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The Role of Integrin $\alpha_v\beta_3$ in Osteocyte Mechanotransduction

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Abstract

Recent *in vivo* studies have proposed that integrin \( \alpha_\text{v}\beta_3 \) attachments between osteocyte cell processes and the extracellular matrix may facilitate mechanosensation in bone. However the role of these attachments in osteocyte biochemical response to mechanical stimulus has yet to be investigated. With this in mind, the objective of this study was to determine the effect of blocking integrin \( \alpha_\text{v}\beta_3 \) function on the biochemical response of osteocytes to mechanical stimulus. Antagonists specific to integrin subunit \( \beta_3 \) were used to block integrin \( \alpha_\text{v}\beta_3 \) on MLO-Y4 mouse osteocytes. After treatment, cells were subjected to laminar oscillatory fluid flow stimulus (1 Pa, 1 Hz) for 1 hour. Fluorescent staining was performed to visualise cell morphology. Prostaglandin E\(_2\) (PGE\(_2\)) release was assayed using an enzyme immunoassay and qRT-PCR was used to analyse the relative expression of cyclooxygenase-2 (COX-2), receptor activator of NF-\(\kappa\)B ligand (RANKL) and osteoprotegerin (OPG). Our results show that blocking integrin \( \alpha_\text{v}\beta_3 \) disrupts osteocyte morphology, causing a reduction in spread area and process retraction. Integrin \( \alpha_\text{v}\beta_3 \) blocking also disrupted COX-2 expression and PGE\(_2\) release in response to fluid shear stress. Taken together, the results of this study indicate that integrin \( \alpha_\text{v}\beta_3 \) is essential for the maintenance of osteocyte cell processes and also for mechanosensation and mechanotransduction by osteocytes. A better understanding of this process may lead to the development of novel treatments for bone pathologies where mechanosensitivity is thought to be compromised.
Highlights:

• We study how integrin αVβ3 antagonists affect osteocyte response to shear stress

• Treatment altered osteocyte morphology resulting in a reduction in cell spread area

• Antagonists disrupted expression of COX-2 and PGE₂ release in response to flow
Introduction

Bone is an adaptive tissue that is constantly remodelling as it seeks to achieve an optimal balance of strength and mass in response to the functional demands of its mechanical environment (1-3). Typically an increase in loading above habitual levels, such as at the onset of weight bearing exercise, results in a shift in the balance of remodelling towards bone formation (4-7). Likewise a decrease in loading levels, as seen with paralysis or exposure to microgravity, results in bone resorption (8-11). While the phenomena of bone adaption to its loading environment has been long observed (12), the mechanism by which bone cells sense mechanical stimulus and produce a corresponding biochemical response is not yet fully understood. A greater understanding of this process may lead to strategies for preventing undesirable bone loss or novel treatments for bone diseases where defects in mechanotransduction are thought to be a factor.

While remodelling is ultimately realised through the coordinated activities of osteoclasts, which resorb bone, and osteoblasts, bone forming cells, the osteocyte is thought to be the cell that is responsible for sensing mechanical stimulus and transducing this stimulus through biochemical signals to orchestrate the regulation of bone structure (13-16). This hypothesis has been strengthened by studies, which have demonstrated that mechanical loading and unloading alters in vivo osteocyte activity (17-20). Additionally, a study by Tatsumi et al. found that mice in which the majority of osteocytes had been ablated were resistant to unloading-induced bone loss (21).

Osteocytes are embedded within the mineralised matrix of bone and comprising approximately 90% of the cell population within the tissue (22, 23). These cells have a distinctive morphology characterised by long dendritic processes that form a network,
known as the lacuna-canalicular network, connecting neighbouring osteocytes, osteoblasts and bone lining cells. It is believed that compression generated in bone during physical activity causes interstitial fluid to flow through this network and that osteocytes are stimulated by the shear stresses generated by this flow (24-27). Supporting this hypothesis, several studies have shown that osteocytes respond to fluid shear in vitro by altering the expression levels of genes involved in the regulation of osteoblast and osteoclast activity, such as cyclooxygenase-2 (COX-2), receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) (28-36). COX-2 is necessary for the synthesis of prostaglandin E$_2$ (PGE$_2$), which is produced by osteocytes in response to fluid flow and leads to an up-regulation of bone formation (37, 38). RANKL stimulates osteoclast precursors to differentiate towards an osteoclast type phenotype, whereas OPG is a soluble decoy receptor that prevents RANKL from binding to osteoclast precursors (32, 39, 40).

