# Differential ß3 and ß1 integrin expression in bone marrow and cortical bone of estrogen deficient rats

**Title:** Differential $\beta_3$ and $\beta_1$ integrin expression in bone marrow and cortical bone of estrogen deficient rats.

M. Voisin [1], L.M. McNamara [1]

[1] Biomechanics Research Centre (BMEC), Biomedical Engineering, College of Engineering and Informatics, National University of Ireland Galway, Ireland.

**Running title:** $\beta_3$ and $\beta_1$ integrins in ovariectomized bone

**Address for correspondence:**

Dr Laoise McNamara,

Biomechanics Research Centre,

Discipline of Biomedical Engineering,

National University of Ireland, Galway,

Galway,

Ireland.

laoise.mcnamara@nuigalway.ie

Tel: +353 91 492251

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**Abstract**

Integrin-based ($\beta_3$) attachments to the extracellular matrix (ECM) on osteocyte cell processes have recently been proposed to play an important role in facilitating osteocyte mechanosensation. However, it is not yet known whether integrin expression is altered in the mechanoregulatory osteocytes during osteoporosis. The objective of this study was to test the hypothesis that the expression of integrin-based mechanosensory complexes ($\beta_1$ and $\beta_3$ integrins) is altered as a direct response to estrogen deficiency, in an estrogen deficient animal model of osteoporosis. Four weeks post-operatively, immunohistochemistry was used to detect for $\beta_1$ and $\beta_3$ integrin subunits in bone tissue and marrow of ovariectomized (OVX) (n=4) and SHAM (n=4) operated animals. A tartrate resistant acid phosphatase (TRAP) control stain was performed to quantify the presence of osteoclasts in the bone marrow and bone surfaces. Image analysis was performed to quantify expression patterns in different biological compartments, i.e bone marrow, endosteum, and cortical bone. Our results showed that $\beta_1$ integrins were ubiquitously expressed throughout the bone and marrow, for both OVX and SHAM groups. $\beta_3$ integrin subunit expression was lower in bone cells from osteoporotic animals compared to controls, whereas $\beta_3$ expression in marrow cells did not differ significantly between groups. At the endosteum no difference was observed in $\beta_3$ integrin subunit expression. As expected, the number of osteoclasts was higher in the OVX group validating an imbalance in bone remodelling. We propose that a reduction in $\beta_3$ integrin expression in osteocytes might impair mechanosensation by bone cells during estrogen deficiency.

**Keywords**: Bone, osteoblast, osteocyte, integrin, marrow, mechanosensors, immunohistochemistry.

**Introduction**:

Bone is a complex and constantly remodelling connective tissue that can adapt its structure and composition in response to changes in physical activity. This adaptive process relies on the presence of cells that can appraise the local mechanical environment (mechanosensors) and elicit a biochemical signalling response (mechanotransduction) to initiate tissue adaptation when the mechanical environment is not favourable. All cells of the osteoblast lineage (osteoprogenitors, osteoblasts, osteocytes) are capable of transducing mechanical signals into biochemical stimuli (el Haj et al., 1990), but osteocytes are the most widely accepted candidates (Burger and Klein-Nulend, 1999; Bonewald, 2011). A recent study demonstrated that osteocytes are influential for stimulating osteogenesis in multipotent stromal cells (MSCs) through direct biochemical signalling (Birmingham et al., 2012), which further corroborates their putative role as regulators of bone biology.

The precise mechanisms by which osteoblasts or osteocytes can sense mechanical stimuli are unclear but various mechanosensory organelles, namely integrin receptors, gap junctions and primary cilia, have been identified in bone tissue and these organelles likely facilitate mechanosensation in bone tissue (Yellowley et al., 2000; Whitfield, 2008; McNamara et al., 2009). Integrins are a family of cell-surface trans-membrane proteins, consisting of α and β subunits, which bind the internal cytoskeleton to extracellular matrix proteins (Hynes, 1992). It is believed that integrins and their ligands act together to mediate cell-cell interactions and sense mechanical stimuli for many cells of the body by activating intracellular signalling pathways and eliciting a biochemical response (Puklin-Faucher and Sheetz, 2009). Integrins are ubiquitously found in bone tissue and cells (Horton and Davies, 1989; Clover et al., 1992a). In osteoclasts, integrins $\alpha_v\beta_1$, $\alpha_2\beta_1$ and $\alpha_v\beta_3$ mediate cell–matrix interactions during bone resorption (Horton et al., 1991; Engleman et al., 1997), whereas osteoblasts and osteocytes express $\beta_1$ in concert with $\alpha_1$, $\alpha_2$, $\alpha_3$, $\alpha_4$ and $\alpha_5$ (Horton and Davies, 1989; Clover et al., 1992a; Grzesik and Robey, 1994)$\beta_3$ integrins are associated with $\alpha_v$ in osteoblasts (Gronthos et al., 1997) and osteocytes (Thi et al., 2013). Recent immunohistochemistry studies of osteocyte cell processes have revealed that integrin $\alpha_v\beta_3$ concentrations occur along osteocyte cell processes *in vivo* and it has been proposed that these integrin-based ($\alpha_v\beta_3$) attachments to the extracellular matrix (ECM) on osteocyte cell processes may facilitate osteocyte mechanosensation (Wang et al., 2007; McNamara et al., 2009; Thi et al., 2013). *In vitro* cell culture studies have demonstrated that blocking of integrins $\beta_1$ and $\alpha_v\beta_3$ disrupts osteoblast and osteocyte responses to fluid flow (Lee et al., 2008; Litzenberger et al., 2010a; Haugh et al., 2015).

