, αGA (Figure 3). To our knowledge, with the exception of the immunostaining data by Krenacs and Rosendaal (56), this is the first report that MKs can form functional GJs, and that GJIC between MKs and OBs occurs and allows for the transfer of small molecules from MKs to OBs as demonstrated by the transfer of calcein-AM

(Figure 3).

Interestingly, our data also demonstrated that MK-OB GJIC does not enhance OB proliferation, but in fact inhibits it. As shown in Figure 4, the relative increase in OB proliferation when MKs were added was greater in the presence of αGA than in the presence of vehicle control (250% vs. 53%, respectively) suggesting that using GJ uncoupling agents enhances MK-induced OB proliferation. In these studies 2 separate GJ uncoupling agents were used, αGA and oleamide. The mode of action of αGA is complex but is primarily thought to act indirectly on the GJ through a series of phosphorylation changes in Cx43. Oleamide, on the other hand, is thought to act by dissolving membrane lipids, changing membrane fluidity, and contracting the channel (82, 83). Although 2 separate GJ uncoupling agents were used in this study and their modes of action vary, both inhibitors have also been implicated in non-GJ signaling pathways as well as in inhibiting GJIC. Importantly, the fact that our previous studies demonstrate that MK conditioned medium is unable to elicit an increase in OB proliferation (8) indicates that several of the non-GJ related signaling pathways can be ruled out (e.g. ATP release through hemichannels). Therefore, while not definitive, the combination of the data presented here using two separate agents known to strongly inhibit GJIC, as well as the previously published data with MK conditioned medium and transwell membrane studies, strongly suggests that the inhibitor data is reflective of blocking MK-OB GJIC.

While our initial goal in this study was to determine whether GJIC was responsible for the MK-mediated increase in OB proliferation, to conduct a more rigorous study we also examined the influence of MK-mediated GJIC on OB differentiation. Specifically we examined OB expression of type I collagen, osteocalcin, and alkaline phosphatase. In addition we performed 2 functional in vitro assays of alkaline phosphatase activity and mineralization (calcium deposition). Surprisingly, we found that MKs inhibited all markers of OB differentiation. These data were unexpected as Bord et al (14) had previously published findings

demonstrating that MKs enhanced OB expression of collagen (COL1 A1) and osteoprotegerin (OPG). These apparent contradictory findings may be explained by experimental differences, specifically the duration of the cultures studied. In their study, OB mRNA was examined 1 day following co-culture (14). In the studies presented here, MKs have been cultured with OBs for 14 days. Our cultures had a confluent monolayer with mineralizing nodules present in the OB control cultures. In the study by Bord et al (14), the cultures are much less mature and this difference alone could easily account for the apparent discrepancy. Alternatively, with longer culture durations, it is possible that MKs could be causing OBs to undergo apoptosis. While specific apoptosis experiments, such as tunnel staining, were not conducted, three lines of evidence suggest that apoptosis is not responsible for the MK-mediated reduction in OB differentiation measures. First, we have previously published that co-culture of OB with MKs for 6 days resulted in increased numbers of proliferating OB (8). Second, visual observation of the OBs would not suggest an apoptotic or even necrotic action. There was an intact monolayer, there were no floating cells to speak of, and cells were piling up and forming normal nodules, just not to the extent observed in cultures not containing MKs. Finally, we had comparable levels of soluble protein in OBs from control cultures or cultures containing MKs (data not shown).

With regard to the influence of GJ uncoupling agents on OB differentiation, when OBs were cultured with αGA, all of the OB differentiation markers examined were markedly reduced (the effect was more pronounced than the MK-mediated reduction). Thus, in order to ascertain the effects of GJIC on MK-mediated alterations in OB differentiation we used the cultures of OB treated with αGA (OB+αGA) as a control for the OB+MK+αGA cultures. When making this comparison, MKs did not alter OB mRNA expression of alkaline phosphatase, type I collagen, or osteocalcin. Nor did MKs alter alkaline phosphatase activity in these cultures. However, there was a significant increase in calcium deposition. It is important to note that although bound calcium levels were elevated, microscopic observation demonstrated that while both OB cultures