While it has been demonstrated that osteocytes are responsive to fluid shear, the exact mechanism by which mechanical stimulus is transduced into a biochemical signal is not yet fully understood. Recent studies have proposed that integrins may play an important role in osteocyte mechanotransduction (41-43). Integrins are transmembrane proteins, composed of $\alpha$ and $\beta$ subunits, that are predominantly responsible for coupling the cytoskeleton to the extracellular matrix (ECM) (44, 45) and have been found to play an important role in mechanosensing in several different cell types (46-49). In vitro studies of bone cells have shown that blocking integrins $\beta_1$ and $\alpha_\beta_3$ disrupts osteoblast expression of COX-2 and OPN in response to fluid flow (50). Additionally, a study by Litzenberger et al. found that osteocytes expressing a dominant negative form of integrin $\beta_1$ exhibited a significantly diminished response to fluid flow (43).
Transmission electron imaging and immunohistochemistry studies of osteocyte cell processes have provided evidence that integrin-based (αvβ3) attachments to ECM on osteocyte cell processes may facilitate osteocyte mechanosensation \(41, 42\). A recent study by Thi et al. has shown that blocking integrin αvβ3 prevents intracellular Ca\(^{2+}\) signalling in response to fluid stimulus applied to the processes \(51\). However, it is not yet known if integrin αvβ3 attachments are required for fluid flow induced changes in osteocyte gene expression and PGE\(_2\) secretion. Importantly, previous studies have found that Ca\(^{2+}\) and PGE\(_2\) signalling may occur via different mechanisms \(43, 52\). With this in mind, the objective of this study is to determine the role of integrin αvβ3 in PGE\(_2\) signalling by osteocytes in response to fluid shear stress.

**Materials and Methods**

*Cell Culture and Integrin Blocking*

MLO-Y4 mouse osteocytes \(30, 53\) were cultured on type I collagen (Sigma-Aldrich) coated T-75 flasks (Sarstedt) in α-minimum essential medium (α-MEM) supplemented with 2.5 % fetal calf serum (FCS), 2.5 % fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all Sigma-Aldrich). Cells were maintained in a humidified atmosphere at 37 °C, 5 % CO\(_2\) and passaged at a ratio of 1:5 prior to experiments. At 70% confluence MLO-Y4 cells were detached using trypsin–ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich), seeded onto collagen coated slides \(200,000\) cells per slide, \(10^4\) cells/cm\(^2\) and maintained for 48 hours prior to experimentation. In order to ensure that the experimental approach resulted in specific blocking of integrin αvβ3 and that any changes in cell response were not due to the side-effects of a particular treatment, two independent antagonists were used to block
interactions between integrin $\alpha_v\beta_3$ and the collagen substrates; (1) a monoclonal antibody against human integrin subunit $\beta_3$ (clone 2C9.G2, BD Pharmingen) and (2) a small molecule inhibitor of integrin $\alpha_v\beta_3$ (IntegriSense 750). This antibody has been used in previous studies to block integrin-substrate interactions with MC3T3-E1 mouse osteoblast like cells (54) and IntegriSense has previously been used to block integrin $\alpha_v\beta_3$ with MLO-Y4s (51).

Thirty minutes before exposure to fluid flow, media was removed from the quadriPerm plates (Sarstedt) containing the cell seeded slides and replaced with 1 mL of media containing 25 $\mu$g/mL anti-$\beta_3$ antibody or 0.5 $\mu$M IntegriSense. Control samples were cultured in 1 mL standard media. After 30 minutes slides were placed into a parallel-plate flow system, described in further detail below, and subjected to oscillatory fluid flow for 1 hour. Samples were analysed directly after 1 hour of Oscillatory Fluid Flow (OFF) or after maintenance in identical static culture. All experiments were repeated three times using a minimum of 4 samples per group (n=3).

