Strain uses gap junctions to reverse stimulation of osteoblast proliferation by osteocytes

Rosemary F.L. Suswillo | Behzad Javaheri | Simon C.F. Rawlinson | Gary P. Dowthwaite | Lance E. Lanyon | Andrew A. Pitsillides

Identifying mechanisms by which cells of the osteoblastic lineage communicate in vivo is complicated by the mineralised matrix that encases osteocytes, and thus, vital mechanoadaptive processes used to achieve load-bearing integrity remain unresolved. We have used the coculture of immunomagnetically purified osteocytes and primary osteoblasts from both embryonic chick long bone and calvariae to examine these mechanisms. We exploited the fact that purified osteocytes are postmitotic to examine both their effect on proliferation of primary osteoblasts and the role of gap junctions in such communication. We found that chick long bone osteocytes significantly increased basal proliferation of primary osteoblasts derived from an identical source (tibiotarsi). Using a gap junction inhibitor, 18β-glycyrrhetinic acid, we also demonstrated that this osteocyte-related increase in osteoblast proliferation was not reliant on functional gap junctions. In contrast, osteocytes purified from calvarial bone failed to modify basal proliferation of primary osteoblasts, but long bone osteocytes preserved their proproliferative action upon calvarial-derived primary osteoblasts.

We also showed that coincubated purified osteocytes exerted a marked inhibitory action on mechanical strain–related increases in proliferation of primary osteoblasts and that this action was abrogated in the presence of a gap junction inhibitor. These data reveal regulatory differences between purified osteocytes derived from functionally distinct bones and provide evidence for mechanisms by which purified osteocytes communicate with primary osteoblasts to coordinate their activity.

**KEYWORDS**
coculture, gap junctions, mechanical strain, osteoblasts, osteocytes

Moreover, within minutes following mechanical strain, osteocytes upregulate endothelial NO synthase and cyclooxygenase 2 expression, which in turn stimulate NO and prostaglandin E\textsubscript{2} (PGE\textsubscript{2}) release, respectively. These are important early cellular changes that regulate downstream events including production of anabolic factors such as insulin-like growth factor 1 and matrix molecules including dentin matrix protein 1. On the basis of such studies, it has frequently been proposed that osteocyte sensitivity to applied loads, sensed either as strain, flow, or some other sequelae of loading, provides the controlling input in the postulated “mechanostat,” which confers bone with its mechanoadaptive capacity.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

Copyright © 2017 The Authors Cell Biochemistry and Function published by John Wiley & Sons Ltd.
Osteocytes are confined to lacunae, however, and can make little if any direct contribution to the architectural adaptive bone (re)modeling activities that load-related strains might stimulate. It is assumed therefore that their influence is achieved via their control of the remodelling activity of osteoclasts (via osteoblasts and lining cells) and osteoblasts on the bone surface. A potential route by which osteocytes could influence the behaviour of overlying osteoblasts in response to external mechanical stimuli is via the passage of small molecules through the osteoblast: osteocyte network of gap junctions or via molecules secreted into the intralacunar fluid. This fluid bathes osteocytes and the bone-facing processes of osteoblasts and lining cells, and its movement through canaliculi results from the pressure differentials induced by dynamic loads. The repetitive bending of the bone matrix is thought to generate a “pumping” action forcing fluid to the bone surface and subsequent dynamic shear strains on osteocyte processes.10,23–28

Previous studies have shown that gap junctions are expressed in all different types of bone cells23–35 and are likely candidates for chemical information transfer between bone cells,36,37 providing evidence that gap junction communications are potentially important in mechanotransduction.35,37–42

Recent studies have examined the effects of fluid shear applied to an osteocytic cell line derived from long bone (MLO-Y4)35,37,41–47 and shown that at least some consequences of this stimulation can be transmitted via gap junctions to otherwise unstimulated osteoblasts.48 Indeed, connexin 43 hemichannels have been postulated to serve a central function in fluid shear–induced PGE2 release from the MLO-Y4 osteocytic cell line.49 Moreover, other studies have demonstrated that fluid flow increases the gap junction expression and function in the osteocytic MLO-Y4 cells.35,41,45 In addition, mechanical stimulation also results in the opening of connexin 43 hemichannels and release of PGE250 and adenosine triphosphate46 from the osteocytes.

Despite the attractive characteristics of these proposed models, there is, to our knowledge, little if any data that have demonstrated that purified osteocytes purified directly from different bones can exert any regulatory influence upon the behaviour of osteoblast. Furthermore, the mechanisms coordinating these interactions either under basal conditions or in response to mechanical strain also remain the subject of some speculation. In the present study, we have examined these osteocyte-osteoblast interactions and the role of gap junction–mediated communication further. This has been achieved by investigating, for the first time, the influence exerted by purified embryonic chick bone osteocytes upon the proliferation of primary osteoblasts in direct contact coculture. We have also examined whether osteocyte-osteoblast communication is modified by pharmacological blockade on functional gap junctions, both under these basal conditions and following application of mechanical strain in vitro. It has previously been shown that calvarial bone explants do not respond to mechanical loads,51 and so initial studies examined whether the source from which osteocytes were purified determined their influence upon primary osteoblast behaviour. Our studies reinforce differences between purified osteocytes derived from functionally distinct bones. In addition, they demonstrate that purified osteocytes can regulate behaviour of primary osteoblasts and that the functional outcome of this communication differs markedly when the proliferative response of osteoblasts to mechanical strain is examined.

