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Mechanically stimulated bone cells secrete paracrine factors that regulate osteoprogenitor recruitment, proliferation, and differentiation

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ABSTRACT

Bone formation requires the recruitment, proliferation and osteogenic differentiation of mesenchymal progenitors. A potent stimulus driving this process is mechanical loading, yet the signalling mechanisms underpinning this are incompletely understood. The objective of this study was to investigate the role of the mechanically-stimulated osteocyte and osteoblast secretome in coordinating progenitor contributions to bone formation. Initially osteocytes (MLO-Y4) and osteoblasts (MC3T3) were mechanically stimulated for 24hrs and secreted factors within the conditioned media were collected and used to evaluate mesenchymal stem cell (MSC) and osteoblast recruitment, proliferation and osteogenesis. Paracrine factors secreted by mechanically stimulated osteocytes significantly enhanced MSC migration, proliferation and osteogenesis and furthermore significantly increased osteoblast migration and proliferation when compared to factors secreted by statically cultured osteocytes. Secondly, paracrine factors secreted by mechanically stimulated osteoblasts significantly enhanced MSC migration but surprisingly, in contrast to the osteocyte secretome, inhibited MSC proliferation when compared to factors secreted by statically cultured osteoblasts. A similar trend was observed in osteoblasts. This study provides new information on mechanically driven signalling mechanisms in bone and highlights a contrasting secretome between cells at different stages in the bone lineage, furthering our understanding of loading-induced bone formation and indirect biophysical regulation of osteoprogenitors.

1. Introduction

Osteoporosis is a silent bone disease that can result in low-energy impact fractures with significant morbidity and associated mortality rates [1]. Treatment of osteoporosis has traditionally aimed to offset bone loss through medications that inhibit catabolism [2]. However, bone anabolic therapeutics may act as an important adjunct for advanced osteoporosis to regenerate lost tissue [3,4]. Given the limited lifespan of the osteoblast [5], bone anabolism requires the continued replenishment of the exhausted osteoblast from an osteoprogenitor population [6–8]. However, little is known regarding the biochemical cues that mediate replenishment from the stem cell niche. Therefore, an increased understanding of bone anabolistic regulatory mechanisms that enhance osteoprogenitor recruitment, proliferation and osteogenesis is required [9].

A potent regulator of bone anabolism is mechanical loading [10]. This involves a complex interplay between multiple cell types, with osteogenic cues believed to be propagated to osteoprogenitors via upstream mechanosensor cells. Recent evidence suggests that this upstream role is predominantly fulfilled by osteocytes [11,12], which are ideally numbered and positioned within their lacuno-canalicular network to coordinate mechanically-mediated secretion of biological signalling factors [12–14]. The mechanism by which osteocytes communicate with neighbouring precursor and/

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or effector cells is a topic of significant ongoing research [15]. It has been demonstrated that osteocytes communicate with osteoblasts via gap junctions [16], and osteoclasts via a paracrine mechanism [17,18], in both static and loaded conditions to regulate the bone remodelling cycle. Furthermore, it has also been shown that osteocytes can induce stem cell osteogenesis in static conditions [19], and recently we have demonstrated that mechanically stimulated osteocytes can induce osteogenic gene expression in MSCs via a paracrine mechanism [20]. These data taken together form a strong argument for the role of the osteocyte as a co-ordinator of bone formation via control of precursor cells, particularly in response to mechanical stimulation. In spite of the mounting evidence of the osteocyte as the key regulator of this process, there is little knowledge of its interplay with the stem cell niche, particularly under physically loaded conditions [15].

Interestingly, bone anabolism in response to loading is maintained in mouse models with depleted osteocytes suggesting a potential coordination by another mechanosensitive cell [21]. Such anabolism may be regulated by osteoblasts which are known to be mechanically sensitive [22] and interact with other cell types [23]. Osteoblasts at the site of loading would be expected to interact with neighbouring and recruited bone forming cells resulting in a co-ordinated anabolic response to loading [24]. Given the lineage progression of osteoblasts towards osteocytes one could infer a comparable secretome between osteoblasts and osteocytes although to date, there is an absence of data indicating the cellular effects of the osteoblast secretome upon bone forming cells.

