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A role for the primary cilium in paracrine signaling between mechanically stimulated osteocytes and mesenchymal stem cells

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ABSTRACT

Bone turnover is a mechanically regulated process, coordinated in part by the network of mechanosensitive osteocytes residing within the tissue. The recruitment and bone forming activity of the mesenchymal derived osteoblast is determined by numerous factors including mechanical loading. It is therefore somewhat surprising that although mechanically regulated signaling between the coordinating osteocytes and mesenchymal stem cells (MSCs) should exist, to date it has not been directly demonstrated. In this study, conditioned media from mechanically stimulated osteocytes (MLO-Y4 cell line) was collected and added to MSCs (C3H10T1/2 cell line). The addition of mechanically stimulated osteocyte conditioned media resulted in a significant upregulation of the osteogenic genes OPN and COX-2 in MSCs compared to statically cultured conditioned media, demonstrating a novel paracrine signaling mechanism between the two cell types. The same mechanically conditioned media did not alter gene expression in osteoblasts (MC3T3 cell line), and mechanically stimulated osteoblast conditioned media did not alter gene expression in MSCs demonstrating that this signaling is unique to osteocytes and MSCs. Finally, the upregulation in osteogenic genes in MSCs was not observed if primary cilia formation was inhibited prior to mechanical stimulation of the osteocyte. In summary, the results of this study indicate that soluble factors secreted by osteocytes in response to mechanical stimulation can enhance osteogenic gene expression in MSCs demonstrating a novel, unique signaling mechanism and introduces a role for the primary cilium in flow mediated paracrine signaling in bone thereby highlighting the cilium as a potential target for therapeutics aimed at enhancing bone formation.

1. Introduction

Bone turnover is a tightly coupled process of bone formation by osteoblasts (which derive from the mesenchymal lineage) and bone resorbing osteoclasts (which derive from the hematopoietic lineage). The recruitment, proliferation and bone forming activity of the mesenchymal stem cell (MSC) derived osteoblast is negotiated by numerous factors including hormonal, neuronal, immunological and mechanical [1,2]. In particular, mechanical loading has been shown to be a potent regulator of bone turnover, with over-loading resulting in increased bone formation and unloading resulting in bone resorption. Osteocytes are the most abundant cell type in bone and are largely believed to be coordinators of this mechanically induced bone remodeling [2]. Thus, it is somewhat surprising that although mechanically regulated signaling between osteocytes and osteoblast progenitors should exist, to date it has not been directly demonstrated.

The mechanical loading of bone and subsequently the cells residing within, stimulates the secretion of signaling molecules which has been shown to regulate the behavior of osteoblasts and osteoclasts [1,3]. Loading induced fluid flow within bone is believed to mechanically stimulate osteocytes and also act as a powerful mechanism to enhance molecular transport. A recent pioneering study by Tatsumi et al. provided compelling evidence that osteocytes are essential transducers of mechanical signals in bone, by demonstrating that mice, which had 80% of their osteocytes ablated, do not lose bone in a hind limb suspension model [2]. In addition, isolated osteocytes have been shown to be highly mechanosensitive [4] and can alter the production and secretion of signaling molecules such as PGE_2, ATP and NO in response to an applied physical stimulus [5,6]. Molecules secreted by osteocytes have been shown to regulate the activity of numerous cell types [7]. Conditioned media from fluid flow stimulated osteocytes inhibits osteoclastogenesis and can enhance the differentiation of osteoblasts [8,9]. However, physical contact between

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the osteocyte cell process and the osteoblast via gap junctions was a prerequisite for this response [10]. Given that osteocytes are spatially distinct from the marrow cavity, signaling between mechanically stimulated osteocytes and osteoblast progenitors within the marrow is likely to be paracrine in nature.