and OB+MK cultures showed typical nodule formation (albeit at lower levels), none of the cultures treated with αGA (OB+αGA and the OB+MK+αGA) exhibited typical nodule formation (data not shown). Visually no matrix was observed in the cultures treated with αGA. Therefore it is thought that the increase in bound calcium measured in OB+MK+αGA cultures as compared to OB+αGA cultures may be reflective of higher background levels as opposed to actual calcium deposition into the matrix. As indicated in Figure 4, there were significantly more proliferating OBs in OB+MK+αGA as compared to OB+αGA cultures, which could account for the higher background levels. In addition, the measured calcium levels were negligible in cultures treated with αGA as compared to untreated cultures. Further, the observation that typical nodule formation is reduced in αGA treated cultures is consistent with the gene expression and alkaline phosphatase activity, suggesting that αGA treatment itself inhibits OB differentiation. Thus, with the possible exception of the bound calcium, the OB differentiation parameters evaluated in this study were unaltered by GJIC, suggesting that future studies will need to explore alternative signaling mechanisms.

As with most in vitro model systems, there are limitations which must be addressed. First, we have taken 2 separate cell types which are present in the BM cavity and cultured them together to assess regulatory mechanisms. Clearly within the marrow cavity there are many other cells, most of which can also form GJs (84-87) and the GJ-mediated interactions of these cells may also be important in skeletal regulation. As mentioned earlier, although our data implicates a role for GJ in MK-mediated changes in OB proliferation, the GJ uncouplers used here have also been implicated in non-GJ signaling pathways. Finally, although our data clearly demonstrate that the relative increase in OB proliferation as stimulated by MKs is higher in cultures treated with αGA as compared to those without αGA (250% vs. 53%, respectively), this may reflect that MKs can increase OB proliferation by a mechanism independent of GJIC. Indeed, examination of the data contained in Figure 4 demonstrates that OB proliferation is only 14% higher in

OB+MK+αGA cultures as compared to OB+MK cultures. Thus, this data may suggest that MK-induced OB proliferation can reach a maximum level and that this maximum level is independent of GJIC. However, our statistical analyses indicate that OB proliferation in OB+MK+αGA cultures is significantly different from that observed in the OB+MK cultures ($p=0.048$). Specifically, our 2-way factorial ANOVA demonstrates that there is an interaction between MKs and αGA indicating that the response to MKs is influenced by the presence of αGA, and vice versa. As described previously, we performed pair-wise Bonferroni comparisons after finding a significant statistical interaction to control for the elevated family-wise error associated with performing multiple comparisons. This revealed a p-value of 0.048 and so, although we cannot rule out non-GJ signaling pathways, treatment of OB+MK cultures with αGA significantly enhances OB proliferation in vitro.

In conclusion, the data present here demonstrate 4 critical findings. First, MKs and OBs can communicate via GJIC. Second, both proliferation and differentiation of OBs is inhibited when cells are treated with αGA. This finding is consistent with the reports of other investigators (88-90). Third, MKs inhibit OB differentiation in vitro when cultured for extended durations. Fourth, GJIC inhibits MK-mediated enhancement of OB proliferation but does not appear to alter MK-mediated reductions in OB differentiation. Importantly, these data suggest that specific inhibition of MK-OB GJIC may increase OB proliferation.

5.B. MK Maturity and Longevity

Here we compare characteristics of GATA-1 deficient MKs to wild-type C57BL/6 MKs in an attempt to isolate the characteristic(s) of these mutant cells that contributes to the high bone mass phenotype seen in GATA-1 deficient mice (8). As much attention has been given to the fact that GATA-1 deficient mice have increased MK number, we first cultured increasing number of MKs, or increasing concentrations of MK CM, with OBs and OC precursors respectively, to study the effect of increased MK number or increased MK CM concentration on bone cells. We

then looked at the effect of MK stage of maturation on the same populations, and finally looked at MK life span.

As Figure 8 demonstrates, 2500 MKs cause an approximate doubling of the OB population, with MKs cultured from GATA-1 deficient and wild-type MK having almost the exact same effect. When the number of MKs in co-culture was doubled (5000 MKs), OB proliferation increased further, and no significant differences were detected between the ability of C57BL/6 or GATA-1 deficient MKs to enhance OB proliferation. Similarly, as Figure 9 shows, both C57BL/6 and GATA-1 deficient MK CM were equally able to inhibit OC formation. These data, on first glance, appear to support the idea that MK number alone can elicit a response in bone cells.