The objective of this study was therefore to investigate the role of mechanically stimulated osteocytes and osteoblasts in directing the behaviour of bone forming cells. The hypothesis of this study is that soluble factors secreted by mechanically stimulated osteocytes will regulate MSC recruitment, proliferation and osteogenesis. We postulate that these same secreted factors will, in addition, regulate the recruitment and proliferation of osteoblasts. We also hypothesise that osteoblasts, in turn, will also regulate the recruitment and proliferation of bone forming cells in response to loading. By obtaining a greater understanding of the mechanisms underpinning bone formation through mechanical loading, we will further our understanding of bone physiology and which may ultimately direct us to novel anabolic bone therapeutics.

2. Materials and methods

2.1. Cell culture

Three cell lines were used in this study. Firstly, the MLO-Y4 cell line which is a murine derived model of an osteocyte (gift from Dr. Lynda Bonewald, University of Missouri–Kansas City, MO, USA). Secondly, the murine MC3T3 cell line (ATCC, Manassas, VA) which has pre-osteoblastic characteristics and finally, the C3H10T1/2 cell line (ATCC, Manassas, VA) which is a murine mesenchymal progenitor. MLO-Y4 cells were cultured on rat tail collagen (BD Biosciences, Bedford, MA) coated cell culture plastic with α-Modified Eagle's Media (α-MEM) supplemented with 5% calf serum (CS), 5% fetal bovine serum (FBS), 1% L-Glutamine, and 2% penicillin–streptomycin (P/S). MC3T3 cells were cultured in α-MEM supplemented with 10% FBS, 1% L-Glutamine and 2% P/S. C3H10T1/2 cells were cultured in D-MEM low glucose supplemented with 10% FBS, 1% L-Glutamine and 2% P/S (All media supplements from Sigma–Aldrich unless otherwise stated).

2.2. Cellular mechanical stimulation

The generation of dynamic fluid flow was achieved through the utilisation of a rocking platform which oscillated at a frequency of 0.5 Hz and with a tilt angle of 7° consistent with previously published work [20,25]. This system is used in order to generate large amounts of conditioned media when compared to other experimental apparatus such as the parallel flow plate chamber [26]. MLO-Y4 cells were cultured in T-175 flasks with a total of 10 ml media in each flask. Those undergoing mechanical stimulation were placed on the platform for a 24hr period. Mechanical stimulation of MC3T3 cells was also carried out as described above.

2.3. Experimental setup

For each experimental assay described below, MLO-Y4 cells were seeded at a density of 1,200cells/cm2 on type-1 collagen (0.15 mg/ml, BD) coated T-175 flasks (Sarstedt, Nuembrecht, Germany) and cultured in normal growth media under standard conditions (37C, 5% carbon dioxide). 72hr following seeding, cells were washed, the media were changed and the cells were cultured statically (No Flow) or mechanically stimulated (Flow) for an additional 24hr. After this period the osteocyte conditioned media were collected. A media control, lacking any cellular exposure, was also generated (Media Control). All media samples were centrifuged at 1,200 rpm for 5 min to remove any cellular debris from the conditioned media. Experiments were repeated using MC3T3 cells as above. For MC3T3 experiments, T-175 flasks were not coated with collagen and the media control consisted of MC3T3 growth media.

2.4. Effect of mechanically stimulated cell media upon migration

Cellular chemotaxis in response to conditioned media collected from mechanically stimulated cells was assessed by utilising a Boyden chamber technique. Well inserts were placed into a 12-well plate and the cells to be studied (C3H10T1/2 or MC3T3) were cultured on such inserts for 2 h in their normal appropriate media. After this time, the inserts were removed and placed into wells containing MLO-Y4 conditioned media from each group (Flow, No Flow and Media Control) for a period of 18hr. Following this incubation period, the inserts were removed and fixed with 10% formalin solution before staining with haematoxylin. Light microscopy (Nikon Eclipse 90i, Nikon), using NIS Element software was then used to calculate the number of migrated cells.