2 MATERIALS AND METHODS

2.1 Cell isolation from embryonic chick bones

Purified osteocyte and primary osteoblasts were derived from both parietal bones and tibiotarsi using modification to methods used previously,18,52–56 with primary osteoblasts derived and allowed to expand in culture prior to osteocyte purification. Briefly, parietal bones of the calvaria and tibiotarsal bones (see Figure 1) were removed from 18-day-old chick embryos and cleared of all attendant soft tissue and periostea. Medullary cavities were flushed with a Dulbecco phosphate-buffered saline (lacking calcium and magnesium [PBS−]; Invitrogen, Paisley, UK) and resident bone cells were then dissociated using an adaptation to the method devised by van der Plas and Nijweide.55 This involved 3 sequential digestions of bone segments with 1-mg/mL collagenase type 1 (Clostridium histolyticum, Sigma, Dorset, UK) in PBS− followed by 4mM ethylenediaminetetraacetic acid (Sigma) in PBS−. Digestion was stopped by incubation with 10% heat-inactivated chick serum in a Hank balanced salt solution (HBSS) (Invitrogen), and each of these 3 consecutive fractions was centrifuged (800 g, 4°C for 5 min) and then resuspended in heat-inactivated chick serum in an HBSS on ice and combined, resupined, and resuspended to a single-cell suspension in PBS− containing 4mM ethylenediaminetetraacetic acid and 0.5% bovine serum albumin (BSA; Fraction V, Sigma) (PEB) to produce a mixed bone-derived cell population.

Osteocytes were purified from this mixed population using Ob7.3(5) mouse antichick osteocyte antibody as previously described (Nijweide, Leiden, the Netherlands).52 This involved addition of Ob7.3(5) to the cell suspension and incubation for 15 minutes on a “rotaspin” at 4°C. After centrifugation (800 g, 4°C for 5 min), cells were resuspended, washed in PEB, and incubated for 15 minutes at 4°C with MACS goat antimouse IgG microbeads (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). These cell suspensions were next passed through a prewashed mini-MACS column with a magnetic collar (Miltenyi Biotech GmbH) and cells expressing epitopes bound by the Ob7.3(5) antibody (Ob7.3(5)+) and beads retained within the column, whilst other unbound cell types passed through to be collected (Ob7.3(5)− cells). The column was washed with 5 volumes of PBS/BSA to remove all weakly bound, trapped, or otherwise unspecifically retained cells, and finally, removal of the mini-MACS column from the magnetic field allowed Ob7.3(5)+ cells to be eluted. These methods achieved enrichment of chick osteocytes in samples immunomagnetically purified using monoclonal Ob7.3(5) antibody.

Enriched primary osteoblast cultures were obtained from the Ob7.3(5)+ cell preparation by immunomagnetic depletion of fibroblasts (using an antifibroblast antibody) to produce a osteoblast population of fibroblast-depleted Ob7.3(5)+ cells. Using long bones and calvariae as primary sources therefore allowed Ob7.3(5)+ osteoblasts (LOBs and COBs) and Ob7.3(5)+ osteocytes (LOCs and COCs) to be separated.
2.2 | Cell culture and characterisation

All bone-derived cells were cultured in Dulbecco Modified Eagle Medium minus phenol red, 5% heat-inactivated chick serum, 2mM L-glutamine, 50 μg/mL gentamicin (Invitrogen); 50 μg/mL L-ascorbic acid; 5.6 mM glucose (Sigma). Samples of each of the Ob7.3(5)+ and Ob7.3(5)− cells had their phenotype confirmed by examination of morphology using scanning electron microscopy, immunocytochemical labelling with the Ob7.3(5) antibody, alkaline phosphatase activity, and in vitro mineralisation. For immunocytochemistry, cells seeded onto glass coverslips were incubated with 0.25% BSA in HBSS for 5 minutes to block nonspecific binding, incubated for 30 minutes at room temperature with Ob7.3(5) diluted 1:5 with BSA/HBSS, and washed and fixed in 4% buffered formaldehyde (VWR/Merck) in HBSS for 10 min at 4°C. The cells were then washed in 0.25% BSA in HBSS prior to 30-minute incubation at room temperature in horse antimouse biotinylated secondary antibody (Vector Labs Limited, Peterborough, UK) (1:100 in BSA/PBS). After being washed, cells were incubated in the dark for 30 minutes at room temperature with streptavidin-coupled CY-3 (Vector, 1:500 in BSA/PBS), washed, and mounted in DAKO fluorescent mounting medium (DAKO Corporation, Carpinteria, CA, USA). Nuclear counterstaining was achieved using 4′,6-diamidino-2-phenylindole. Control samples were treated similarly but were incubated in the absence of either primary or secondary antibody.

Alkaline phosphatase activity was assessed using the Naphthol AS-BI and Fast Blue BB method57 and was followed by counterstaining with a Meyer haemalum (VWR/Merck). Mineralisation potential was assessed with and without the addition of 50 μg/mL l-ascorbic acid and 10mM β-glycerophosphate (Sigma). These samples were reacted with alkaline phosphatase, counterstained with von Kossa and Safranin O (VWR/Merck), and visualised using an Olympus BH-2 microscope.

