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The Role of Gap Junctions in Megakaryocyte-Mediated Osteoblast Proliferation and Differentiation

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Abstract

Gap junctions (GJs) are membrane-spanning channels that facilitate intercellular communication by allowing small signaling molecules (e.g. calcium ions, inositol phosphates, and cyclic nucleotides) to pass from cell to cell. Over the past two decades, many studies have described a role for GJ intercellular communication (GJIC) in the proliferation and differentiation of many cells, including bone cells. Recently, we reported that megakaryocytes (MKs) enhance osteoblast (OB) proliferation by a juxtacrine signaling mechanism. Here we determine whether that response is facilitated by GJIC. First we demonstrate that MKs express connexin 43 (Cx43), the predominant GJ protein expressed by bone cells, including OBs. Next, we provide data showing that MKs can communicate with OBs via GJIC, and that the addition of two distinct GJ uncouplers, 18 α -glycyrrhetic acid (α GA) or oleamide, inhibits this communication. We then demonstrate that inhibiting MK-mediated GJIC further enhances the ability of MK to stimulate OB proliferation. Finally, we show that while culturing MKs with OBs reduces gene expression of several differentiation markers/matrix proteins (type I collagen, osteocalcin, and alkaline phosphatase), reduces alkaline phosphatase enzymatic activity, and decreases mineralization in OBs, blocking GJIC does not result in MK-induced reductions in OB gene expression, enzymatic levels, or mineralized nodule formation. Overall, these data provide evidence that GJIC between MKs and OBs is functional, and that inhibiting GJIC in MK-OB cultures enhances OB proliferation without apparently altering differentiation when compared to similarly treated OB cultures. Thus, these observations regarding MK-OB GJIC inhibition may provide insight regarding potential novel targets for anabolic bone formation.

Keywords

connexin 43; Cx43; GJIC; mouse

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Introduction

Bone is a dynamic tissue system in a constant state of flux. Old tissue is continuously resorbed by osteoclasts (OCs), as new bone is simultaneously laid down by OBs [1]. This is a highly regulated process in which alterations in resorption or formation lead to an imbalance in bone mass. In bone loss diseases such as osteoporosis, bone resorption exceeds formation. Therefore, therapeutic treatments should aim to decrease resorption and/or increase formation.

Unfortunately, there are few successful means to induce anabolic bone formation. Recently we [2] and others [3,4] demonstrated that MKs can act to stimulate OB proliferation and differentiation. However, the specific mechanisms by which MKs stimulate OB proliferation and differentiation remain unclear. Our previous studies demonstrated that MK conditioned medium did not enhance OB proliferation [2]. Similarly, our studies also showed that in a transwell co-culture system (where MKs were separated from OBs with a 0.1 μ m membrane), MKs did not enhance OB proliferation [2]. These data suggest that MK secreted factors such as cytokines as growth factors are not responsible for the induction in OB proliferation, rather MKs stimulate OB proliferation through some sort of a 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) direct transmission from the cytoplasm of one cell to the cytoplasm of a second cell. GJIC is an example of the latter mechanism. Because GJIC can also be blocked with 0.4 μ m 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 [5], 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 was responsible for the MK-induced enhancement in OB proliferation. Here, we demonstrate that MKs do express Cx43, that they can in fact communicate with OBs through GJs, and we continue to elucidate the mechanisms by which MKs interact with OBs.

Materials and Methods

Preparation of Fetal Liver Derived MKs

Murine MKs were prepared as previously described [2,6]. 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 2 $\times$ 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 [7]. 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 [8]. 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.

Preparation of neonatal calvarial cells (OB)

Murine calvarial cells were prepared as previously described [9]. Our technique was a modification of the basic method described by Wong and Cohn [10]. Briefly, calvaria from mice less than 48 hours old were pretreated 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 [9,11,12]. Freshly prepared OBs were used for all studies.

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 [13]. 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).