The primary cilium is a solitary cellular extension, which has recently emerged in recent times as a nexus of extracellular signal sensing regulating numerous tissues including bone [11–13]. Extending from the surface of a cell in an antenna like fashion, the cilium has been shown to act as a mechanical sensor in cell types including kidney and liver epithelial cells, endothelial cells and, more recently, in bone cells [14,15]. Removal of the primary cilium in osteoblasts and osteocytes has been shown to inhibit mechanically induced increases in COX-2 mRNA expression [15,16]. COX-2 is a synthase required for prostaglandin-E_2 (PGE_2) production which is known to be a key signaling protein produced by osteocytes [17]. Furthermore, specific removal of primary cilia from bone cells *in vivo* results in reduced loading induced bone formation [18]. Taken together, these data demonstrate that the primary cilium plays an important role in bone turnover by partially mediating bone cell mechanosensing and transmitting this stimulus into a biochemical bone forming response.

Given that mechanical loading of bone results in the recruitment and osteogenic differentiation of MSCs and the osteocyte has been proposed as a coordinator of mechanically regulated bone turnover it is reasonable to assume that mechanically stimulated osteocytes signal to MSCs within the marrow cavity triggering a bone forming response. In this study we demonstrate that mechanically stimulated osteocytes signal to MSCs in a paracrine manner enhancing osteogenic gene expression. Furthermore, we show that this signaling is specific to osteocytes, as mechanically stimulated osteoblasts did not elicit a similar response in MSCs. Finally we demonstrate that the osteocyte primary cilium is required for mechanosensation and subsequent secretion of soluble osteogenic factors, thereby enhancing MSC differentiation.

2. Materials and methods

2.1. Cell culture

Three cell lines were used in this study; the MLO-Y4 cell line (gift from Lynda Bonewald, U. of Missouri) which has characteristics similar to osteocytes; the MC3T3-E1 cell line which is a model of an osteoblast; and the C3H10T1/2 cell line which is a mesenchymal progenitor. MLO-Y4 cells were maintained in α-MEM supplemented with 5% fetal bovine serum (FBS), 5% calf serum (CS) and 1% penicillin–streptomycin (P/S). MC3T3-E1 cells were maintained in α-MEM supplemented with 10% FBS and 1% P/S and C3H10T1/2 cells were maintained in DMEM low glucose supplemented with 10% FBS and 1% P/S.

For each experiment, MLO-Y4 cells were seeded at 4000 cells/cm^2 on type-1 collagen (0.15 mg/ml, BD) coated flasks. 48 h following seeding, media was changed and the cells were cultured statically or mechanically stimulated (see below) for an additional 24 h at which point the osteocyte conditioned media was collected. MC3T3 and C3H10T1/2 cells were seeded at 2000 and 1000 cells/cm^2, respectively 48 h prior to receiving osteocyte conditioned media.

2.2. Cellular mechanical stimulation

Dynamic fluid flow was generated by placing rectangular (82 × 92 mm; 10 ml of media) flasks on a rocking platform which oscillated at a frequency of 0.5 Hz and with an amplitude of 1.5 cm (Fig. 1A). This system has been shown to generate spatiotemporal fluid flow induced shear stress across a layer of cells [19] that is partially representative of that experienced by osteocytes within the lacuno-canalicular network in bone. Most importantly for this experiment, this system prevents dilution of soluble factors, which are secreted by cells under flow, and is therefore ideally suited for the study of paracrine signaling mechanisms among mechanically stimulated cells. In all experiments, fluid flow was applied for a duration of 24 h.