Osteoporosis is a debilitating bone disease, most commonly manifested in women following the menopause when estrogen production is deficient, which is associated with severe bone loss and fractures of the

hip, wrist or vertebrae. Clinical hormone and bisphosphonate drug treatments only reduce fracture susceptibility by approximately 50% (Randell et al., 2002). This may be owing to the fact that, although extensive research has been undertaken, the mechanisms initiating the disease are poorly understood. Osteoblasts and osteocytes possess receptors for estrogen (Braidman et al., 2001), which plays a role in the normal response of bone cells to loading (Lanyon, 1996), and osteoblastic cells deprived of estrogen display deficient osteogenic responses to mechanical stimuli *in vitro* (Sterck et al., 1998; Jessop et al., 2004). Mechanical loading, representative of loads occurring during postmenopausal osteoporosis, also leads to a downregulation of estrogen receptor α ($ER_\alpha$) expression in osteocytes (Ehrlich et al., 2002). Furthermore the proliferative effects of mechanical strain have been shown to be mediated by ERα for osteoblastic cells *in vitro* (Zaman et al., 2000; Galea et al., 2013). In addition the organisation of the osteocyte network is altered (Knothe Tate et al., 2004) and some studies reported that osteocyte density decreased in aging bone (Qiu et al., 2002) whereas others reported that it increased in post menopausal bone (Mullender et al., 1996), and has been shown to strongly depend on the skeletal site (Hernandez et al., 2004). Such changes might be related to changes in mechanosensation, perhaps through integrin-based attachments to the extracellular matrix, but this has never been demonstrated.

Integrins, in particular $\alpha_v\beta_3$, have been shown *in vitro* and *in vivo* to play an important role in osteoporosis, as they are involved with osteoclast functionality (Li et al., 1995; Lane et al., 2005). It is known that the presence of estrogen increases expression of $\beta_3$ integrin through mRNA stabilisation in preosteoclasts (Li et al., 1995). $\beta_3$ integrin is also involved in early bone fracture repair and $\beta_3$-null mice exhibit higher levels of bone formation and cartilage maturation (Hu et al., 2010). MSCs isolated from osteoporotic patients downregulate expression of $\alpha_2$ integrin, but not $\alpha_1$ and $\alpha_{11}$, other known subunits associated with $\beta_1$ in collagen receptors (Popov et al., 2011). It is however not yet clear whether integrin expression is altered in the mechanoregulatory cells of the bone tissue (particularly osteocytes). Given the recent findings of the important role of $\beta_1$ and $\alpha_v\beta_3$ for osteoblast and osteocyte mechanosensation (Wang et al., 2007; Lee et al., 2008; McNamara et al., 2009; Litzenberger et al., 2010a), it is important to understand whether integrin expression is altered in osteoblasts and osteocytes of bone tissue during estrogen deficiency. Thus, the objective of this study was to delineate whether the expression of integrin-based mechanosensory complexes is altered as a direct response to estrogen deficiency, in an animal model of osteoporosis.

**Materials and methods**:

***Animal model:***

All animal work was performed in accordance with the guidelines of the Animal Care and Research Ethics Committee (ACREC) procedures, National University of Ireland, Galway, and under licence from the Irish Department of Health and Children and in compliance with the Council of European Union directive 86/609. For these studies bone tissue was harvested from an ovariectomized rat model, which is an Food and Drug Administration (FDA) approved estrogen depletion-induced osteoporosis (type I post-menopausal) model for studies on osteoporosis (Thompson et al., 1995). At the age of 6 months, female Wistar breeder rats (Charles River) were retired and aged until 9 months old. The animals went through a full physiological cycle, and achieved a skeletally mature state. Eight animals were divided into two groups, one consisting of bilaterally ovariectomized animals (n=4), and in the other group, animals were subjected to a sham operation (n=4). Ovariectomies and sham operation were performed using a dorsal thoracolumbar entry (at Charles River). Animals were housed in pairs, food and water was dispensed *ad libitum* and the environment was enriched to reduce level of stress **(**structure and substrate, manipulanda and novel foods). A 12 hour light cycle, air humidity (40-70%) and temperature (19-23˚C) were monitored and controlled. After operation and recovery, activity was normal and animals did not show signs of infections. Four weeks post ovariectomy; animals did not show any difference in weight (487.25 ± 77.7 g for OVX animals vs. 490 ± 91.6 g for SHAM controls, $p = 0.9645$).

Animals were sacrificed four weeks post-operation, when the effects of early osteoporosis would be expected (Keiler et al., 2012). Tibia and femora were harvested from ovariectomized animals and sham-operated controls after euthanasia by increasing rates of $CO_2$. Verification of SHAM or OVX status was achieved by identifying the presence or absence of ovaries during sampling. After collection, tissues were immersed for 48H in fixative (4% w/v paraformaldehyde (Sigma Aldrich) in phosphate buffered saline (PBS, Sigma Aldrich) solution, at 4˚C.

After an extensive rinse in PBS, tissue samples were decalcified in a 10% w/v solution of ethylenediaminetetraacetic acid (EDTA, Sigma Aldrich) in deionised water ($dH_2O$) at a pH of 7.5. The decalcification endpoint was determined using an oxalate test and a physical probing test. After decalcification, samples were rinsed in water overnight, to drain excess EDTA. Afterwards, a tissue processor (Leica ASP300) routine protocol was used (formalin, grade of ethanol, xylenes and paraffin immersion) and samples were embedded in paraffin (Leica EG1150H).