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 Cyclor 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 ΔC_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 $\Delta\Delta C_T$ must be calculated. $\Delta\Delta C_T$ for this example is the absolute value of the difference between the ΔC_T for the MK and the OB or $|\Delta C_T \text{ MK} - \Delta C_T \text{ OB}|$. The relative difference (fold increase or decrease) in expression is calculated as $2^{-\Delta\Delta C_T}$. The following was the 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' GCTGATCATTCACGTTTT

Alkaline phosphatase reverse primer: 5' CTGGGCCTGGTAGTTGTTGT

Type I collagen forward primer: 5' CAGGGAAGCCTCTTTCTCCT

Type I collagen reverse primer: 5' ACGTCCTGGTGAAGTTGGTC

GAPDH forward primer: 5' CGTGGGGCTGCCAGAACAT

GAPDH reverse primer: 5' TCTCCAGGCGGCACGTCAGA

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 [14]. Our protocol was adapted from Ziambaras et al [15]. 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 37°C. 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 37°C. 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 [16], 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 18 α -glycyrrhetic acid (α GA, Sigma, St. Louis, MO) or oleamide (Sigma) to assess the ability of a GJ uncouplers to inhibit MK-OB GJIC. In these experiments α GA or oleamide was added to the receiving cells (OBs) 30 minutes prior to dropping donor cells (MKs also pretreated with inhibitors) into cultures.

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 α GA (50 μ M, 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 37°C 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 [17], our preliminary studies demonstrated that the inhibitory response of α GA 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 μ Ci/well; 5-8 Ci/mmol) added during the last 16 hours of culture [18]. To assess OB proliferation alone, MK were removed from wells (4 washes) prior to measuring incorporation of 3 H-thymidine [2].

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 α GA (40-50 μ M,

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 37°C in α -MEM supplemented with 2% FCS and 50 μ g/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 α GA/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.

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) [19]. Briefly, cells were washed 2 $\times$ 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).

Quantative Analysis of Calcium Deposition

Calcium deposition was assessed by eluting Alizarin Red S from cell monolayers as previously described by Stanford et al [20]. 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).

Statistical Analysis

Two-way factorial analyses of variances were used to compare groups, with MKs and α GA 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.

Results

MK 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 showed 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 1).

MKs Form Functional GJ 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 2A), 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).

α GA 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, α GA and oleamide [17,21]. α GA 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 2B shows that GJIC remained unchanged with 10 μ M of α GA. However, with 20 - 50 μ M of α GA, MK-OB GJIC was reduced (Figures 2C-2E), and with 100 μ M of α GA, significant inhibition of MK-OB GJIC was observed (Figure 2F). 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 μ M of oleamide resulted in a significant reduction in GJIC, 25% and 30% respectively (data not shown).

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 μ M of α GA (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 in Figure 3, addition of MKs to OB cultures significantly enhances OB proliferation by greater than 50% over a 3 day period of time [2]. Next, we show that OB proliferation was significantly reduced when OBs were cultured with α GA in the absence of MKs. There was approximately a 50% reduction in OB proliferation when OB were cultured with α GA. This suggests that OBs communicate with each other via GJIC to increase their own proliferation, and that α GA 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 α GA, OB proliferation is enhanced even further than when MKs alone are added to OBs (compared to the appropriate control, OB treated with α GA, there was approximately a 250% increase in OB proliferation). Although MK-mediated proliferation was increased only a modest 14% in cultures containing α GA as compared to those without α GA, 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 α GA allows MKs to exert signals for proliferation unopposed by the inhibitory GJ signals.

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+ α GA, and OB+MK+ α GA, 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 in Figure 4, a greater than 50% reduction in type I collagen, osteocalcin, and alkaline phosphatase gene expression was observed in OB cells when MKs were culture 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 α GA. 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 α GA, expression of the genes examined was unaltered in OBs.

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 5, 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 α GA, alkaline phosphatase activity was further reduced. Like, gene expression, when MKs were added to OB cultures containing α GA, alkaline phosphatase activity remained unaltered.

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 6). When OBs were cultured with α GA calcium deposition was further reduced. However, unlike the other measures of differentiation, when MKs were added to OB cultures containing α GA, calcium deposition significantly increased. This latter trend is similar to what was observed in OB proliferation studies.