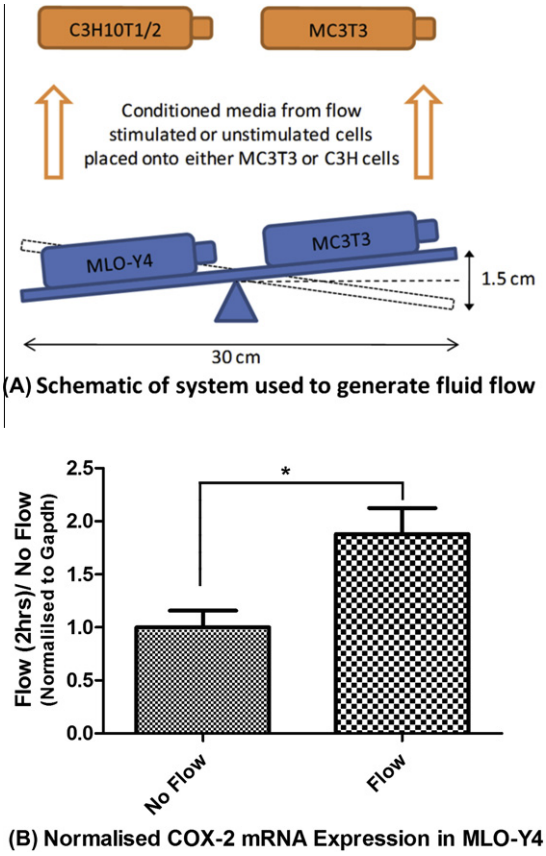

Fig. 1. (A) Experimental design and schematic of system used to generate low magnitude spatiotemporal fluid flow. Both MLO-Y4 and MC3T3 cells were subjected to dynamic fluid flow stimulation by placing rectangular flasks on a rocking platform. (B) mRNA levels of COX-2 in MLO-Y4 cells following 2 h of fluid flow using the system illustrated in (A). * indicates flow mRNA levels is significantly higher than no flow levels ($p < 0.05$).

2.3. RNA interference

The formation of primary cilia was inhibited using siRNA targeting Polaris (component of intraflagellar transport required for ciliogenesis [20]). MLO-Y4 cells were transfected with 20 μM siRNA targeting Polaris (sequence: 5′-CCAGAAACAGATGAGGACGACCTTT-3′) or 20 μM scrambled control siRNA (Invitrogen) using Lipofectamine 2000 (Invitrogen). Medium was changed 8 h after the transfection, and experiments were performed 48 h following transfection. Scrambled siRNA and siRNA targeting Polaris had no significant effect on cellular morphology. Successful knockdown of Polaris mRNA was validated using qRT-PCR (see below).

2.4. Real-time RT-PCR

Total RNA was extracted using Tri-Reagent (Sigma) and cDNA was synthesized using Taqman reverse transcriptase (Applied Biosystems). The resulting cDNA was subjected to real-time PCR analysis using the relative standard curve method (ABI Prism 7900

sequence detection system) as in Malone et al. [16]. Taqman PCR Master Mix and 20× Primers and Probes for GAPDH (4352339E), COX-2 (Mm00478374_m1), OPN (Mm00436767_m1), RUNX2 (Mm00501584_m1) and Polaris (Mm00493675_m1) were obtained from Applied Biosystems. GAPDH was used as an endogenous control in all experiments. All sample and standards were run in triplicate.

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism (Version 5.0) software with 3–10 samples analyzed within each experimental group. All data are expressed as means ± standard error. Two-way ANOVA was used for analysis of variance with a Bonferroni post-hoc test to compare between groups. For two sample comparisons, a student's *t*-test was used.

3. Results

3.1. Cellular mechanical stimulation applied via a rocking platform is sufficient to elicit an osteogenic response in osteocytes

Two hours of mechanical stimulation using the system described above resulted in a two-fold upregulation of COX-2 gene expression in MLO-Y4 cells demonstrating that this system is capable of eliciting a mechanically regulated response in this cell line (Fig. 1B).

3.2. Conditioned media from mechanically stimulated osteocytes promotes the osteogenic differentiation of MSCs but not osteoblasts

Mechanically stimulated osteocyte conditioned media significantly upregulated OPN mRNA levels after both 2 and 24 h treatment and COX-2 mRNA levels after 24 h treatment in the C3H10T1/2 cell line compared to cells treated with unstimulated conditioned media. No change was found in RUNX-2 mRNA expression at any time point (Fig. 2). The same mechanically stimulated osteocyte conditioned media did not alter mRNA expression in MC3T3 cells compared to MC3T3 cells treated with unstimulated osteocyte conditioned media.