Samples were cut longitudinally and embedded. Sections were cut from the inside to the outside of the bone out, with a thickness of 5 µm, using a microtome (Leica RM2235). Slices were collected on SuperFrost® Plus slides (Menzel Glaser), and stored at room temperature, until staining.

***Immunohistochemistry for integrin-based mechanosensors in vivo***

This study focuses on the study of integrin subunit rather than full heterodimers. Integrin $\beta_3$ is found in only a few heterodimeric forms *in vivo* and *in vitro* (Takada et al., 2007), and is only found under the form of $\alpha_v\beta_3$ in osteocytes and osteoblasts (Hughes et al., 1993; Grzesik and Robey, 1994) (Clover et al., 1992b; Gronthos et al., 1997), and osteoclasts (Rodan and Rodan, 1997) (Nesbitt et al., 1993). The $\beta_1$ subunit has been shown to be under a variety of forms including $\alpha_2\beta_1$(Olivares-Navarrete et al., 2008), $\alpha 1\beta_1$ , $\alpha_3\beta_1$, $\alpha_4\beta_1$ and $\alpha_5\beta_1$ (Saito et al., 1994; Anselme, 2000). Nevertheless, it is commonly studied as a subunit in order to understand osteoblast and osteocyte biology *in vivo* (Zimmerman et al., 2000) and *in vitro* (Yeh et al., 2010)(Litzenberger et al., 2010b). Furthermore, the $\beta_1$ subunit is involved in the response of osteoblasts to mechanical stimulation in the form of fluid shear stress (Litzenberger et al., 2010b)(Pavalko et al., 1998). For these reasons we chose to study $\beta_3$ and $\beta_1$ subunits.

Slides were dewaxed overnight at 60˚C. Slides were then cleared in Xylenes (Sigma Aldrich), three times for five minutes each, then rehydrated in decreasing grades of ethanol (100%, 90%, 70%, 50%, 30%) (Fisher), twice for two minutes each, and rinsed in deionised water.

Antigen retrieval was performed using proteinase K (20 µg /mL, Sigma Aldrich), in Tris EDTA buffer (TE) (50mM Tris base (Sigma Aldrich), 1mM EDTA, 0.5% TritonX-100 (Sigma Aldrich), pH=7.5), for ten minutes at 37 ˚C, followed by 10 minutes cooling and rinsing twice for 2 minutes each in PBS-Tween (Sigma Aldrich) 0.5% v/v. Blocking and antibody diluent were made using Normal Goat Serum (NGS) 3% w/v/ Bovine Serum Albumin (BSA) 1% w/v in PBS. Blocking was allowed for 1 hour at room temperature.

After blocking, sections were incubated with antibodies against $\beta_1$ integrin (Merck Millipore AB1952, 1/100), or against $\beta_3$ integrin (Merck Millipore 04-1060, 1/50)), overnight at 4˚C. Then samples were rinsed three times for 10 minutes in PBS /BSA 1% w/v. Secondary antibody (Goat dylight 488 anti rabbit, Jackson ImmunoResearch 115-485-209, 1/200) was then incubated with the samples for 1 hour at room temperature in a humidification chamber, in the dark. To evaluate specificity, controls containing antibody diluent only, instead of primary antibodies, were used with the secondary antibody as described above. Cells from control animals, bone marrow cells for $\beta_1$ and megakaryocytes for $\beta_3$, served as positive validation for the primary antibodies. Finally, three rinses, for 10 minutes each in PBS BSA 1% w/v, were performed, in the dark, and slides were

mounted with a propidium iodide (PI) containing mounting media (Sigma Aldrich) with coverslips (#1 thickness, Menzel Glaser).

***Confocal microscopy:***

Slides were imaged on an inverted confocal laser-scanning microscope (LSM 510; Zeiss, Germany). The image acquisition software used was AIM 4.2 (Zeiss, Germany). High magnification (63X, oil immersion objectives) micrographs were obtained, using a low scan speed (7 s), averaging 8 images, using a 1024x1024 pixel definition. An argon laser (488 nm, 514 nm) was used to excite Dylight 488 and PI. The field of view was 142.86 µm * 142.86 µm.

***Immunohistochemistry for Tartrate resistant acid phosphatase (TRAP):***

To identify the presence of osteoclasts at the endosteum, immunohistochemistry targeting TRAP (also known as ACP5) was used. Slides were processed as previously described and samples were quenched for endogenous peroxidase with 1 % hydrogen peroxide (Sigma) for 5 minutes. Antigen retrieval was the same as previously described. For detection of TRAP on tissues, a goat anti TRAP antibody (Santa Cruz sc-30833, 1/100) was used in conjunction with a matching biotinylated anti goat secondary antibody and AB reagents (Santa Cruz, sc-2023), as per manufacturer's instructions. The blocking solution provided was applied for one hour at room temperature, followed by incubation with anti-TRAP antibody overnight at 4˚C. Then samples were rinsed three times for 5 minutes in PBS, and incubated with secondary antibody (1/66) for 45 minutes at room temperature. Samples were then rinsed three times for 5 minutes in PBS, and AB enzyme reagent was applied for 30 minutes. After three rinses for 5 minutes in PBS, peroxidase substrate was added for 20 minutes. Slides were counterstained with Haematoxylin (Sigma) for 2 minutes, rinsed, dehydrated using ascending grades of ethanol, cleared in Xylenes and mounted with DPX.