Oscillatory Fluid Flow Experiments

Laminar oscillatory fluid flow (OFF) was applied using a custom-designed parallel plate system, which consisted of a syringe pump (NE-1600, New Era Pump Systems, Farmingdale, NY), parallel plate chambers, and individual media reservoirs (Figure 1A) connected via gas-permeable, platinum-cured silicone tubing (Cole-Parmer, Vernon Hills, IL). Cell-seeded slides were placed into a slot in the bottom surface of the flow path within the parallel plate chambers, which was 140 mm long, 18 mm wide and had a height of 0.2 mm. Laminar oscillatory fluid flow (OFF) was applied using the syringe pump to generate a sinusoidal flow profile with a peak flow rate of 9.2 mL/min and a
frequency of 1 Hz. This flow profile produced a peak shear stress of 1 Pa as determined according to equation 1:

\[ Q = \frac{6\mu \tau}{wh^2} \]

where \( Q \) is the flow rate, \( w \) is the chamber width, \( h \) is the chamber height, \( \tau \) is the shear stress and \( \mu \) is the dynamic viscosity of the media (55, 56). In order to confirm the flow regime within the parallel plate flow chamber system, a computational fluid dynamics (CFD) study was carried out using ANSYS CFX. The CFD model discretised the entire fluid system including inlet and outlet tubing using approximately 4.5 million tetrahedral elements, with significant local refinement in the channel region to adequately resolve pressure and WSS gradients at the boundary layer. A no-slip boundary condition was assumed for all chamber walls while a velocity-inlet and pressure-outlet boundary conditions are assigned. The solution was calculated using a finite volume approach under steady-flow conditions to a convergence criterion of \( 1 \times 10^{-4} \). Figure 1B shows a largely homogeneous distribution of wall shear stress of \( \tau = 1 \) Pa being imparted on the bottom plate of the channel and Figure 1C shows the development of laminar flow within the parallel plate chamber.

**Histological Staining and Microscopy**

Immunofluorescent staining was used in order to visualise the distribution of integrins \( \beta_1 \) and \( \beta_3 \), and to investigate changes in cell morphology due to integrin antagonists and exposure to OFF. Samples were washed with PBS and fixed using 4% paraformaldehyde. Samples were then permeabilised using 0.1% Triton X in a solution of 1% bovine serum albumin (BSA) in PBS for 5 min at 4 °C (all Sigma-Aldrich). This was followed by blocking of non-specific binding sites using 1% goat serum for 60
minutes at room temperature (Jackson ImmunoResearch). Samples were then incubated with rabbit anti-mouse antibodies against either integrins $\beta_1$ (1:200) or $\beta_3$ (1:100) for 60 minutes at room temperature (AB1952 and 04-1060, Millipore). Samples were then washed using a 1 % BSA solution before incubation for 60 minutes at room temperature with a Dylight™ 488 conjugated goat anti-rabbit secondary antibody at a dilution of 1:200 (Jackson ImmunoResearch). After secondary staining, the actin cytoskeleton was stained using a 1.5 ng/µL phalloidin-tetramethylrhodamine B isothiocyanate (TRITC) solution (Sigma-Aldrich). Cell-seeded cover slips were then mounted onto slides using a hard set mounting media containing 4’,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories) and visualised using a Carl Zeiss LSM510 META confocal microscope (integrins and actin) or an Olympus IX50 inverted fluorescence microscope (actin and DAPI). Cell spread area was quantified from thresholded images using ImageJ (NIH). Individual cells were manually selected, the edges of the cells detected were automatically by ImageJ and the bounded cell area was calculated using standard ImageJ functions.

**RNA Isolation and quantitative Reverse Transcription-PCR**

qRT-PCR was used to analyse the relative expression of COX-2, RANKL and OPG, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the house-keeping reference gene. Loaded groups were harvested directly after the application of OFF, with the static control samples harvested at the same time point. RNA was extracted from the samples by lysing in 1 mL of Tri-reagent reagent (Sigma-Aldrich), followed by chloroform extraction. RNA was purified using Qiagen RNeasy columns following the manufacturer’s instructions (Qiagen). RNA yield and purity were assessed using a
nanodrop spectrophotometer (ND-1000, Thermo Scientific), with 260/280 ratios of > 1.8 for all samples. cDNA was synthesised using a ENZA RNA isolation kit (Omega Bio-tek). qPCR was then carried out on the resultant cDNA using a Taqman master mix kit (Applied Biosystems) and a StepOnePlus™ system (Applied Biosystems). Taqman primers for COX-2 (Mm00478374_m1), RANKL (Mm00441906_m1), OPG (Mm00435452_m1), and GAPDH (Mm99999915_g1) were obtained from Applied Biosystems. qRT-PCR data was analysed using the comparative $2^{-\Delta\Delta Ct}$ method as described previously (57), with the static samples used as the calibrator.