To verify that this response was attributable to molecules specifically secreted by osteocytes, conditioned media from mechanically stimulated MC3T3 cells was used to treat C3H10T1/2 and MC3T3 cells for 2 and 24 h. No significant effect was found

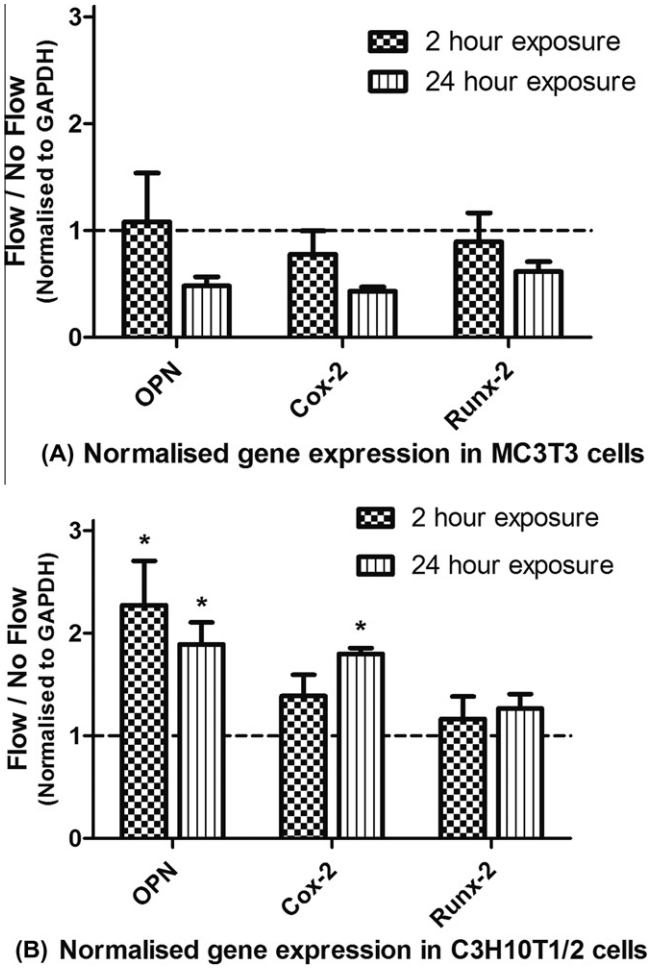

Fig. 2. mRNA levels of OPN, COX-2 and RUNX-2 in (A) MC3T3 cells and (B) C3H10T1/2 cells after either 2 or 24 h of supplementation with flow stimulated osteocyte conditioned media (CM). Expression levels from cells supplemented with flow stimulated CM has been normalized to those supplemented with CM from cells not subjected to flow stimulation. * indicates flow expression is significantly higher than no flow expression ($p < 0.05$).