***Image analysis and Quantitative analysis of integrin expression:***

ImageJ (http://rsbweb.nih.gov/ij/) was used to analyse micrographs and quantify cell number and protein expression. Integrin expression was analysed in at least two stained sections from each bone, stained in two separate experiments, and a minimum of 10 fields were imaged per section.

Micrographs were converted into 8 bit images, and each channel was analysed separately. Nuclei were marked individually (polynuclear cells were counted once only), the "Analyse particle" command was then used, and that score was recorded to give the total nuclei number. The same technique was applied to the protein staining: a positive score was given for each positively stained cell . A percentage positive of integrin expressing cells was then calculated.

***Quantitative analysis of TRAP staining:***

Positively stained cells at the endosteum were counted as osteoclasts. A total perimeter of 4 mm was analysed per sample (n=4 per group), and the number of total osteoclasts was reported per mm of cortical endosteum, to compare osteoclast frequency between SHAM and OVX groups.

***Statistics:***

A Shapiro Wilk test was used to confirm that the data was following a normal distribution. Results are expressed as means ± standard deviation. The data was compared using a Welch two-tailed unpaired Student's t-test and statistical significance was taken at $p<0.05$. All statistical analysis was performed using the R statistical suite (www.**r**-project.org).

**Results:**

***Immunohistochemistry controls in vivo:***

Cells within the bone marrow acted as positive control for $\beta_1$ integrin (Fig. 1a, arrow).and polykaryonic cells from the marrow as positive controls for $\beta_3$ integrin (Fig. 1b, arrow). Negative controls replacing primary antibody by antibody diluent containing normal goat serum did not show any unspecific staining (Fig. 1c).

***Immunohistochemistry for $\beta_1$ integrin-based mechanosensors in vivo:***

In both sham operated and ovariectomized rats, the distribution of $\beta_1$ integrin subunit was observed indiscriminately in the marrow, on mono and polynuclear cells (Figure 2a and c, arrows). The $\beta_1$ integrin subunit was also found on bone-lining cells (Fig 3a, arrow), and osteocytes (Figure and d, arrows). There was no significant difference in $\beta_1$ expression between the SHAM and OVX groups in either the bone marrow, or for bone lining cells and osteocytes (Fig. 2e).

***Immunohistochemistry for $\beta_3$ integrin-based mechanosensors in vivo:***

In sham operated animals, $\beta_3$ integrin was identified in multi and mononucleated cells of the marrow (Fig. 3a). $\beta_3$ integrin was also found in cells at the endosteum, where osteoblasts, lining cells, endosteal macrophages and osteoclasts are situated, (Fig 3b) and on osteocytes of cortical bone (Fig. 3b). In mature bone tissue, osteocytes are the only cells present within the lacunae and display a very distinguishable morphology, with an elongated cell body and cell processes, and were thereby identified by their physical location and morphology. Adipocytes within the bone marrow displayed clusters of $\beta_3$ integrin adhesion (Fig. 4a). In the bone marrow, adipocytes display a very distinguishable morphology, and were identified by their physical location, shape, and the presence of large voids (where the centrally lipid vacuole was located before extraction). In ovariectomized animals, the distribution of $\beta_3$ integrin subunit was detected in the marrow, where it was apparent in polynuclear cells, mononucleated cells (Fig. 3c), and adipocytes (Fig. 4b). $\beta_3$ integrin was also expressed in osteocytes from cortical bone (Fig. 3d), and at the interface between the bone and marrow (Fig. 3d). Here also clusters of $\beta_3$ integrin were found to interface between adipocytes and their neighbouring cells (Fig. 4b).

$\beta_3$ expression was not statistically different between the osteoporotic and control bone marrow (34.27±12.06 % vs. 30.72±4.36 %, p= 0.6108) (Fig. 5a). $\beta_3$ integrin expression was significantly lower in osteoporotic cortical bone tissue compared to that of sham-operated controls (23.50± 5.17% vs. 37.5± 8.73%, p = 0.041) (Fig.5a).

The difference between $\beta_3$ expression in the entire marrow compartment was not statistically different to $\beta_3$ expression in the bone lining cells (i.e < 20µm from the bone and marrow interface) but in both cases the lining zone expressed higher amounts of $\beta_3$ integrin than the cells in the entire marrow compartment (Fig. 5b). At the lining between marrow and bone (<20µm from the bone and marrow interface), $\beta_3$ integrin expression was similar in OVX bone tissue compared to the control bone tissue (64.68 ± 17.73% vs. 57.45 ± 11.49 %, p = 0.5235). The expression of $\beta_3$ in adipocytes showed no difference between groups (50.39±13.86 % for OVX vs. 61.36±26.94 % for SHAM, p = 0.5753) (Fig. 5b).

***Immunohistochemistry for TRAP in vivo:***

Positively TRAP stained osteoclasts were visualised on both SHAM and OVX samples, at the lining between bone and bone marrow (Fig.6a and 6b). The TRAP distribution was intracellular and intense staining was well defined at the endosteum. As part of the validation, negative controls replacing primary antibody by normal serum were used and did not show any specific staining (Fig. 6c)

To obtain an accurate representation of numbers, the same length of total endosteum per animal (4 mm) was measured in both SHAM and OVX samples, and TRAP positive cells were counted. Results showed that OVX samples had significantly more osteoclasts than the SHAM, 5.64 ± 1.8 vs. 1.50 ± 0.92 /mm, p = 0.0122 (Fig. 6d). The total number of osteoclasts counted for OVX samples was 99 and the total for SHAM samples was 24.