**PGE$_2$ Enzyme Immunoassay**

After exposure to fluid flow or static conditions, samples were removed from the chambers and placed into quadriPerm culture plates (Sarstedt). The slides were then covered with 1 mL of media and incubated at 37 °C, 5 % CO$_2$ for 1 hour. After incubation, the media was aspirated from the slides and stored at -80 °C until analysis. The PGE$_2$ content of the media samples were measured using an enzyme immunoassay (EIA) system according to the manufacturer’s instructions (KO18-HX1, Arbor Assays, MI, USA). PGE$_2$ release was normalised to DNA content using the Hoechst 33258 DNA assay, which fluorescently labels double-stranded DNA (Sigma-Aldrich), according to a previously published protocol (58). Briefly, cells were lysed by three cycles of freeze–thawing in molecular grade water. The cell lysate was mixed with a Hoechst solution and incubated in the dark for 10 minutes. Fluorescence was measured at an emission of 460 nm and an excitation of 365 nm using an absorbance spectrophotometer (Synergy HT, BioTek). Calf thymus DNA (Sigma-Aldrich) was used as a standard.
**Statistical Analysis**

Results are expressed as mean ± standard deviation. Two-way analysis of variance (ANOVA) were used to evaluate the results, with exposure to OFF and blocking treatment as the independent factors, followed by pairwise multiple comparison procedures (Tukey test). Statistical significance was declared at p≤0.05.

**Results**

*Integrin Distribution within MLO-Y4 Osteocytes*

Immunofluorescent staining was used to investigate the distribution of integrins within MLO-Y4s. Staining of integrin subunits β₁ (Figure 2A and B) and β₃ (Figure 2C and D) revealed punctate clusters of integrins distributed on the cell surface and also within the cell processes.

*The Effect of OFF and Integrin Blocking on Cell Morphology*

Fluorescent staining was used to investigate the effect of integrin αᵥβ₃ blocking and OFF on osteocyte morphology (Figure 3). Control samples under static conditions had a dendritic morphology, with cells possessing several cell processes characteristic of osteocytes (Figure 3A and B). After exposure to flow the cells displayed a similar morphology to static controls, with an average cell spread area of 425 μm before and 346 μm after flow (Figure 3C). Blocking integrin αᵥβ₃ on MLO-Y4 cells resulted in an increased population of cells that displayed a reduced spread area, 227-82 μm compared to 346-463 μm in the control group under both static and flow conditions (Figure 3D-I). Furthermore following blocking of integrin αᵥβ₃ a number of cells that
were observed to have no cell processes (Fig 3D-I), whereas the cells in the control group were seen to have cell processes (Fig 3A-C). These results indicate that integrin $\alpha_v\beta_3$ is necessary for the development of a normal osteocyte morphology and cell processes.

**The Effect of OFF and Integrin Blocking on MLO-Y4 Gene Expression**

Figure 4A shows COX-2 expression in response to OFF. The application of 1 hour of OFF to control cells was found to cause a 6.2 fold increase in COX-2 expression compared to static controls ($p<0.05$). However, there was no significant difference in COX-2 expression observed between cells exposed to OFF and the static controls after treatment with the anti-$\beta_3$ antibody or IntegriSense (Figure 4A).

Figure 4B shows RANKL expression in response to OFF. RANKL expression in control cells was not found to change with exposure to OFF in either the control or treatment groups (Figure 4B). Figure 4C shows OPG expression in response to OFF. Similarly to RANKL expression, no significant changes in OPG expression were observed with exposure to OFF in either the control or treatment groups.

**The Effect of OFF and Integrin Blocking on PGE$_2$ Release**

Figure 5 shows PGE$_2$ release from MLO-Y4 osteocytes in response to OFF. In the untreated controls, PGE$_2$ release increased significantly from 3.8 to 10.1 pg/ng DNA with exposure to OFF ($p<0.05$). In the groups that were incubated with the integrin antagonists there was no significant difference in PGE$_2$ release between cells exposed to OFF and the static controls after treatment with the anti-$\beta_3$ antibody or IntegriSense.