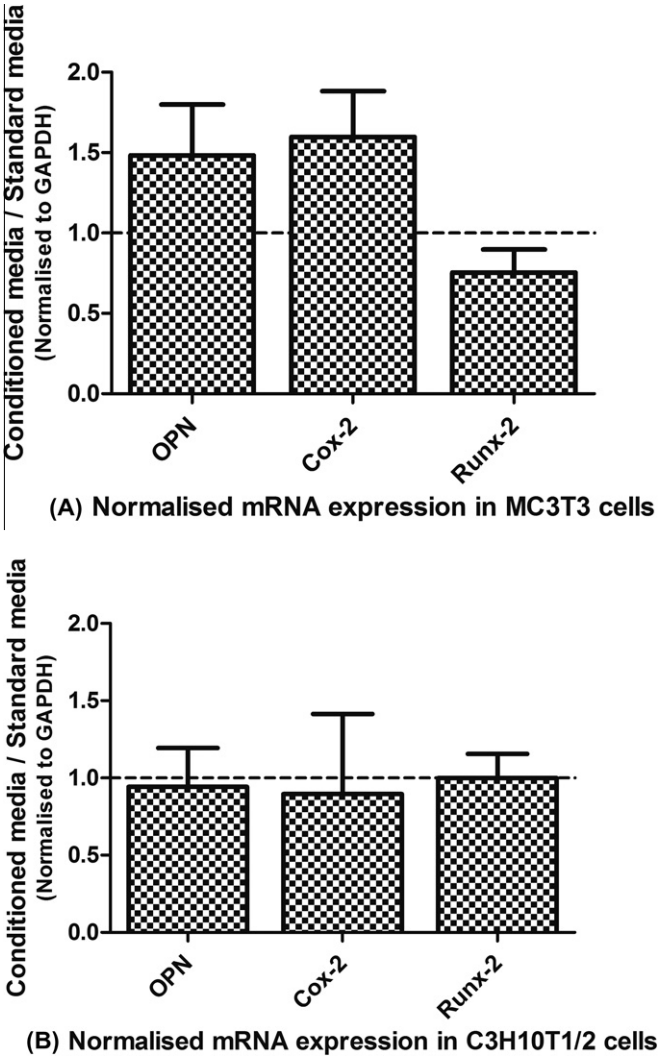

Fig. 3. mRNA levels of OPN, COX-2 and RUNX-2 in (A) MC3T3 cells and (B) C3H10T1/2 cells. Expression levels from cells supplemented with conditioned media from MLO-Y4 cells have been normalized to those supplemented with standard MLO-Y4 growth media.

on the expression levels of all genes investigated at both time points and in either cell type (Fig. S1).

3.3. Conditioned media from statically cultured osteocytes does not promote osteogenic differentiation of osteoblasts or MSCs

Media was conditioned by statically cultured MLO-Y4 cells for 24 h and placed onto either MC3T3 or C3H10T1/2 cells. After 24 h of treatment with osteocyte conditioned media, there was no significant change in OPN, COX-2 and RUNX-2 mRNA expression in either the MC3T3 or C3H10T1/2 lines compared to standard MLO-Y4 growth media (Fig. 3).

3.4. Transfection of MLO-Y4 cells with siRNA targeting POLARIS significantly downregulates POLARIS mRNA expression

mRNA expression of POLARIS was reduced by 95% in cells treated with siRNA targeting Polaris compared to cells treated with a scrambled siRNA control (Fig. 4A). We have previously shown that this reduction in mRNA corresponds to reduced protein expression and number of ciliated cells [15,16].