**Discussion**:

The objective of this study was to delineate whether the distribution of integrin-based mechanosensory complexes (i.e $\beta_1$ or $\beta_3$ integrin receptors) was altered during osteoporosis. Our results showed that $\beta_1$ was equally expressed in marrow and cortical bone of control and osteoporotic animals, four weeks post ovariectomy. In the bone marrow and at the endosteum, there was no difference was in $\beta_3$ integrin expression. However, TRAP staining for osteoclasts showed a marked increased at the osteoporotic endosteum, and, as it is well accepted that osteoclasts and their committed progenitors express a high levels of $\beta_3$ integrin, these results suggest that changes in $\beta_3$ integrin expression may be variable across the marrow cell population. Interestingly, $\beta_3$ integrin receptors were differentially expressed between control cortical bone and osteoporotic animals, specifically $\beta_3$ expression decreased in the osteoporotic osteocytes.

There are certain limitations to this study that should be considered. Firstly, although the ovariectomized rat model is a widely approved and utilised model of osteoporosis (Thompson et al., 1995), it shows dissimilarities from human biology, such as the absence of developed Haversian canals, and the fact that rats do not experience natural menopause, but this it is commonly chemically or chirurgically induced. Nonetheless, the similarities of the model to the human disease, including increased rate of bone turnover, an initial rapid bone loss phase followed by secondary slower bone loss, and similar skeletal response to therapeutic agents, make it suitable for providing an insight into human disease mechanisms. Also, despite the absence of dual staining for lineage specific markers, such as Dentin Matrix Protein 1 (DMP1) or Sclerostin (Sost) for osteocytes (Franz-Odendaal et al., 2006), cell phenotype can be strongly inferred by the physical location and morphology of the cells within the marrow and cortical bone, particularly for adipocytes and osteocytes. Longer time points need to be considered in future studies to see if these changes can be confirmed at later stages of the disease, or to investigate whether regulatory mechanisms are triggered to counterbalance this effect of estrogen depletion on $\beta_3$ integrin receptor expression in bone cells. Furthermore the amount of $\beta_3$ integrin expressed in osteoblasts and lining cells of the endosteum was not assessed directly. Dual staining could have been employed, using $\beta_3$ integrin and osteoblasts markers, but would have likely also quantified early osteocytes (Dallas and Bonewald, 2010). Another way to circumvent this problem would have been to extract and study primary osteoblasts. However, this process would likely disrupted membrane-expressed adhesion markers, as the process involves enzymatic and mechanical treatments and takes the cells out of their environment, changing the design from *in vivo*, niche related study to primary, *in vitro* 2D cell study (Declercq et al., 2004). Finally, this study is based on immunohistochemistry methods alone as this allowed evaluation of the precise spatial distribution of the integrins of interest. Other antibody based methods such as western blotting could have been considered, but it would give a gross estimation of protein expression, without the means to discriminate between cell populations. Indeed, most separation methods rely on flushing marrow out of the cavity to retrieve marrow cells, or by sequential enzymatic digestion to retrieve osteoblastic cells (Declercq et al., 2004), which will either include heterogeneous cell phenotypes, include early osteocytes or exclude early osteoblastic cells (Dallas and Bonewald, 2010). Real time polymerase chain reaction could also be used to study transcripts of interest, but the same limitation applies in terms of cell population heterogeneity. Lastly, these techniques would take the cells out of their natural environment, changing the design from an *in vivo*, niche-related study to an *in vitro* 2D primary cell study. *In situ* hybridization remains a possibility for future studies; however, at present there are

currently no available probes for the integrins of interest in rat as most of the commercially available probes are directed against human targets.

The results of this study show that the bone marrow of control bones expressed high levels of $\beta_1$ integrin for different cell types. Interestingly we found that there was no difference in $\beta_1$ expression during estrogen deficiency, whereas a recent study showed that $\alpha_2$ integrin, as part of a $\alpha_2\beta_1$ heterodimer, was down regulated, unlike other $\beta_1$ heterodimers ($\alpha_1\beta_1$ and $\alpha_{11}\beta_1$), in osteoporotic versus aged human patients (Popov et al., 2011). In the present study global $\beta_1$ integrin subunit expression was analysed, rather than the presence of specific heterodimers, therefore it may be that only a small subset of $\beta_1$ receptors for collagen or non collagenous proteins are modified during disease (e.g. $\alpha_1\beta_1$, $\alpha_2\beta_1$ or $\alpha_{11}\beta_1$ collagen receptors, $\alpha_1\beta_1$ receptors for fibronectin or $\alpha_1\beta_1$ receptors for laminin) either in the marrow or the cortical bone. It is also possible that such changes, related to the modifications in ECM, such as collagen biochemical modifications (due to altered gene expression, degradation, crosslinking) (Young, 2003) and contents (Bailey et al., 1999)) occur at a later phase of the disease, whereas the changes observed here appeared within four weeks after abrupt withdrawal of estrogen, at an early stage of the disease. In a mouse model, localized expression of the $\beta_3$ subunit integrin has been identified at the site of osteocyte processes, very likely under the form of the $\alpha_v\beta_3$ heterodimer (McNamara et al., 2009). In the current study we did not observe particular $\beta_3$ staining along cell processes. This might be explained by the fact that the previous study involved younger animals with immature skeleton whereas here, older, skeletally mature animals were used. However, the findings are similar regarding the expression of $\beta_3$ in the vicinity of osteocytes, and for the $\beta_1$ ubiquitous expression, which may imply that age or maturity of the skeleton is not a discriminating factor for the expression of $\beta_1$ integrin.