**Discussion**
The results of our experiments show that blocking integrin \( \alpha_v\beta_3 \) disrupts osteocyte morphology preventing cell spreading and the development of the cell processes that are characteristic of osteocytes. Importantly, it was shown that blocking of integrin \( \alpha_v\beta_3 \) disrupted the expression of COX-2 and PGE\(_2\) release in response to fluid shear stress. Taken together, these results suggest that integrin \( \alpha_v\beta_3 \) plays an important role in mechanotransduction by osteocytes.

The findings of this study reveal the importance of integrin \( \alpha_v\beta_3 \) for osteocyte mechanotransduction \textit{in vitro}, using a monolayer of MLO-Y4 cells exposed to fluid shear stress using a custom designed parallel plate flow chamber. However, it is important to note that these \textit{in vitro} conditions are a simplification of the complex 3D environment of osteocytes embedded within bone tissue \textit{in vivo}. In a monolayer cells can only interact with the extracellular matrix in one plane, whereas the density of cellular-matrix interactions would likely be significantly different in the 3D environment of the lacuno canalicular network \textit{in vivo}. Nonetheless, \textit{in vitro} experimentation using parallel plate flow chambers allows us to reduce the number of experimental variables and avoids the difficulties of disrupting integrins in an animal model. Additionally, the MLO-Y4 cell line may behave differently to primary osteocytes. However, it is difficult to obtain a pure population of primary osteocytes from bone tissue and MLO-Y4s are a well-established model of osteocytes, which have been used extensively in studies of osteocyte mechanobiology (30-32, 34, 35, 43).

Qualitative observations of the distribution of integrin \( \alpha_v\beta_3 \) in MLO-Y4s under static conditions found in our immunohistochemistry (Figure 2) are in keeping with staining of the distribution of integrin \( \alpha_v\beta_3 \) in rat osteocytes cultured \textit{in vitro} (59). Additionally, we also found no alignment of osteocytes in response to oscillatory fluid
Osteocyte expression of COX-2 was upregulated (~6 fold) in response to OFF, similar to previous studies which have shown that COX-2 expression increases by 2 to 6 fold in response to fluid shear (32, 34, 43, 60). However in contrast to previous findings (32, 43), we did not observe significant changes in RANKL or OPG expression in response to OFF in either the control or treatment groups. It is possible that integrin β1 attachment is required for COX-2 and RANKL/OPG expression, while integrin αvβ3 is required for regulation of COX-2 expression but does not play a role in the regulation of RANKL/OPG expression. Interestingly, dissimilar roles of specific integrins in osteocyte Ca^{2+} signalling have previously been reported (43, 51). Litzenberger et al. found that blocking integrin β1 on MLO-Y4s did not alter Ca^{2+} signalling in response to a fluid flow shear stress stimulus (43). However Thi et al. found that blocking integrin αvβ3 with IntegriSense resulted in greatly diminished signalling in response to fluid-induced shear stress (51). Furthermore, the expression of RANKL and OPG are frequently presented as a ratio (32, 43), making it difficult to determine the consistency of the response of individual genes to stimulus when considering the results of such studies. Additionally, it should be noted that the variety of experimental flow chamber designs used to stimulate cells makes direct comparisons difficult. Indeed, recent work has shown that wide ranges in the pressure drop across the flow chamber exist in parallel plate flow chambers that confer the same fluid shear stress, and that this pressure may have a significant effect on the response of cells to fluid shear (61-63), thus making it difficult to directly compare previous studies.

Previous studies have proposed that osteocytes sense mechanical loading through integrin αvβ3 based attachments along cell processes and have demonstrated
that blocking integrin $\alpha_v\beta_3$ disturbs Ca$^{2+}$ signalling (41, 42, 51). Our results show that blocking integrin $\alpha_v\beta_3$ also reduces cell spread area and COX2 and PGE$_2$ signalling in response to fluid flow. A previous study has shown that MLO-Y4 osteocytes in which the integrin $\beta_1$ subunit is disrupted maintain a normal morphology (43), therefore the results of our study suggest a distinct role for integrin $\alpha_v\beta_3$ in the development of osteocyte cell processes. Integrin $\alpha_v\beta_3$ is known to play an important role in cell motility for many cells (64), however as osteocytes are encased with a highly mineralised tissue the cell body is stationary. Interestingly, recent work has found that the connections between osteocytes do not appear to be permanent but rather the processes are repeatedly extended and retracted (65). This may explain why in vivo studies have reported that integrin $\alpha_v\beta_3$ is localised exclusively along osteocyte processes and not the cell body (42). As a result of this localisation of integrin $\alpha_v\beta_3$ on osteocyte process in vivo and the increased fluid shear stresses that have been predicted along cell processes, it has been hypothesised that integrin $\alpha_v\beta_3$ plays a major role in osteocyte mechanotransduction (41, 42).