2.3 | Coculture and assessment of proliferation

Proliferation was assessed in both monoculture and in cocultures with cells seeded at a density of 20 000 total cells per well, in 24-well plates (Nalge Nunc International). Preliminary experiments established an optimum osteocyte-to-osteoblast ratio of 4:1 and that primary osteoblasts should be allowed to adhere prior to the addition of osteocytes in coculture, prior to being allowed to settle overnight. After overnight serum depletion to synchronise proliferative activity, cultures were pulsed with methyl L[5-3H]-thymidine (1 μCi/mL per well, Amersham International, UK) and incubated for 18 hours. Rates of DNA synthesis were assessed by measuring [5-3H]-thymidine incorporation as previously described.58,59 Briefly, cells were washed 3 times with ice-cold PBS−, detached from the substrate with 0.25% trypsin (Sigma) and 100 μL of carrier DNA solution (1 μg of salmon sperm DNA; Sigma/1-μL PBS) and 1 mL 10% trichloroacetic acid (TCA) added. The samples were vortexed and incubated for 16 hours at 4°C, and the TCA-insoluble fraction was recovered by 3 sequential 1500 g centrifugations at 4°C, for 30 minutes, and washed with ice-cold 5% TCA. The resultant pellet was dried with ice-cold 90% ethanol and dissolved in a 20% formic acid (VWR/Merck) and 80% ACSII scintillant mixture (Amersham, Buckinghamshire, UK). Radioactive disintegrations per
minute were counted using a 1214 Rackbeta liquid scintillation counter (LKB Wallac, London, UK). In experiments examining the effects of gap junction blockade, cells were incubated in a medium containing 20μM 18β-glycyrrhetinic acid (Sigma).60

2.4 Application of mechanical strain

Mechanical strain was applied to cells seeded onto specially prepared (Nalge Nunc International, Naperville, Illinois) plastic cell culture–treated slides (75 × 25 mm), maintained in charcoal dextran (VWR/Merck) stripped medium and allowed to equilibrate in a humidified atmosphere of 95% air/5% CO₂ after being placed in a custom-designed jig, which loaded the strips in 4-point bending.61 Each strip was subjected to 600 cycles of applied load at 1 Hz, with each cycle producing a maximum longitudinal strain on the surface of the strip of 3000με. Control cells, on otherwise unperturbed slides, were treated identically but without strain stimulation. Following the period of strain, the slides were returned to plastic dishes, together with their surrounding medium and incubated for a further 18 hours after the addition of 1 μCi/mL of [3H]–thymidine (an index of cell proliferation)62–68 to the medium.

2.5 Statistical analysis

Statistical analyses were performed using either Microsoft Excel or GraphPad Prism 6 (GraphPad Software, Inc., San Diego, California). Data are presented as mean ± SEM and were considered statistically significant when \( P \leq .05 \). A 2-sample, unpaired \( t \) test was used to compare means between control and treated groups.

3 RESULTS

3.1 Morphologically characteristic phenotypes retained in vitro in purified osteocytes

Using scanning electron microscopy, we found that Ob7.3(5)+ cells from both calvarial and tibiotarsal bones were generally smaller, exhibited a lower cytoplasmic area, had a distinct stellate appearance, and contained many more long slender processes radiating from a central cell body (Figure 2A and B). Furthermore, efficiency of purification and phenotypic stability were first confirmed by comparing Ob7.3(5) monoclonal antibody immunolabelling, which demonstrated negligible immunocytochemical labelling of isolated primary osteoblasts (Figure 2C), and unambiguous positive expression of the Ob7.3(5)-directed epitope in osteocytes (Figure 2D and Table 1). This confirms the persistence of the osteocyte phenotype in Ob7.3(5)+ cells in vitro.

Another characteristic of resident osteocytes is their postmitotic phenotype. To verify this postmitotic behaviour of immuno-magnetically purified Ob7.3(5)+ cells, we compared their proliferation rates to Ob7.3(5) primary osteoblasts by measuring [3H]–thymidine incorporation. We found that Ob7.3(5)+ purified from both calvariae and tibiotarsi showed negligible proliferation compared with Ob7.3(5)+ cells and that the latter showed similar rates of proliferation irrespective of whether they were sourced from tibiotarsi or calvariae.
cells as COB (calvarial) and LOB (long bone).

osteocyte reverse proliferative influence of purified osteocytes through coculture with long bone purified osteocytes (Figure 4A). Surprisingly, however, we found that mechanical strain application increases proliferation of primary osteoblasts cultured alone.

We found, consistent with previous studies, that mechanical strain induces strain or fluid flow in vivo.1,2,4 Evidence for osteoblast regulation by purified osteocytes was therefore sought, and we found that the coculture of LOC with homogenic LOB significantly enhanced proliferation to levels greater than the expected sum attributable to osteoblasts and osteocytes cultured independently (LOB = 20,909; LOC = 394; LOB + LOC = 29,863 dpm; P ≤ .05; Figure 2F).

In contrast, homogenic calvarial cocultures (COB + COC) did not show similar enhancement in proliferation (Figure 2F).