Our results regarding the presence of $\beta_3$ integrin in the marrow converge with previous studies that have shown that $\alpha_v\beta_3$ integrin is strongly linked to osteoclastogenesis and osteoclastic resorptive activity, as $\alpha_v\beta_3$ forms the sealing zone for resorption pit formation (Li et al., 1995) and so far $\beta_3$ integrin has been reported only under the form of $\alpha_v\beta_3$ in osteoclasts. It might be expected that $\beta_3$ expression would be higher in the bone marrow of osteoporotic animals, which are known to have increased osteoclastogenesis and bone resorption (D'Amelio et al., 2008), therefore our findings of no difference in $\beta_3$ expression between control and osteoporotic tissues in the bone marrow or at the bone/marrow interface might at first appear to be confounding. However, our TRAP data confirms that mature osteoclasts were indeed more numerous in OVX samples, confirming the increased osteoclastic activity state of ovariectomised animals.. Total osteoblast number has been shown, in an immature ovariectomised rat model, to be equivalent 4 weeks post operatively, and decreased over

time, compared to matched sham controls (Lei et al., 2009). Another study, in mature ovariectomised rats, did not show any statistical differences in osteoblast surface five weeks post operatively (Li et al., 2009). Therefore, we propose that, due to the presence of increased numbers of osteoclasts at the endosteal surface, and the similarity in $\beta_3$ protein expression between groups, the amount of $\beta_3$ integrin expressed by other cells, i.e lining cells, and osteal tissue macrophages (osteomacs) (Chang et al., 2008), osteoblasts and early osteocytes may be decreased in OVX samples compared to controls. If this were the case, this finding would be in keeping with the results reported here for the cortical bone, which showed lower levels of $\beta_3$ integrin expression in the osteocyte population. However further studies are required to definitively address this question.

$\beta_3$ integrin is present in the marrow on a variety of cells, from both hematopoietic (megakaryoblasts and megakaryocytes, and platelet precursors) and stromal lineage (Mesenchymal stem cells (MSCs) or bone marrow stromal cells (BMSCs)). Although thought to be few in numbers in the bone marrow, MSCs and BMSCs express integrins in a variety of subunits and heterodimers, including $\beta_1$ and $\beta_3$ subunits. Integrins are known to be essential to MCSs and BMSCs for adhesion and differentiation (Docheva et al., 2007). Therefore it is possible that the lack of differences in $\beta_3$ integrin expression between the marrow population of OVX and CON groups might also be explained by modifications in $\beta_3$ integrin expression in other non-hematopoietic bone marrow cells. Specifically, we propose that an increase in $\beta_3$ integrin due to osteoclastogenesis and a decrease in the osteoblast precursors may balance each other and explain why no difference was seen in the bone marrow during osteoporosis. However, further study including lineage and stage specific markers is required to definitely investigate the differential expression of $\beta_3$ integrins in cells of the bone marrow compartment and at the endosteum.

It is intriguing to speculate on the cause of differences in $\beta_3$ integrin expression in the osteocytes of bone tissue arising from estrogen depletion, as seen in this study. One of the main effects of the menopause is a dramatic reduction in estrogen levels, with levels of estradiol varying from 50 - 300 pg/mL, at the peak of their cycle, to lower than 20 pg/mL after the menopause. In a pre-menopausal situation, estrogens bind to estrogen receptors (ERs) within the cells. Upon binding ERs change conformation, adopt their active form and translocate to the nucleus, where they become transcriptionally active, regulating gene promoters (Nilsson et al., 2001). In post-menopausal conditions, when the estrogen hormone is deficient, the ERs are located within the cell (cytoplasm and nucleus) where they remain in an inactive form (no transcriptional activity). Estrogen, through transcription factors called homeobox genes (i.e. HOXA10), stabilises mRNA coding for $\beta_3$ integrin in other cell types, i.e the endometrium cells where it increases integrin mediated adhesion (Daftary et al., 2002).

HOXA10 was also demonstrated as necessary for the transcription of the gene coding for $\beta_3$ integrin, and the expression of the $\beta_3$ protein during differentiation of myeloid cells in bone marrow (Bei et al., 2007). Interestingly, HOXA10 was shown to regulate and support osteoblast differentiation in bone, where it induces expression of the RUNX2 transcription factor (Hassan et al., 2007). Recent microRNA (miRNA) profiling experiments on osteoporotic MSCs have shown that HOXA10 is downregulated by specific miRNAs elicited during osteoporosis (miR-705), shifting MSC differentiation from osteoblastic to adipogenic. This correlates with the fact that the fat cell content in osteoporotic bone marrow is higher than controls (Justesen et al., 2001) and a study also showed that HOXA10 deficient osteoblasts differentiated improperly (Liao et al., 2013). Therefore we propose that differences in $\beta_3$ integrin expression in the osteocytes of bone tissue are regulated by changes in HOXA10 during estrogen depletion. We show here that those changes go beyond the bone marrow biological niche and extend to the cortical bone where osteocytes are present and might play an important role in the overall bone loss cascade.

It has been suggested that $\alpha_v\beta_3$ may facilitate mechanosensation at the osteocyte cell processes level *in vivo* (McNamara et al., 2009). A recent in vitro study showed that osteocyte cell processes are mechanosensitive through discrete attachment sites provided by $\alpha_v\beta_3$ integrin (Thi et al., 2013), and another study showed that blocking of $\alpha_v\beta_3$ resulted in altered cell morphologyand decreased biochemical responses to fluid flow (Haugh et al., 2015). Therefore a decrease in $\beta_3$ integrin in osteocytes, as evidenced in this study, may affect mechanosensing and thereby contribute to bone loss at the later stage of estrogen deficiency.