Discussion

GJs are pores or channels that span the cellular membrane and which 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 [22], each with a different molecular permeability and a unique capability to interact with specific other connexins [23]. Different tissue types express different Cxs, with Cx43 being the predominant GJ protein expressed by bone cells [24]. OBs, OCs and osteocytes have all been shown to express Cx43, but Cx45 and Cx46 have also been detected [24]. Importantly, in one study, in addition to cells of the OB lineage, MKs were also labeled for Cx43 when immunostains were performed on human and mouse bone marrow biopsies [5]. This suggests that MKs may also be able to communicate through GJs. The combination of the fact that MKs stained positively for Cx43 and that GJIC can be blocked by 0.4 μ m transwell membranes (unpublished observation, H.J.D and A.F.T.) led us to study 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 [2]. Our previous studies have shown that MKs can enhance OB proliferation by up to 6-fold in vitro and that MK secreted growth factors or cytokines are not responsible for the enhancement in OB proliferation.

In this study we demonstrated that MKs expressed Cx43 (Figure 1), that functional GJs formed between MKs and OBs (Figure 2), and that GJIC between MKs and OBs could be significantly

inhibited with exposure to a GJ uncoupler, α GA (Figure 2). To our knowledge, with the exception of the immunostaining data by Krenacs and Rosendaal [5], 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 2).

Interestingly, our data also demonstrated that MK-OB GJIC does not enhance OB proliferation, but in fact inhibits it. As shown in Figure 3, the relative increase in OB proliferation when MK were added was greater in the presence of α GA 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, α GA and oleamide. The mode of action of α GA 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 [25,26]. 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 [2] 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, alkaline phosphatase activity and mineralization (calcium deposition). Unexpectedly, we found that MKs inhibited all markers of OB differentiation. These data were unexpected as Bord et al [4] 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 [4]. In the studies presented here, MKs have been cultured with OBs for 14 days. In the cultures described here a confluent monolayer is observed with mineralizing nodules present in the OB control cultures. In the study by Bord et al [4], the cultures are much less mature than those described here 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 [2]. Second, visual observation of the OBs would not suggest an apoptotic or even necrotic action, as an intact monolayer was present, 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 α GA, 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 α GA (OB+ α GA) as a control for the OB+MK+ α GA 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 with respect to the latter measurement, that although bound calcium levels were elevated, microscopic observation demonstrated that while OB cultures showed typical nodule formation and that OB+MK cultures also showed typical nodule formation albeit at lower levels, none of the cultures treated with α GA (OB+ α GA and the OB+MK+ α GA) exhibited typical nodule formation (data not shown). Visually no matrix was observed in the cultures treated with α GA. Therefore it is thought that the increase in bound calcium measured in OB+MK+ α GA cultures as compared to OB+ α GA cultures may be reflective of higher background levels as opposed to calcium deposition into matrix. As indicated in Figure 3, there were significantly more proliferating OBs in OB+MK+ α GA as compared to OB+ α GA cultures, which could account for the higher background levels. In addition, the measured calcium levels were negligible in cultures treated with α GA as compared to untreated cultures. Further, the observation that typical nodule formation is reduced in α GA treated cultures is consistent with the gene expression and alkaline phosphatase activity, suggesting that α GA 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 bone marrow 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 [27-30] 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 α GA as compared to those without α GA (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 3 demonstrates that OB proliferation is only 14% higher in OB+MK+ α GA 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+ α GA 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 α GA indicating that the response to MKs is influenced by the presence of α GA, 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 α GA 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 α GA. This finding is consistent with the reports of other investigators [31-33]. 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. Increasing OB proliferation without altering differentiation (as demonstrated here) may provide a potential novel anabolic therapeutic treatment approach for bone loss diseases such as osteoporosis. In summary, both

OB and MK cells possess functional GJs, enabling them to communicate. These findings suggest that MK-mediated GJIC may be an important factor to consider when examining the role of MKs in regulating skeletal homeostasis and an exciting new therapeutic target.

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Figure 1.

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 ($2^{-\Delta\Delta C_t}$ 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 $\Delta\Delta C_t$ value.

Figure 2.

Histograms from FACS analysis of OBs after dual-labeled MKs were dropped onto OBs in parachute assay and GJ were allowed to form. 2A shows the percentage of OBs in which calcein-Am was transferred from MKs. 2B-2F shows the percentage of OBs in which calcein-Am was transferred from MKs when cultures were treated with various concentrations of α GA (10-100 μ M). 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.

Figure 3.

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.

Figure 4.

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 ($2^{-\Delta\Delta C_t}$ 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 $\Delta\Delta C_t$ 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).

Figure 5.

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.

Figure 6.

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.