2.5. Effect of mechanically stimulated cell media upon proliferation

To assess changes in proliferation in response to conditioned media collected from mechanically stimulated cells, cells to be studied (C3H10T1/2 or MC3T3) were seeded at a density of 31,000cells/cm2. Cells were cultured for a 24hr period, washed 3x with PBS and treated with conditioned media from the Flow, No Flow, or Media Control groups. After a further period of 72hrs the cells were centrifuged at 1,200 rpm for 5 min, media aspirated and lysis buffer (TritonTM X-1oo, Sigma–Aldrich, USA) added to each well. Following three freeze-thaw cycles the DNA concentration in each well was calculated using the PicoGreen assay (Invitrogen, USA).

2.6. Effect of mechanically stimulated cell media upon osteogenesis

Osteogenesis was determined using C3H10T1/2 cells seeded at a density of 31,000cells/cm2. Cells were cultured for a 24hr period, washed 3x with PBS and incubated with conditioned media from the Flow, No Flow, or Media Control groups. An additional positive control study group was prepared and incubated with standard osteocyte media with added osteogenic supplements - Dexamethasone 100 nM, Ascorbic acid 2-P 50 μM, and β-

glycerophosphate 10 mM (all Sigma–Aldrich, USA). Groups were cultured for 22 days with media changes every 3–4 days with freshly generated conditioned media prepared as described above. Final calcium concentration was determined using a photometric assay kit (Stanbio, USA).

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism (V5.0) (GraphPad Software, CA, USA) software. All data are expressed as means ± standard error. One-way analysis of variance (ANOVA) was used with a Bonferroni test to compare between groups. A probability value of 95% ($p < 0.05$) was used to determine significance.

3. Results

3.1. Osteocytes regulate MSC recruitment, proliferation and osteogenesis

To determine the effect of mechanically regulated soluble factors secreted by osteocytes upon MSC chemotaxis, proliferation and osteogenesis, MSCs were treated with MLO-Y4 conditioned media taken from mechanically stimulated (Flow) cells. Statically cultured (No Flow) and a growth media (Media Control) were also included as controls (see Fig. 1).

The factors released upon mechanical stimulation of MLO-Y4 cells elicit a chemotactic response in the C3H10T1/2 mesenchymal progenitor cells. The level of migration was significantly higher for the mechanically stimulated (Flow) compared to the statically conditioned (No Flow), 128 ± 9.7% increase ($n = 12$, $p < 0.05$), and Media Control groups, 94.6 ± 38.4% increase ($n = 9$-12, $p < 0.05$). No significant difference was found between the No Flow and Media Control groups. There was a significant increase in stem cell proliferation observed after a 72 h period of exposure to mechanically stimulated MLO-Y4 conditioned Flow compared to No Flow media, 56.6 ± 1.1% increase ($n = 9$, $p < 0.001$), and the Media Control, 21.8 ± 2.1% increase ($n = 9$, $p < 0.001$). There was also a significant difference observed between the No Flow and media control group, 22.1 ± 1% decrease ($n = 9$, $p < 0.001$). Regarding osteogenesis, the Flow group conditioned media resulted in significantly greater mineral when compared to the No flow, 31.6 ± 1.9% increase ($n = 3$, $p < 0.001$), and media control groups, 65.3 ± 1.2% increase ($n = 3$, $p > 0.001$). There was no significant difference between the No Flow group and the Media Control. In addition, the total level of mineral produced by the Flow group conditioned media was directly comparable to the level of the osteogenic supplemented media positive control.

These data taken together indicate that upon mechanical stimulation, osteocytes release factors capable of recruiting, stimulating proliferation, and directing the osteogenesis of MSCs.

3.2. Osteocytes regulate osteoblast chemotaxis and proliferation

To determine the effects of mechanically regulated soluble factors secreted by osteocytes upon osteoblast chemotaxis and proliferation, osteoblasts were treated with MLO-Y4 conditioned media taken from mechanically stimulated (Flow) cells. Statically cultured (No Flow) and a growth media (Media Control) were also included as controls (see Fig. 2).