The results of this study suggest that integrin expression could act as a marker of osteoporosis as early as 4 weeks post ovariectomy deficiency in rats. From a therapeutic point of view, the $\alpha_v\beta_3$ ligand, Arg-Gly-Asp (RGD) mimetic, has been shown to prevent the rapid bone loss that accompanies estrogen withdrawal (Engleman et al., 1997). In the light of the results presented here, it would be important to reassess the activity of $\alpha_v\beta_3$ antagonists, as they might also operate by targeting osteocyte function. The results of this study suggest that $\alpha_v\beta_3$ may prove to be an important target for pharmacological intervention during osteoporosis, by means of action through osteocytes and osteoblast rather that osteoclasts binding alone.

**Conclusions**

This study sheds light on the relationship between bone estrogen deficiency and mechanosensor expression *in vivo*, providing a possible link between hormone depletion, cell adhesion and mechanosensation. We provide a quantitative assessment of $\beta_1$ and $\beta_3$ integrin distribution in bone marrow cells and osteocytes

during osteoporosis. We show the importance of the $\beta_3$ integrin receptor and the modification of this mechanosome in cortical bone. Additionally, we propose that $\beta_3$ could serve as a marker of early osteoporosis, and that it should be carefully considered as a therapeutic target.

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**References:**


Anselme K. 2000. Osteoblast adhesion on biomaterials. Biomaterials 21:667-681.

Bailey AJ, Sims TJ, Ebbesen EN, Mansell JP, Thomsen JS, Mosekilde L. 1999. Age-Related Changes in the Biochemical Properties of Human Cancellous Bone Collagen: Relationship to Bone Strength. Calcif Tissue Int 65:203-210.

Bei L, Lu Y, Bellis SL, Zhou W, Horvath E, Eklund EA. 2007. Identification of a HoxA10 Activation Domain Necessary for Transcription of the Gene Encoding β3 Integrin during Myeloid Differentiation. Journal of Biological Chemistry 282:16846-16859.

Birmingham E, Niebur GL, McHugh PE, Shaw G, Barry FP, McNamara LM. 2012. Osteogenic differentiation of mesenchymal stem cells is regulated by osteocyte and osteoblast cells in a simplified bone niche. Eur Cell Mater 23:13-27.

Bonewald LF. 2011. The amazing osteocyte. J Bone Miner Res 26:229-238.

Braidman IP, Hainey L, Batra G, Selby PL, Saunders PT, Hoyland JA. 2001. Localization of estrogen receptor beta protein expression in adult human bone. J Bone Miner Res 16:214-220.

Burger EH, Klein-Nulend J. 1999. Mechanotransduction in bone--role of the lacuno-canalicular network. FASEB J 13 Suppl:S101-112.

Chang MK, Raggatt L-J, Alexander KA, Kuliwaba JS, Fazzalari NL, Schroder K, Maylin ER, Ripoll VM, Hume DA, Pettit AR. 2008. Osteal Tissue Macrophages Are Intercalated throughout Human and Mouse Bone Lining Tissues and Regulate Osteoblast Function In Vitro and In Vivo. The Journal of Immunology 181:1232-1244.

Clover J, Dodds R, Gowen M. 1992a. Integrin subunit expression by human osteoblasts and osteoclasts in situ and in culture. J Cell Sci 103 ( Pt 1):267-271.

Clover J, Dodds RA, Gowen M. 1992b. Integrin subunit expression by human osteoblasts and osteoclasts in situ and in culture. J Cell Sci 103:267-271.

D'Amelio P, Grimaldi A, Di Bella S, Brianza SZM, Cristofaro MA, Tamone C, Giribaldi G, Ulliers D, Pescarmona GP, Isaia G. 2008. Estrogen deficiency increases osteoclastogenesis up-regulating T cells activity: A key mechanism in osteoporosis. Bone 43:92-100.

Daftary GS, Troy PJ, Bagot CN, Young SL, Taylor HS. 2002. Direct Regulation of β3-Integrin Subunit Gene Expression by HOXA10 in Endometrial Cells. Molecular Endocrinology 16:571-579.

Dallas SL, Bonewald LF. 2010. Dynamics of the transition from osteoblast to osteocyte. Annals of the New York Academy of Sciences 1192:437-443.

Declercq H, Van den Vreken N, De Maeyer E, Verbeeck R, Schacht E, De Ridder L, Cornelissen M. 2004. Isolation, proliferation and differentiation of osteoblastic cells to study cell/biomaterial interactions: comparison of different isolation techniques and source. Biomaterials 25:757-768.

Docheva D, Popov C, Mutschler W, Schieker M. 2007. Human mesenchymal stem cells in contact with their environment: surface characteristics and the integrin system. Journal of Cellular and Molecular Medicine 11:21-38.

Ehrlich PJ, Noble BS, Jessop HL, Stevens HY, Mosley JR, Lanyon LE. 2002. The Effect of In Vivo Mechanical Loading on Estrogen Receptor α Expression in Rat Ulnar Osteocytes. Journal of Bone and Mineral Research 17:1646-1655.

el Haj AJ, Minter SL, Rawlinson SC, Suswillo R, Lanyon LE. 1990. Cellular responses to mechanical loading in vitro. Journal of Bone and Mineral Research 5:923-932.