Our results show that blocking of the integrin $\alpha_v\beta_3$ abrogated the biochemical response (COX-2, PGE$_2$) of MLO-Y4 osteocytes to OFF. This data suggests that in the absence of integrin $\alpha_v\beta_3$ attachments to the collagen substrate both osteocyte structure and the regulation of bone cell activity through the COX-2/PGE2 signalling pathway are disrupted. It has been previously reported that disruption of the cytoskeleton abrogated the response of MLO-Y4 osteocytes to fluid shear (31). Additionally, Litzenberger et al. have found that blocking integrin $\beta_1$ also abrogates the response of osteocytes to mechanical stimulus (43). Litzenberger et al. transfected cells with a dominant negative fragment of the integrin $\beta_1$ subunit, which could not bind to the
extracellular matrix. Transfection did not alter cellular morphology, yet changes in COX-2 and RANKL/OPG expression in response to fluid shear were reported. Interestingly, Ca$^{2+}$ signalling was not altered by transfection. Thi et al. have shown that blocking integrin $\alpha_v\beta_3$ disrupts Ca$^{2+}$ signalling is response to stimulus. These results indicate different roles for integrins $\alpha_v\beta_3$ and $\beta_1$ in the response of osteocytes to fluid shear stress, as blocking integrin $\alpha_v\beta_3$ disrupts both PGE$_2$ release and Ca$^{2+}$ signalling whereas blocking integrin $\beta_1$ only disrupts PGE$_2$ release. Therefore we propose that, due to the localisation of $\alpha_v\beta_3$ along cell processes in vivo (42) and the importance for osteocyte response to stimulus demonstrated in this study, integrin $\alpha_v\beta_3$ may play a significant contributory role in osteocyte mechanobiology.

**Conclusions**

Osteocytes are believed to be the orchestrator of bone formation and resorption through the regulation of both osteoclast and osteoblast activity (15). The results of this study show that blocking integrin $\alpha_v\beta_3$ disrupts osteocyte morphology, in particular the mechanosensory cell processes. Furthermore blocking integrin $\alpha_v\beta_3$ alters the biochemical response of osteocytes to mechanical stimulus. A better understanding of mechanosensation in osteocytes may lead to the development of novel treatments for bone pathologies where mechanosensitivity is thought to be compromised.

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MLO-Y4 cell line used in this study was received as a kind gift from Professor Lynda Bonewald (School of Dentistry, University of Missouri, Kansas City, MO, U.S.A.).

References:


**Figure Captions:**

**Figure 1** (A) Illustration of parallel plate apparatus: (i) syringe-pump, (ii) parallel plate chamber, (iii) media reservoir and (iv) filtered opening to allow gas exchange. (B) Illustration of the flow path within the chambers showing a CFD simulation of the wall shear stress distribution and (v) an overlay showing the location of the cell seeded slide. (C) Illustration of the fluid velocity streamlines within the chamber showing the transition to laminar flow.

**Figure 2:** Immunofluorescent imaging of integrins on MLO-Y4s under static conditions. (A&B) Integrin β1 (green) and actin (red). (C&D) Integrin β3 (green) and actin (red). The boxes in images A and C highlight the areas magnified in images C and D.

**Figure 3:** Fluorescent imaging of MLO-Y4s directly after control and integrin αvβ3 antagonist treatments (A,D&G) and after 1 hour of static (B,E&H) or oscillatory flow conditions (C,F&I). Cell nuclei are stained with DAPI (blue) and actin is stained with phalloidin-TRITC (red). White arrows highlight cells showing a reduced spread area and retraction of processes. Cell spread area was quantified using ImageJ.
**Figure 4:** Expression of (A) COX-2, (B) RANKL and (C) OPG in MLO-Y4s under static and oscillatory flow conditions in control and integrin $\alpha_v\beta_3$ antagonist treated cells. *p<0.05 versus all other groups.

**Figure 5:** PGE$_2$ release by MLO-Y4s under static and oscillatory flow conditions in control and integrin $\alpha_v\beta_3$ antagonist treated cells. *p<0.05 versus all other groups.