Soluble factors released by mechanically stimulated MLO-Y4s elicit a chemotactic response in MC3T3 cells. Flow media generated a significantly greater level of migration in comparison to No Flow MLO-Y4 conditioned media, 98.7 ± 5.4% increase ($n = 12$, $p < 0.01$), and growth media control groups, 46.5 ± 0.8% increase ($n = 12$, $p < 0.5$). There was a decrease in migration in the No Flow group when compared to the Media Control, 26.27 ± 21.2% ($n = 12$).

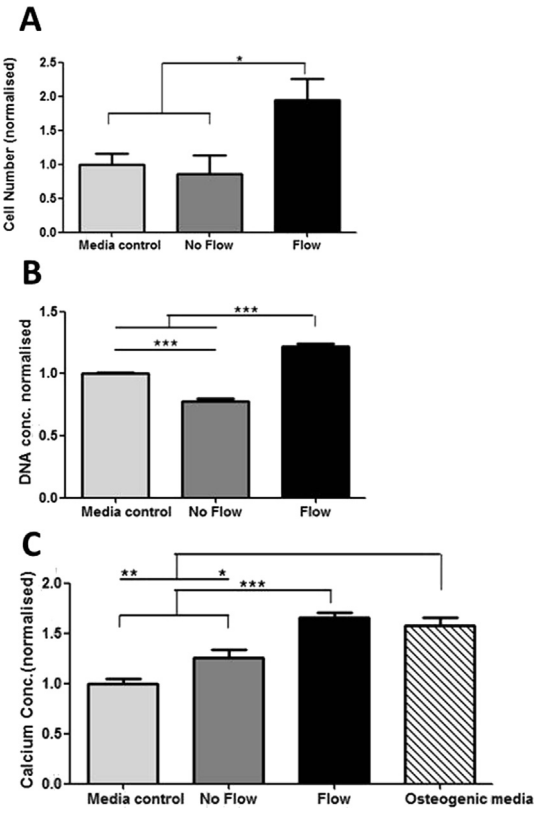

Fig. 1. The effect of mechanically stimulated osteocyte conditioned media on mesenchymal stem cell **(A)** migration, **(B)** proliferation, and **(C)** osteogenic differentiation compared to statically cultured osteocyte conditioned media and no cell controls.

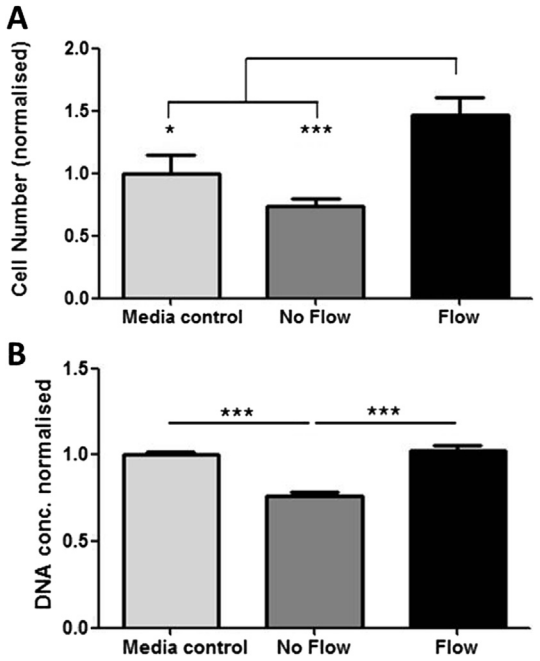

Fig. 2. The effect of mechanically stimulated osteocyte conditioned media on osteoblast **(A)** migration, and **(B)** proliferation compared to statically cultured osteocyte conditioned media and no cell controls.

There was also a significant increase in the level of proliferation in the MC3T3 cells exposed to Flow versus No Flow conditioned media respectively, 35.2 ± 1.5% increase ($n = 9$, $p < 0.001$). A significant difference was observed between the No Flow compared to the media control which demonstrated a 23.9 ± 1.3% ($n = 9$, $p < 0.001$) drop in total DNA content. Given that osteocyte conditioned media was capable of directing osteogenesis of stem cells through to a mineralizing phenotype, osteogenesis was not investigated in the pre-osteoblastic cells.