To address whether this osteocyte-induced promotion of primary osteoblast proliferation is selective to purified osteocytes from long bones or an inherent characteristic of long bone osteoblasts, we also assessed proliferation rates in heterogenic cocultures (LOB + COC and COB + LOC). This showed that cocultures of COB with LOC showed significantly higher 3H-thymidine incorporation levels than the sum of independent cultures (COB = 17,741; LOC = 554; COB + LOC = 22,065; P ≤ .04; Figure 3). Such coculture-related enhancement was not evident, however, when LOBs were cultured with heterogenic COCs (LOB = 19,073; COC = 109; LOB + COC = 21,719; P = .1; Figure 3), suggesting a selective influence of LOC on primary osteoblast proliferation. Together, these data indicate that osteocytes purified from long bones, but not those from calvariae, stimulate the basal proliferation of primary osteoblasts derived from either bone source.

3.2 Stimulation of primary osteoblast proliferation through coculture with long bone purified osteocytes

It is broadly held that osteocytes act as mediators of the changes in (re)modelling induced by load-induced strain or fluid flow in vivo.1,2,4–8 Evidence for osteoblast regulation by purified osteocytes was therefore sought, and we found that the coculture of LOC with homogenic LOB significantly enhanced proliferation to levels greater than the expected sum attributable to osteoblasts and osteocytes cultured independently (LOB = 20,909; LOC = 394; LOB + LOC = 29,863 dpm; P ≤ .05; Figure 2F). In contrast, homogenic calvarial cocultures (COB + COC) did not show similar enhancement in proliferation (Figure 2F).

To address whether this osteocyte-induced promotion of primary osteoblast proliferation is selective to purified osteocytes from long bones or an inherent characteristic of long bone osteoblasts, we also assessed proliferation rates in heterogenic cocultures (LOB + COC and COB + LOC). This showed that cocultures of COB with LOC showed significantly higher 3H-thymidine incorporation levels than the sum of independent cultures (COB = 17,741; LOC = 554; COB + LOC = 22,065; P ≤ .04; Figure 3). Such coculture-related enhancement was not evident, however, when LOBs were cultured with heterogenic COCs (LOB = 19,073; COC = 109; LOB + COC = 21,719; P = .1; Figure 3), suggesting a selective influence of LOC on primary osteoblast proliferation. Together, these data indicate that osteocytes purified from long bones, but not those from calvariae, stimulate the basal proliferation of primary osteoblasts derived from either bone source.

3.3 Mechanical strain use of gap junctions to reverse proliferative influence of purified osteocytes on primary osteoblasts; gap junction–independent osteocyte-related osteoblast proliferation

We found, consistent with previous studies, that mechanical strain application increases proliferation of primary osteoblasts cultured alone (Figure 4A). Surprisingly, however, we found that mechanical strain exposure evoked significant suppression of LOB proliferation when they were cocultured with LOC (Figure 4). To determine whether gap junction communication is required for LOC-induced LOB proliferation and whether it contributes to bone cell responses to mechanical strain, we examined the effect of the gap junction blocker, β-glycyrrhetinic acid (β-GA), in homogenic long bone cocultures (LOC + LOB) both with and without mechanical strain stimulation. This revealed that LOC-induced LOB proliferation in the absence of a mechanical strain stimulus was unaffected by β-GA (Figure 4A) but, in contrast, that the mechanical strain–related suppression of osteocyte-induced LOB proliferation was abrogated, and indeed reversed, in the presence of β-GA (Figure 4B).

Thus, treatment of mechanically strained cocultures (LOB + LOC + strain) with β-GA results in similar levels of osteocyte-induced proliferation as untreated, nonstrained LOC + LOB cocultures (Figure 4B). These data suggest that the influence of the purified osteocytes to regulating strain-induced proliferation of primary osteoblasts is mediated by functional gap junctions but that their promotion of primary osteoblast proliferation in the absence of a strain stimulus is achieved independently of gap junction–mediated communication.

4 DISCUSSION

Monoculture systems are used most commonly to examine the mechanisms involved in bone cell biology. However, bone is an organ containing various cell types, and attempts to better replicate in vivo relationships have recently been shown to be pertinent.69 Osteocytes are considered as the mechanosensors of bone.1–13 It is frequently asserted that they act to influence osteoblast (and osteoclast)
behaviour to control remodelling activity to ensure mechanical competence, but evidence for this contention is sparse and unequivocal proof is lacking. A recent study performed in chick and mouse calvarial parietal bone shows, through FRAP analysis, the existence of cell-to-cell communication via gap junctions in the 3D morphology of the osteocyte network. Our use of primary osteoblast-osteocyte cocultures, however, reveals additional relationships that would otherwise not be apparent in monoculture.