The next parameter we studied was MK maturation stage. In our previous studies of MK effect on OBs and OCs, no attempt was made to separate out MKs based on stage of maturity, yet 2 of the 4 aforementioned mouse models with high bone mass phenotypes have documented defects in MK maturation. Mice deficient in NF-E2 and GATA-1 transcription factors have a developmental arrest in MK differentiation, resulting in the accumulation of immature MKs (8). Further, immature MKs from NF-E2 deficient mice have significantly reduced numbers of granules (6), while the MKs from GATA-1 deficient mice are so immature as to have few if any specific granules, inhibiting if not precluding their ability to hold and later secrete proteins like wild-type MKs (7). However, in the TPO overexpressing mice, there are increased numbers of MKs at all stages of differentiation, and these mice have increased bone mass (3-5). As the former mouse models imply that increases in the number of immature MKs may be a requisite for increases in bone mass; perhaps the TPO overexpressing mice have sufficient numbers of immature MKs to produce this high bone mass phenotype. MK maturation stage is also an important parameter to consider because when we culture fetal livers in vitro for MK isolation, C57BL/6 cultures resemble cultures from GATA-1 deficient fetal livers. Our FACS analysis of these cultures in vitro demonstrated that at day 3 they were virtually identical (data not shown),

and at day 4 some slight differences were seen. Therefore if our in vitro C57BL/6 and GATA-1 deficient MK cultures are virtually identical in subpopulation distribution, it would not be unreasonable for these MKs to elicit a similar response. Thus like our prior assumption with GATA-1 deficient and NF-E2 deficient mice, perhaps the reason C57BL/6 MKs induce OB proliferation in vitro is because they are a more immature cell population. Here we directly address this issue by sorting MKs from C57BL/6 wild-type animals into populations based on stage of differentiation, and co-culture these subpopulations with OBs and OCs to study the effect of MK maturation on these separated cells. For these studies we examined BSA separated MKs, as well as flow cytometry separated megakaryoblasts, immature MKs, and mature MKs. Figures 10 and 11, respectively show that the degree of OB proliferation induced, or OC formation inhibited, was not significantly different if immature MKs, mature MKs, or BSA separated MKs were used. Megakaryoblasts, however, had no proliferative effect on OBs nor inhibitory effect on OCs. It should be noted that for these maturation studies we chose to analyze only one time point for OB proliferation (day 3), and used only the lower cell number (2500). Similarly, we only used the lower concentration of MK CM (3% vol:vol). This was done because recovery of sorted subpopulations of MKs was low. In general it is difficult to sort large cells such as MKs, and sorting is done more slowly. Added to this we only obtained approximately $1x10^6$ BSA separated MKs per pregnant mouse. These BSA separated MKs are approximately 90-95% immature and mature MKs and the contaminating cells are megakaryoblasts and other fetal liver cells (57). Thus, many mice and long sorting times are required to obtain sufficient numbers of cells in each subpopulation. To further enhance our ability to obtain sufficient numbers of cells in each subpopulation we did the following. We extended our fetal liver culture duration (stimulated with TPO) from 3-4 days to 5 days to enhance the number of mature MKs obtained. On the other hand, to enhance megakaryoblasts we cultured fetal liver cells for 3 days without TPO. This is important, as both C57BL/6 and GATA-1 deficient fetal liver cultures stimulated with TPO appear similar in their relative distribution of megakaryoblasts and immature MK if they are

collected on day 3, but on day 4 the distribution is starting to change with an increase in mature MK in C57BL/6 cultures (data not shown). These technical issues aside, it appears that in vitro mature and immature MKs equivalently influence OBs and OCs while megakaryoblasts do not exert an influence.

We now turn to our data on MK longevity. Of note, we chose to culture MKs alone, with TPO, with OBs, and with TPO and OBs (Figure 12). We thought it vital to co-culture MKs with OBs, for just as MKs have a positive proliferative effect on OBs, OBs similarly stimulate megakaryopoiesis (8, 36). Since our focus is on the interaction of the hematopoietic and bone cell systems, we wanted to create an in vitro environment that more closely resembled the natural in vivo BM environment. To our knowledge this is the first formal attempt to quantify MK lifespan in vitro when cultured with TPO and OBs. In reference to longevity the most striking data was seen when MKs were cultured in the presence of OBs and TPO. In the presence of both OBs and TPO, viable C57BL/6 MK number peaked at day 20 and declined precipitously after that, with only a few viable MKs present at day 28, and none detected at day 34. This result is in agreement with work done by one group who studied MK lifespan in vitro, and found when cultured with TPO the number of mature MKs peaked at days 12-15, with the majority of MKs beginning to show markers of apoptosis at days 18-21 (23). We found similar results when MKs were co-cultured with OBs in the presence of TPO, but in the absence of OBs our wild-type MKs did not survive as long as the above group reports. This may be explained by the fact that we cultured our cells in osteogenic media as opposed to MK culture media. The GATA-1 deficient MK population co-cultured with OBs under the influence of TPO also peaks around day 20. Of note, the peak observed in both populations is due to the impurity of the population. For approximately 5-10% of the sample is megakaryoblasts and as they proliferate in response to TPO the populations of both wild-type and GATA-1 deficient MKs initially increases, and only begins to decline after these cells have presumably finished responding. However, at day 34 when no mature C57BL/6 MKs were detected, there were more than 200 viable GATA-1 deficient MKs