These data taken together indicate that mechanical stimulation of osteocytes triggers the release of soluble factors that can recruit and stimulate the proliferation of osteoblasts.

3.3. Osteoblasts regulate MSC chemotaxis and proliferation

To determine the effect of soluble factors secreted by mechanically stimulated osteoblastic cells upon MSC chemotaxis and proliferation, MSCs were treated with MC3T3 conditioned media taken from mechanically stimulated (Flow) and statically cultured (No Flow) cells. A growth media group (Media control) was also included (Fig. 3).

The factors secreted by mechanically stimulated osteoblasts elicit a chemotactic response in the C3H10T1/2 mesenchymal progenitor cells. Migration was significantly higher for the mechanically stimulated (Flow) versus the statically conditioned (No Flow) groups, 105.5 ± 4.8% increase ($n = 3$, $p < 0.001$), and the media control group, 49.4 ± 0.9% fold increase. The proliferation studies carried out using MC3T3 conditioned media showed contrasting results to that of the osteocyte conditioned media experiments presented above. The application of mechanically stimulated conditioned MC3T3 conditioned media was found to reduce the level of MSC proliferation when compared to both the statically conditioned, 51.7 ± 2.3% decrease ($n = 9$, $p < 0.001$), and to the Media Control group, 59.5 ± 4% decrease ($n = 9$, $p < 0.001$). There was also a drop in the No Flow group when compared to the Media Control, 16.1 ± 7.5% decrease ($n = 9$, $p < 0.05$).

These data taken together indicate that upon mechanical stimulation, osteoblasts release paracrine factors capable of recruiting and, in contrast to osteocyte factors, inhibit proliferation of MSCs. This therefore suggests that the bone cell secretome may alter during lineage progression.

3.4. Osteoblasts regulate chemotaxis and proliferation of osteoblasts

To investigate the effects of soluble factors secreted by mechanically stimulated osteoblasts upon osteoblast chemotaxis and proliferation, MC3T3 were treated with MC3T3 conditioned media taken from mechanically stimulated (Flow) and statically cultured (No Flow) cells in addition to a growth media control (Media Control) (Fig. 4).

Regarding chemotaxis, the MC3T3 Flow group elicited a migratory trend compared to the No Flow, 23.4 ± 13.7% increase ($n = 5–6$), and Media Control groups, 189.44 ± 10.48% increase ($n = 5$, $p < 0.001$). Interestingly, MC3T3 conditioned media (No Flow) alone was found to enhance recruitment of other osteoblasts, 134.5% ± 3.5% ($n = 5–6$) increase, when compared to the Media Controls. Regarding proliferation, there was a reduction in proliferation of osteoblasts in the Flow group when compared to No Flow, 59.0 ± 0.5% decrease ($n = 9$, $p < 0.001$) and media controls, 57.0 ± 1.4% ($n = 9$, $p < 0.001$).

These data together indicate that statically cultured osteoblasts release soluble factors capable of recruiting fellow osteoblasts. In addition, upon osteoblast mechanical stimulation this recruitment is further enhanced. Regarding proliferation, these data illustrate an inhibition of proliferation due to soluble factors released upon mechanical stimulation of osteoblasts.