Using coculture, we sought evidence that purified osteocytes contribute to regulating primary osteoblast behaviour and whether any such contribution was reliant upon functional gap junctions. We found that postmitotic purified osteocytes were indeed capable of stimulating enhanced rates of proliferation by primary osteoblasts. Our data showed that osteocytes purified from mechanically responsive long bone, but not those from nonresponsive skull bones, exhibit a capacity to promote proliferation of primary osteoblasts. Using a pharmacological blocker (18β-glycyrrhetinic acid) under these basal conditions, we also show that this osteocyte-mediated promotion of proliferation of primary osteoblast was not dependent on functional gap junctions. Further to blocking gap junctions, 18β-glycyrrhetinic acid has been reported to exert additional pharmacological actions including on 11β-hydroxysteroid dehydrogenase 1, pannexin channel activity as well as high-mobility group box protein 1 action, and glucocorticoid metabolism. This is a limitation of our studies, and future examination of gap junction requires a more specific method of blocking these junctions. In marked contrast, we found that application of physiological levels of dynamic mechanical strain to cocultured long bone cells efficiently abrogated proliferation of primary osteoblast, with gap junction blockade indicating that strain-related transfer of an inhibitory stimulus between purified osteocytes and primary osteoblasts involves functional communicating gap junctions.

The Ob7.3(5) antibody, which was used herein to isolate osteocytes, has been shown to be specific for the phosphate-regulating gene with homology to endopeptidases on the X chromosome protein that is abundant in osteocytes. We find that cells isolated from chick bone (tibiotarsal or calvarial) using this antibody exhibit low, nonsignificant incorporation of [3H]-thymidine when maintained in monoculture. This demonstrates their postmitotic characteristics and further supports the osteocyte specificity of the antibody and the purity of the resident cells isolated and used herein. Previous studies established and fully optimised this osteocyte immunomagnetic purification, and we, and others, have previously used these purified cells extensively to describe osteocyte in vitro behaviours. Future characterisation using other markers including sclerostin and keratocan would provide additional evidence authenticating this cell population, but the phenotypic features and in vitro behaviour are consistent with terminally differentiated osteocytes. Use of MLO-Y4 osteocyte-like cell lines in our studies would have made it unfeasible to conduct our simple coculture studies as they, like primary osteoblasts, display avid proliferation in monoculture. Use of such a cell line, which was derived from one functional skeletal source (limb bone), would also make comparisons between osteocytes derived from functionally distinct calvarial bones impossible and may have obscured the specific influence that long bone-derived osteocytes exert on osteoblast behaviour. Indeed, the specificity of this influence is emphasised by the fact that osteocyte-induced osteoblast proliferation is achieved in coculture only by osteocytes extracted from tibiotarsi and not by those extracted from calvariae and that osteoblasts derived from either source appear to behave similarly. These data not only rule out the possibility of some noncell, coculture effect but also pinpoint osteocytes derived from load-responsive long bones rather than predominantly protective skull bones as distinct in their promitogenic osteoblast influence. They also suggest that the proliferative response to coculture with long bone-derived osteocytes is retained in primary osteoblasts derived from either bone site. The use of [3H]-thymidine is widely reported as a method to assess proliferative capacity of bone

![FIGURE 4](image-url)
cells; however, it is possible that cell survival is also affected. Although no evidence of an increased rate of cell death was observed in our experimental protocols, further studies to assess apoptosis directly would determine whether lack of proliferation might be associated with increased cell death.

The proproliferative influence of postmitotic osteocytes on cocultured osteoblasts is perhaps most readily interpreted, owing to the lack of apparent gap junction involvement, to suggest the involvement of an osteocyte-derived soluble mediator. There are many candidate soluble mediators that are produced by osteocytes, which may account for this osteoblast stimulation. These include secretory phospholipase A2, which evokes increased PGE2 and prostaglandin I2, whereas mechanically stimulated secretory candidate soluble mediators that are produced by osteocytes, which promote osteoclast formation,82 whereas mechanically stimulated osteocytes subjected to fluid shear strains.48 MLO-Y4 cells inhibit osteoclastogenesis,83 possibly via matrix extracellular phosphoglycoprotein.84 Our studies allow additions to the growing evidence that osteocytes can promote osteoblast proliferation, that this influence can be restricted by the application of strain, and that only the latter, osteocyte strain-related control of osteoblast proliferation is dependent upon gap junctions (Figure 5).69

If our findings were to be directly extrapolated to the in vivo scenario, they would imply that, at rest, osteocytes act via transcellular signalling to maintain an active osteoblast population on the bone surface. Unlike the situation in culture, even at rest, osteocytes are constantly subjected to mechanical inputs, amongst which the most continuous is fluid shear strain stimuli driven by the circulatory system. In stark contrast, however, these proliferative signalling molecules emanating from osteocytes must rather be completely “overruled” in response to mechanical loading of bones, by information transferred to osteoblasts via gap junctions to promote an appropriate (re)modeling event. This implies that there is continual osteocyte-derived signalling to cells on the bone surface maintaining the delicate balance of formation and resorption.

It is worth emphasising that the application of the mechanical strain stimulus is only transient in our studies, and yet this is nevertheless sufficient to significantly restrict the proliferation of osteoblasts induced by osteocytes. We interpret our findings to reflect the mechanism by which osteocytes and mechanical inputs together act to regulate the proliferation of osteoblasts. Thus, with increased loading, inhibition of proliferation would have to precede osteoblast differentiation at locations where new bone is required to withstand increased mechanical demands. An alternative interpretation is that osteocytes exert some hitherto unresolved suppression of the increases in proliferation that normally ensue periods of loading. In conclusion, our studies suggest that purified osteocytes, derived from load-bearing long bones, exert a direct proproliferative influence upon primary osteoblasts and that mechanical strain may use gap junctions to reverse this osteocyte-derived stimulatory effect.