still present. It was not until day 69 that there were no viable GATA-1 deficient MKs detected. Our results are broadly consistent with previous work done by Vyas et al. (23) showing that GATA-1 deficient MKs outlive wild-type MKs. Of note, the GATA-1 deficient MKs cultured with TPO alone in the study by Vyas et al. (23) lived up to 4-weeks, while our GATA-1 deficient MK population cultured with TPO alone did not survive as long, again likely owing to our use of osteogenic culture media. However, in the presence of OBs and TPO we saw a marked increase in longevity of 103% which was similar to the 100-133% increase in longevity reported by Vyas et al. (23) with TPO alone. Thus GATA-1 deficient MKs live significantly longer than their wild-type counterparts when both are cultured with OBs in the presence of TPO. Interestingly, we show here that culturing MKs with TPO and OBs has a synergistic effect on both GATA-1 deficient and wild-type MK lifespan. For when MKs of either type are cultured with TPO alone, there are no viable MK cells by approximately day 9, and with OBs alone there are no viable MK cells by day 15. However, when C56BL/6 MKs are cultured with TPO and OB they live up to 34 days, and GATA-1 deficient MKs survive up to 69 days, highlighting the significant synergistic effect of TPO and OBs together on MK survivability.

Here we confirm prior in vivo observation of mouse models that increased MK number corresponds to a high bone mass phenotype by showing that, to an extent, increasing MK number further increases OB proliferation and inhibits OC formation. However, here we looked closer at the MKs themselves, wondering if in addition to increased number, there was something inherently different about GATA-1 deficient MKs that favored net bone deposition more than wild-type MKs. We looked at stage of maturation and showed that immature and mature MKs have similar effects on osteoblastogenesis and osteoclastogenesis. Finally, we tried to better simulate the in vivo BM environment by co-culturing MKs with OBs in the presence of TPO and demonstrated that GATA-1 deficient MKs significantly outlive C57BL/6 MKs by up 35 days. Thus, in addition to increased MK number, we propose that increased longevity contributes to the increased influence of GATA-1 deficient MKs on skeletal homeostasis. If viable MKs are around

for a longer duration in the BM cavity, there is more opportunity to stimulate OB proliferation and likewise inhibit OC development. Therefore, the increased longevity of GATA-1 deficient MKs may partially explain the high bone mass phenotype seen in GATA-1 deficient mice.

5.C. Conclusions

It is now imminently clear that the hematopoietic and bone lineages are connected not just by proximity, but by functionality, and that MK induced effects on OBs and OCs are multiple and complex. While the exact mechanisms of inhibition in osteoclastogenesis and induction in osteoblastogenesis still need to be further elucidated, here we make important strides toward that goal.

Our first work focused on MK-OB communication, finding that the two lineages are capable of interacting by GJIC. While the net overall effect of MKs on OBs is to increase OB proliferation, here we found a novel inhibitory reaction. We postulate that GJIC between MKs and OBs partially counteracts another juxtacrine mechanism, and showed that when GJIC was inhibited, OB proliferation was enhanced even further. In the second study we focused on MKs in an attempt to uncover what inherent quality contributes to their osteoblastogenic effect. We discovered that mutant GATA-1 MKs significantly outlive wildtype MKs, and that this may contribute to the bone mass phenotype observed in the mutant mice.

By discovering details in the complex interplay of these cells, we take steps towards clinical significance with the distant possibility of therapeutic intervention if we could harness the osteoblastogenic effect of MKs. Specifically, in the GJIC study we found a method of increasing OB proliferation without altering differentiation, which may provide a potential novel anabolic therapeutic treatment approach for bone loss diseases such as osteoporosis.

As we continue to unravel the complexities behind the hematopoietic-bone cell interplay, pharmacologic and even genetic therapies for important public health problems may be discovered.

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