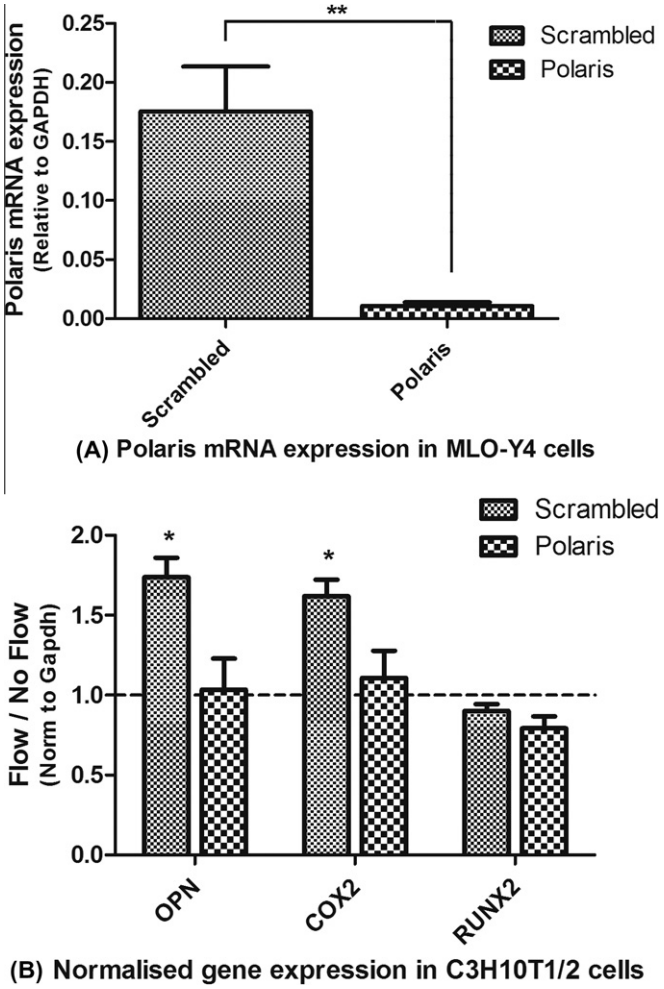

Fig. 4. (A) mRNA levels of Polaris in MLO-Y4 cells treated with scrambled siRNA and siRNA targeting Polaris. (B) mRNA levels of OPN, COX-2 and RUNX-2 in C3H10T1/2 cells after 24 h supplementation of flow stimulated osteocyte conditioned media. Expression levels from cells supplemented with flow stimulated CM from MLO-Y4 cells with (scrambled) and without (Polaris) primary cilia has been normalised to those supplemented with CM from cells not subjected to flow stimulation. *Indicates flow expression is significantly higher than no flow expression.

3.5. Osteogenic differentiation of MSCs in response to mechanically stimulated osteocyte conditioned media is dependent on the osteocyte primary cilium

Osteocytes treated with siRNA targeting POLARIS or scrambled siRNA were exposed to mechanical stimulation for 24 h and the flow stimulated osteocyte conditioned media was placed on C3H10T1/2 cells for 24 h as before. The conditioned media from mechanically stimulated MLO-Y4 cells treated with scrambled siRNA elicited a similar response in the C3H10T1/2 cells as above, significantly upregulating OPN and COX-2 mRNA levels over no flow controls. However, the conditioned media from MLO-Y4 cells treated with siRNA targeting Polaris did not result in any change in mRNA expression in C3H10T1/2 cells (Fig. 4B).

4. Discussion

This study investigated the effect of mechanically stimulated osteocyte conditioned media on the osteogenic differentiation of mesenchymal stem cells. A significant outcome of this study is that soluble factors secreted by mechanically stimulated osteocytes enhanced the osteogenic differentiation of MSCs, yet did not affect gene expression in osteoblasts. In addition, we demonstrated that the osteocyte primary cilium was required for mechanosensation and subsequent secretion of signaling factors from the osteocyte. These data demonstrate, for the first time, a selective paracrine signaling mechanism between terminally differentiated mechanically stimulated osteocytes and mesenchymal stem cells and furthermore contributes to the emerging picture of the role of the primary cilium in bone turnover, thereby highlighting the cilium as a potential target for therapeutic treatments aimed at enhancing bone formation.

Osteogenic differentiation was assayed by increased expression of the markers OPN and COX-2. OPN is a non-collagenous extracellular bone matrix protein that plays important roles in cell adhesion and migration [21]. OPN levels have been shown to be reduced in MSCs isolated from osteoporotic rats [22]. Furthermore, MSC migration has been shown to be significantly reduced following the treatment of MSCs with OPN neutralizing antibodies [23]. COX-2, through its enzymatic production of PGE_2, is critically involved in MSC osteogenic differentiation and bone formation. COX-2 knockout mice demonstrate significantly delayed tibia fracture repair resulting from a reduced capacity for MSC osteogenic differentiation [24]. This defect in differentiation was rescued by the addition of PGE_2. In addition, PGE_2 treatment in rats stimulates cancellous bone formation *in vivo* and correlates with enhanced MSC osteogenic differentiation [25]. Therefore the upregulation of both OPN and COX-2 strongly indicate an enhanced ability of MSCs to adhere, migrate and differentiate into bone forming osteoblasts.