REFERENCES

1. Burger EH, Klein-Nulend J. Responses of bone cells to biomechanical forces in vitro. Adv Dent Res. 1999;13:93–98.

![Figure 5](image-url) Schema depicting osteocyte-derived signalling on osteoblasts. The results suggest that the proproliferative influence of osteocytes upon osteoblasts is reversed by the application of strain and that only this reversal is gap junction mediated.
2. Burger EH, Klein-Nulend J, van der Plas A, Nijweide PJ. Function of osteocytes in bone—its role in mechanotransduction. J Nutr. 1995;125:2020S–2023S.

3. Dallas SL, Zaman G, Pead MJ, Lanyon LE. Early strain-related changes in cultured embryonic chick tibiotarsus parallel those associated with adaptive modeling in vivo. J Bone Miner Res. 1993;8:251–259.

4. Pitsillides AA, Rawlinson SC, Suswillo RF, Bourrin S, Zaman G, Lanyon LE. Mechanical strain-induced NO production by bone cells: a possible role in adaptive bone remodeling? FASEB J. 1995;9:1614–1622.

5. Rawlinson SC, Wheeler-Jones CP, Lanyon LE. Arachidonic acid for loading induced prostacyclin and prostaglandin E2 (PGE2) release from osteoblasts and osteocytes is derived from the activities of different forms of phospholipase A2 (PLA2). Bone. 2000;27:241–247.

6. Rawlinson SCF, El Haj AJ, Minter SL, Tavares IA, Bennett A, Lanyon LE. Load-related increases in prostaglandin production in cores of adult canine cancellous bone in vitro: a role for prostacyclin in adaptive bone remodeling? J Bone Miner Res. 1991;6:1345–1351.

7. Rawlinson SCF, Pitsillides AA, Lanyon LE. Involvement of different ion channels in osteoblasts' and osteocytes' early responses to mechanical strain. Bone. 1996;19:609–614.

8. Zaman G, Jessop HL, Muzylak M, et al. Osteocytes use estrogen receptor alpha to respond to strain but their ERalpha content is regulated by estrogen. J Bone Miner Res. 2006;21:1297–1306.

9. Javaheri B, Stern AR, Lara N, et al. Deletion of a single beta allele in osteocytes abolishes the bone anabolic response to loading. J Bone Miner Res. 2001;16:634S–475.

10. Burger EH, Klein-Nulend J. Mechano-transduction in bone—role of the lacuno-canaliculair network. FASEB J. 1999;13(Suppl):S101–S112.

11. Rawlinson SCF, Pitsillides AA, Suswillo RF, Bourrin S, Zaman G, Lanyon LE. Mechanical strain induces NO production by bone cells: a possible role in adaptive bone remodeling? FASEB J. 1995;9:1614–1622.

12. Burger EH. Pulsating fluid flow increases prostaglandin production by cultured chicken osteocytes. J Bone Miner Metab. 1993;53(Suppl 1):S102–S106. discussion S106–S107.

13. Weinbaum S, Cowin SC, Zeng Y. A model for the excitation of osteocytes in bone by mechanical loading-induced bone fluid shear stresses. J Biomech. 1994;27:339–360.

14. Lanyon LE. Osteocytes, strain detection, bone modeling and remodeling. Calcif Tissue Int. 1993;53(Suppl 1):S102–S106. discussion S106–S107.

15. Skerry TM, Bitsenis L, Chuyen J, Lanyon LE. Early strain-related changes in enzyme activity in osteocytes following bone loading in vivo. J Bone Miner Res. 1989;4:783–788.

16. Ajobi NE, Klein-Nulend J, Nijweide PJ, Vrijheid-Lammers T, Alblas MJ, Burger EH. Pulsating fluid flow increases prostaglandin production by cultured chicken osteocytes—a cytoskeleton-dependent process. Biochem Biophys Res Commun. 1996;225:62–68.

17. Kleinnlend J, Semeins C, Ajobi N, Nijweide P, Burger E. Pulsating fluid flow increases nitric oxide (NO) synthesis by osteocytes but not periosseal fibroblasts-correlation with prostaglandin upregulation. Biochem Biophys Res Commun. 1995;217:640–648.

18. Zaman G, Pitsillides A, Rawlinson S, et al. Mechanical strain stimulates nitric oxide production by rapid activation of endothelial nitric oxide synthase in osteocytes. J Bone Miner Res. 1999;14:1123–1131.

19. Iqbal J, Zaidi M. Molecular regulation of mechanotransduction. Biochem Biophys Res Commun. 2005;328:751–755.

20. Yang W, Lu Y, Kalajic I, et al. Dentin matrix protein 1 gene cis-regulation: role in osteocytes to characterize local responses to mechanical loading in vitro and in vivo. J Biol Chem. 2005;280:20680–20690.

21. Skerry TM. One mechanostat or many? Modifications of the site-specific response of bone to mechanical loading by nature and nurture. J Musculoskelet Neuronal Interact. 2006;6:122–127.

22. Frost HM. The mechanostat: a proposed pathogenic mechanism of osteoporesis and the bone mass effects of mechanical and nonmechanical agents. Bone Miner. 1987;2:73–85.