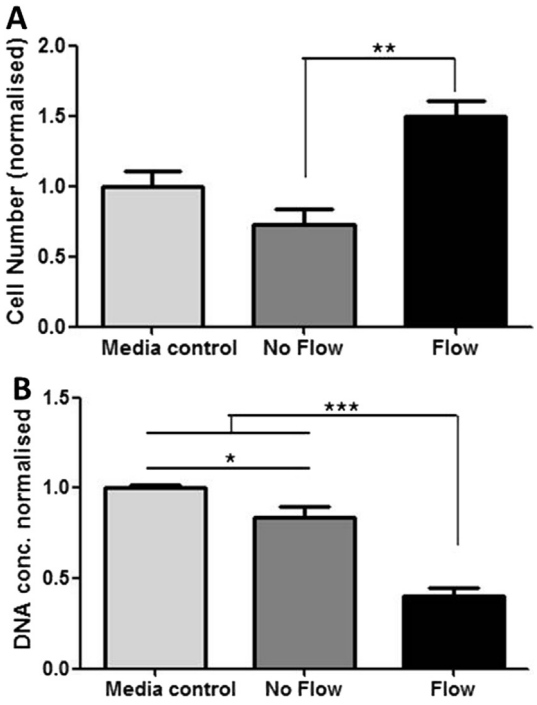

Fig. 3. The effect of mechanically stimulated osteoblast conditioned media on mesenchymal stem cell **(A)** migration, and **(B)** proliferation compared to statically cultured osteoblast conditioned media and no cell controls.

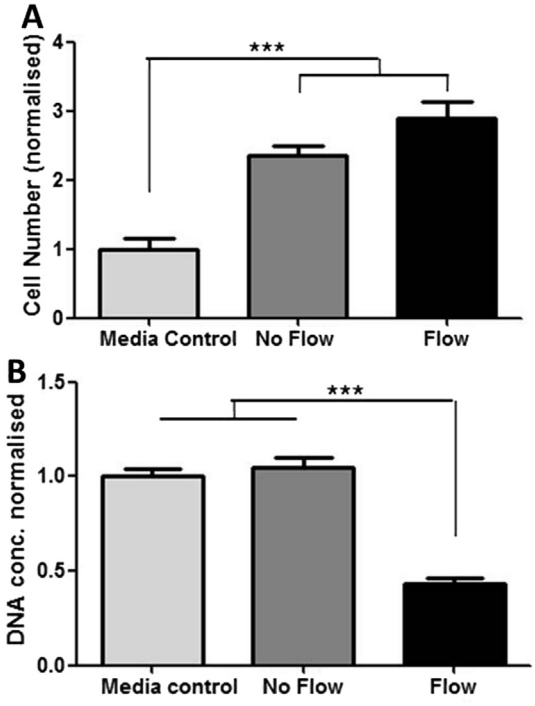

Fig. 4. The effect of mechanically stimulated osteoblast conditioned media on osteoblast **(A)** migration, and **(B)** proliferation compared to statically cultured osteoblast conditioned media and no cell controls.

4. Discussion

Bone formation in response to mechanical loading requires the recruitment, proliferation and differentiation of osteoprogenitors. The overall objective of this study was therefore to investigate the role of osteocytes, and osteoblasts, in mediating these effects in response to mechanical stimulation. A significant outcome of this study is that, for the first time, osteocytes upon mechanical stimulation have been shown to recruit, enhance proliferation, and direct osteogenesis of mesenchymal stem cells. In addition these same factors recruit and stimulate proliferation of osteoblasts. We speculate that upon mechanical stimulation, osteocytes secrete a number of paracrine factors which may be responsible for directing the migration, proliferation and osteogenesis of stem cells to replenish exhausted osteoblasts, enhancing bone formation and strengthening the tissue. Furthermore, we have also shown that osteoblasts, upon mechanical stimulation, can also direct migration of both MSCs and other osteoblasts. However in contrast to osteocytes, we have shown that conditioned medium from mechanically stimulated osteoblasts results in a decreased proliferative rate in MSCs and osteoblasts suggesting a negative feedback mechanism regulating the bone forming process. This study provides new information on mechanically driven signalling mechanisms in bone and highlights a contrasting secretome between cells at different stages in the bone lineage, furthering our understanding of loading-induced bone formation.