Surprisingly, osteocyte conditioned media did not alter mRNA expression of RUNX2 in MSCs. RUNX2 is considered to be a central control transcription factor in the osteoblast phenotype directly binding to and regulating the transcription of numerous osteogenic genes such as osteocalcin [26]. It was originally believed that RUNX2 regulation was solely mediated by mRNA/protein levels. However, data demonstrating biochemical induced increases in osteocalcin and bone sialoprotein expression was not accompanied by comparable changes in levels of RUNX2 mRNA or protein [27]. Recent studies have revealed a high degree of RUNX2 phosphorylation during the differentiation process which may regulate its binding activity [28]. Therefore, although no change was found in RUNX2 mRNA levels in this study, the activity of RUNX2 may be altered by osteocyte conditioned media and was simply not reflected in altered expression level.

Interestingly, the same soluble factors which enhanced osteogenic gene expression in MSCs did not influence the expression of osteogenic genes in osteoblasts. Therefore these data indicate the presence of a novel paracrine signaling mechanism of communication between mechanically stimulated terminally differentiated osteocytes and MSCs bypassing the osteoblast. This finding also provides indirect support for the hypothesis that direct cell-cell physical contact is necessary for signaling between flows stimulated osteocytes and osteoblasts as previous studies have only demonstrated enhanced osteoblast activity when the two cell types are connected via gap junctions [10]. Furthermore, conditioned media from flow stimulated osteoblasts did not significantly affect osteogenic gene expression in MSCs, suggesting that this novel mechanism is unique to osteocytes and MSCs. This therefore provides a possible mechanism by which mechanical loading of bone *in vivo*, resulting in the stimulation of osteocytes, results in the osteogenic differentiation of MSCs within the bone marrow cavity [29].

Osteocytes are largely believed to be mechanosensing cells in bone. However, the exact mechanism by which osteocytes sense biophysical stimuli and translate those stimuli into a biochemical response is still largely unknown. Recently, our lab among others has demonstrated that the osteocyte primary cilium plays an important mechanosensory role in bone turnover [11,13,15,16]. The results of this study also suggest that the osteocyte primary cilium is required to sense spatiotemporal flow and is necessary for the mechanically mediated secretion of signaling molecules from osteocytes resulting in the enhanced osteogenic gene expression in MSCs. This therefore highlights the primary cilium as a potential therapeutic target aimed at enhancing bone formation.

The signaling molecule(s) involved in this osteocyte-MSC signaling mechanism is an area of active research. A likely candidate based on our findings is the COX-2 mediated production of PGE_2. PGE_2 treatment *in vivo* has been shown to stimulate bone formation [30] at a similar rate to that observed in response to mechanical loading [29]. Furthermore, treatment of bone marrow cells with PGE_2 *in vitro* results in increased mineralized nodule formation and enhanced alkaline phosphatase activity [25]. Therefore, based on the upregulation of COX-2 after 2 h of mechanical stimulation in MLO-Y4 cells (Fig. 1B) and on our previous findings that primary cilium removal in osteocytes inhibits the flow mediated increase in COX-2 expression, our data points to PGE_2 as a possible signaling molecule secreted by osteocytes that result in the osteogenic differentiation of MSCs. However future research is required to completely delineate the molecule(s) involved in this signaling mechanism.

In conclusion, this study has demonstrated a novel, selective paracrine signaling mechanism between mechanically stimulated osteocytes and mesenchymal stem cells. This, therefore, suggests a possible mechanism by which mechanical loading of bone and subsequently the osteocytes residing within, results in the osteogenic differentiation of MSCs within the marrow cavity. Furthermore, this study has demonstrated that osteocytes utilize the primary cilium in a mechanosensory role, which upon stimulation results in the secretion of soluble factors which can uniquely enhance the osteogenic differentiation of MSCs. These data contribute to the emerging picture of primary cilia as a nexus of extracellular signal sensing and, once again, highlights the primary cilium as a potential therapeutic target for efforts to prevent bone loss during diseases such as osteoporosis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.07.072.

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