23. Palumbo C, Palazzini S, Marotti G. Morphological study of intercellular junctions during osteocyte differentiation. Bone. 1990;11:401–406.

24. Jones S, Gray C, Sakamaki H, et al. The incidence and size of gap junctions between the bone cells in rat calvaria. Anat Embryol. 1993;187:343–352.

25. Shapiro F. Variable conformation of GAP junctions linking bone cells: a transmission electron microscopic study of linear, stacked linear, curvilinear, oval, and annular junctions. Calcif Tissue Int. 1997;61:285–293.

26. Civitelli R, Beyer EC, Warlow PM, Robertson AJ, Geist ST, Steinberg T. Connexin43 mediates direct intercellular communication in human osteoblast cell networks. J Clin Invest. 1993;91:1888.

27. Donahue HJ, Guilak F, Vander Molen MA, et al. Chondrocytes isolated from mature articular cartilage retain the capacity to form functional gap junctions. J Bone Miner Res. 1995;10:1359–1364.

28. Schwab W, Hofer A, Kasper M. Immunohistochemical distribution of connexin 43 in the cartilage of rats and mice. Histochem J. 1998;30:413–419.

29. Su M, Borke J, Donahue H, et al. Expression of connexin 43 in rat mandibular bone and periodontal ligament (PDL) cells during experimental tooth movement. J Dent Res. 1997;76:1357–1366.

30. Gluhak-Heinrich J, Gu S, Pavlin D, Jiang JX. Mechanical loading stimulates expression of connexin 43 in alveolar bone cells in the tooth movement model. Cell Commun Adhes. 2006;13:115–125.

31. Schirmacher K, Schmitz I, Winterhager E, et al. Characterization of gap junctions between osteoblast-like cells in culture. Calcif Tissue Int. 1992;51:285–290.

32. Chiba H, Sawada N, Oyamada M, et al. Relationship between the expression of the gap junction protein and osteoblast phenotype in a human osteoblastic cell line during cell proliferation. Cell Struct Funct. 1993;18:419–426.

33. Yamaguchi DT, Da M, Lee A, Huang J, Gruber HE. Isolation and characterization of gap junctions in the osteoblastic MC3T3-E1 cell line. J Bone Miner Res. 1994;9:791–803.

34. Kamioka H, Ishihara Y, Ris H, et al. Primary cultures of chick osteocytes retain functional gap junctions between osteocytes and between osteocytes and osteoblasts. Microsc Microanal. 2007;13:108–117.

35. Cheng B, Zhao S, Luo J, Sprague E, Bonewald LF, Jiang JX. Expression of functional gap junctions and regulation by fluid flow in osteocyte-like MLO-Y4 cells. J Bone Miner Res. 2001;16:249–259.

36. Doty SB. Morphological evidence of gap junctions between bone cells. Calcif Tissue Int. 1981;33:509–512.

37. Yellowley CE, Li Z, Zhou Z, Jacobs CR, Donahue HJ. Functional gap junctions between osteocytic and osteoblastic cells. J Bone Miner Res. 2000;15:209–217.

38. Jiang JX, Siller-Machl M, and Jackson AJ, et al. Mechanical stress enhances gap junctional communication between osteoblastic cells. Front Biosci. 2007;12:1450.

39. Ziambaras K, Lecanda F, Steinberg TH, Civitelli R. Cyclic stretch enhances gap junctional communication between osteoblastic cells. J Bone Miner Res. 1998;13:218–228.

40. Batra N, Burra S, Siller-Jackson AJ, et al. Mechanical stress-activated integrin α5β1 induces opening of connexin 43 hemichannels. Proc Natl Acad Sci. 2012;109:3359–3364.

41. Cherian PP, Cheng B, Gu S, Sprague E, Bonewald LF, Jiang JX. Effects of mechanical strain on the function of gap junctions in osteocytes are mediated through the prostaglandin EP2 receptor. J Biol Chem. 2003;278:43146–43156.

42. Uzer G, Pongkkitwitoon S, Ian C, et al. Gap junctional communication in osteocytes is amplified by low intensity vibrations in vitro. PLoS One. 2014;9:e90840.

43. Bonewald LF. Establishment and characterization of an osteocyte-like cell line, MLO-Y4. J Bone Miner Metab. 1999;17:61–65.
44. Kato Y, Windle JJ, Koop BA, Mundy GR, Bonewald LF. Establishment of an osteocyte-like cell line, MLO-Y4. J Bone Miner Res. 1997;12:2014–2023.

45. Alford AJ, Jacobs CR, Donahue HJ. Oscillating fluid flow regulates gap junction communication in osteocytic MLO-Y4 cells by an ERK1/2 MAP kinase-dependent mechanism. Bone. 2003;33:64–70.

46. Genetos DC, Kephart CJ, Zhang Y, Yellowley CE, Donahue HJ. Oscillating fluid flow activation of gap junction hemichannels induces ATP release from MLO-Y4 osteocytes. J Cell Physiol. 2007;212:207–214.

60. Goldberg GS, Moreno AP, Bechberger JF, et al. Evidence that disruption of connexin particle arrangements in gap junction plaques is associated with inhibition of gap junctional communication by a glycyrrhetinic acid derivative. Exp Cell Res. 1996;222:48–53.