Our study demonstrated that the secretome of the mechanically stimulated osteocyte contains paracrine factors which can recruit osteoprogenitors (MSCs and osteoblasts) from the niche to the site of loading, enhance proliferation of progenitors to ensure sufficient numbers and lastly promote the osteogenic lineage commitment of these cells. Whilst little is known regarding the recruitment of stem cells from the niche to a site of loading, these data are supported by the *in vivo* observations by Turner et al. [27], of mechanical loading-induced recruitment of proliferating cells from the marrow to the bone surface enhancing bone formation. Robust proliferative effects of statically cultured osteocyte conditioned media upon bone marrow progenitors and osteoblasts has been demonstrated previously [28], however the enhanced proliferative response observed with the application of mechanically stimulated osteocyte conditioned media indicates that these molecules are mechanically regulated. Regarding differentiation, this data agrees with previous works that demonstrated the osteogenic potential of osteocyte conditioned media upon stem cells [19,28], whilst the enhanced osteogenesis observed with addition of flow media is supported by work previously undertaken in our laboratory demonstrating enhanced early gene expression in MSCs [20]. This *in vitro* demonstration of indirect biophysical regulation of osteoprogenitors further highlights the important upstream mechanosensory role of the osteocyte and the importance of physical loading in regulating bones anabolic response [11,12,15,29].

Interestingly, we also demonstrated that the secretome of the mechanically stimulated osteoblast contains paracrine factors which can recruit osteoprogenitors (MSCs and osteoblasts) from the niche but surprisingly inhibits the proliferation of such progenitors. Therefore, as the osteoblast is recruited to the bone surface, the resident osteoblasts may act to nullify the proliferative effect of the osteocyte secretome and synergistically with the osteocyte enhance differentiation. Such a synergistic mechanism has been demonstrated previously *in vitro* [19]. Similar to osteocytes, mechanically stimulated osteoblasts also secrete factors that lead to an increase in the migration of both progenitor cells and other osteoblasts. Interestingly, even statically conditioned osteoblast media elicited a migratory response when compared to the media control indicating a basal level of osteoblast-mediated osteoblast recruitment, perhaps aiding in the maintenance of a bone osteoblast niche. The contrasting effects of the osteoblasts upon osteoprogenitor proliferation are surprising given the common lineage progression to the osteocyte phenotype. However, our observations are supported by previous findings, where osteoblast conditioned media was found to have a contrasting effect to osteocyte conditioned media [28]. Despite the common ancestry, it is clear that the secretome of the osteoblast and osteocyte are distinct, but may act in a synergistic fashion, to maintain a sufficient number of active bone forming cells at a site of loading ensuring a robust bone anabolistic response.

The biochemical factors within the osteocyte and osteoblast secretome that regulate osteoprogenitor behaviour are poorly understood. There are many signalling molecules secreted by osteocytes in response to mechanical stimulation, including NO, ATP, PGE2, SOST, DKK1, IGF-1, SDF-1, and FGF23, all of which have been linked to bone anabolism [11]. Interestingly, two key osteocyte secreted factors (sclerostin and FGF23) are not produced by MLO-Y4s, indicating they these are not contributing to our findings [30]. Osteoblasts produce a wealth of soluble factors that play diverse roles in bone growth and repair [31,32]. It is clear that the mechanically regulated paracrine mechanisms controlling migration, proliferation and osteoblastic lineage commitment of MSCs and osteoblasts are numerous and complex, involving many cells types and bioactive secreted factors. Although it is unclear what factors are regulating the mechanisms demonstrated herein, this is an active area of research, and may hold the key to identifying novel anabolic therapeutics for osteoporosis.

A limitation to this study was the use of MLO-Y4 osteocyte-like cells instead of primary osteocytes. Whilst these cells are widely used in the study of osteocyte biology, they lack certain osteocyte characteristics such as the production of sclerostin. However, the observation of the above effects, in a sclerostin free experimental system, adds to our understanding of loading-induced bone formation.

In conclusion, this study has demonstrated signalling mechanisms in bone that together, act to increase the number of available bone forming cells at a site of loading. This adds to the growing evidence that osteocytes are co-ordinators of loading-induced bone formation. Furthermore, we have also highlighted the contrasting role of the osteoblast within this process and hypothesise a paracrine feedback mechanism between osteoblasts and the principal signalling osteocytes. These data contribute to furthering our understanding of loading-induced bone formation, highlighting the importance of mechanical cues in maintaining bone density, and in the future, may direct us to novel anabolic bone therapeutics.

Conflict of interest

None.

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