63. Brighton CT, Fisher JR Jr, Levine SE, et al. The biochemical pathway mediating the proliferative response of bone cells to a mechanical stimulus. J Bone Joint Surg Am. 1996;78:1337–1347.

64. Stanford CM, Morcuende JA, Brand RA. Proliferative and phenotypic responses of bone-like cells to mechanical deformation. J Orthop Res. 1995;13:664–670.

67. Tanaka K, Yamaguchi Y, Hakeda Y. Isolated chick osteocytes stimulate DNA and alters their pattern of protein synthesis. J Cell Biol. 1998;15:2177–2182.

68. Damien E, Price JS, Lanyon LE. The estrogen receptor's involvement in osteoblasts' adaptive response to mechanical strain. J Bone Miner Res. 1998;13:1275–1282.

69. Vazquez M, Evans BA, Riccardi D, et al. A new method to investigate how mechanical loading of osteocytes controls osteoblasts. Front Endocrinol (Lausanne). 2014;5:208

70. Sugawara Y, Ando R, Kamioka H, et al. The three-dimensional morphometry and cell-cell communication of the osteocyte network in chick and mouse embryonic calvaria. Calcif Tissue Int. 2011;88:416–424.

71. Davidson JS, Baumgarten IM, Harley EH. Reversible inhibition of intercellular junctional communication by glycyrrhetinic acid. Biochim Biophys Acta. 1986;1160:29–30.

72. Irie A, Fukui T, Negishi M, Nagata N, Ichikawa A. Glycyrrhetinic acid bound to 11 beta-hydroxysteroid dehydrogenase in rat liver microsomes. Biochim Biophys Acta. 1992;1160:229–234.

73. Ulmann A, Menard J, Corvol P. Binding of glycyrrhetinic acid to kidney mineralocorticoid and glucocorticoid receptors. Endocrinology. 1975;97:46–51.

74. Bruzzzone R, Barbe MT, Jakob NJ, Monyer H. Pharmacological properties of homomeric and heteromeric pannexin hemichannels expressed in Xenopus oocytes. J Neurochem. 2005;92:1033–1043.

75. Cheng MZ, Rawlinson SC, Pitsillides AA, et al. Human osteoblasts' proliferative responses to strain and 17β-estradiol are mediated by the estrogen receptor and the receptor for insulin-like growth factor I. J Bone Miner Res. 2002;17:1225–1232.

76. Van der Plas A, Mulder R. Identification of osteocytes in osteoblast cultures using a monoclonal antibody specifically directed against osteocytes. Histochemistry. 1984;84:342–347.

77. Van der Plas A, Nijweide PJ. Isolation and purification of osteocytes. Bone Miner Res. 1995;10:493–505.

78. Nijweide PJ, Mulder R. Identification of osteocytes in osteoblast-like cell cultures using a monoclonal antibody specifically directed against osteocytes. Histochemistry. 1984;84:342–347.

79. De Rooij KE, Nijweide PJ. Osteocyte-specific monoclonal antibody MAb OB7.3 is directed against PHEX protein. J Bone Miner Res. 2002;17:845–853.

80. Tanaka-Kamioka K, Kamioka H, Ris H, Lim SS. Osteocyte shape is dependent on actin filaments and osteocyte processes are unique actin-rich projections. J Bone Miner Res. 1998;13:1555–1568.

81. Nijweide PJ, Mulder R. Identification of osteocytes in osteoblast-like cell cultures using a monoclonal antibody specifically directed against osteocytes. Histochemistry. 1984;84:342–347.

82. Zhao S, Zhang YK, Harris S, Ahuja SS, Bonewald LF. MLO-Y4 osteocyte-like cells support osteoclast formation and activation. J Bone Miner Res. 2002;17:2068–2079.

83. Chan ME, Lu XL, Huo B, et al. A trabecular bone explant model of osteocyte-osteoclast co-culture for bone mechanobiology. Cell Mol Biol. 2009;2:405–415.

84. Endsres S, Kratz M, Wunsch S, Jones DB. Zetos: a culture loading system for trabecular bone. Investigation of different loading signal intensities on bovine bone cylinders. J Musculoskeletal Neuronal Interact. 2009;9:173–183.

85. Zhao S, Zhang YK, Harris S, Ahuja SS, Bonewald LF. MLO-Y4 osteocyte-like cells support osteoclast formation and activation. J Bone Miner Res. 2002;17:2068–2079.

86. You J, Reilly GC, Zhen X, et al. Osteopectin gene regulation by oscillatory fluid flow via intracellular calcium mobilization and activation of
mitogen-activated protein kinase in MC3T3-E1 osteoblasts. J Biol Chem. 2001;276:13365–13371.

84. Kulkarni RN, Bakker AD, Everts V, Klein-Nulend J. Inhibition of osteoclastogenesis by mechanically loaded osteocytes: involvement of MEPE. Calcif Tissue Int. 2010;87:461–468.

How to cite this article: Suswillo RFL, Javaheri B, Rawlinson SCF, Dowthwaite GP, Lanyon LE, Pitsillides AA. Strain uses gap junctions to reverse stimulation of osteoblast proliferation by osteocytes. Cell Biochem Funct. 2017;35:56–65. doi: 10.1002/cbf.3245.