Engleman V, Nickols G, Ross F, Horton M, Griggs D, Settle S, Ruminski P, Teitelbaum S. 1997. A peptidomimetic antagonist of the alpha(v)beta3 integrin inhibits bone resorption in vitro and prevents osteoporosis in vivo. The Journal of clinical investigation 99:2284-2292.

Franz-Odendaal TA, Hall BK, Witten PE. 2006. Buried alive: how osteoblasts become osteocytes. Developmental dynamics : an official publication of the American Association of Anatomists 235:176-190.

Galea GL, Meakin LB, Sugiyama T, Zebda N, Sunters A, Taipaleenmaki H, Stein GS, van Wijnen AJ, Lanyon LE, Price JS. 2013. Estrogen Receptor α Mediates Proliferation of Osteoblastic Cells Stimulated by Estrogen and Mechanical Strain, but Their Acute Down-regulation of the Wnt Antagonist Sost Is Mediated by Estrogen Receptor β. Journal of Biological Chemistry 288:9035-9048.

Gronthos S, Stewart K, Graves S, Hay S, Simmons P. 1997. Integrin expression and function on human osteoblast-like cells. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 12:1189-1197.

Grzesik W, Robey P. 1994. Bone matrix RGD glycoproteins: immunolocalization and interaction with human primary osteoblastic bone cells in vitro. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 9:487-496.

Hassan MQ, Tare R, Lee SH, Mandeville M, Weiner B, Montecino M, van Wijnen AJ, Stein JL, Stein GS, Lian JB. 2007. HOXA10 Controls Osteoblastogenesis by Directly Activating Bone Regulatory and Phenotypic Genes. Molecular and cellular biology 27:3337-3352.

Haugh MG, Vaughan TJ, McNamara LM. 2015. The role of integrin alphaVbeta3 in osteocyte mechanotransduction. Journal of the Mechanical Behavior of Biomedical Materials 42:67-75.

Hernandez CJ, Majeska RJ, Schaffler MB. 2004. Osteocyte density in woven bone. Bone 35:1095-1099.

Horton M, Davies J. 1989. Perspectives: adhesion receptors in bone. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 4:803-808.

Horton M, Taylor M, Arnett T, Helfrich M. 1991. Arg-Gly-Asp (RGD) peptides and the anti-vitronectin receptor antibody 23C6 inhibit dentine resorption and cell spreading by osteoclasts. Experimental cell research 195:368-375.

Hu D, Lu C, Sapozhnikova A, Barnett M, Sparrey C, Miclau T, Marcucio RS. 2010. Absence of beta3 integrin accelerates early skeletal repair. Journal of Orthopaedic Research 28:32-37.

Hughes D, Salter D, Dedhar S, Simpson R. 1993. Integrin expression in human bone. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 8:527-533.

Hynes R. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. Cell 69:11-25.

Jessop HL, Suswillo RF, Rawlinson SC, Zaman G, Lee K, Das-Gupta V, Pitsillides AA, Lanyon LE. 2004. Osteoblast-like cells from estrogen receptor alpha knockout mice have deficient responses to mechanical strain. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 19:938-946.

Justesen J, Stenderup K, Ebbesen EN, Mosekilde L, Steiniche T, Kassem M. 2001. Adipocyte tissue volume in bone marrow is increased with aging and in patients with osteoporosis. Biogerontology 2:165-171.

Keiler AM, Zierau O, Vollmer G, Scharnweber D, Bernhardt R. 2012. Estimation of an early meaningful time point of bone parameter changes in application to an osteoporotic rat model with in vivo microcomputed tomography measurements. Laboratory Animals 46:237-244.

Knothe Tate ML, Adamson JR, Tami AE, Bauer TW. 2004. The osteocyte. The international journal of biochemistry & cell biology 36:1-8.

Lane NE, Yao W, Nakamura MC, Humphrey MB, Kimmel D, Huang X, Sheppard D, Ross FP, Teitelbaum SL. 2005. Mice lacking the integrin beta3 subunit have accelerated osteoclast maturation and increased activity in the estrogen-deficient state. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 20:58-66.

Lanyon LE. 1996. Using Functional Loading to Influence Bone Mass and Architecture - Objectives, Mechanisms, and Relationship With Estrogen Of the Mechanically Adaptive Process In Bone. Bone 18:S37-S43.

Lee D-Y, Yeh C-R, Chang S-F, Lee P-L, Chien S, Cheng C-K, Chiu J-J. 2008. Integrin-Mediated Expression of Bone Formation-Related Genes in Osteoblast-Like Cells in Response to Fluid Shear Stress: Roles of Extracellular Matrix, Shc, and Mitogen-Activated Protein Kinase. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 23:1140-1149.

Lei Z, Xiaoying Z, Xingguo L. 2009. Ovariectomy-associated changes in bone mineral density and bone marrow haematopoiesis in rats. International Journal of Experimental Pathology 90:512-519.

Li CF, Ross FP, Cao X, Teitelbaum SL. 1995. Estrogen enhances alpha v beta 3 integrin expression by avian osteoclast precursors via stabilization of beta 3 integrin mRNA. Mol Endocrinol 9:805-813.

Li X, Ominsky MS, Warmington KS, Morony S, Gong J, Cao J, Gao Y, Shalhoub V, Tipton B, Haldankar R. 2009. Sclerostin antibody treatment increases bone formation, bone mass, and bone strength in a rat model of postmenopausal osteoporosis. Journal of Bone and Mineral Research 24:578-588.

Liao L, Yang X, Su X, Hu C, Zhu X, Yang N, Chen X, Shi S, Jin Y. 2013. Redundant miR-3077-5p and miR-705 mediate the shift of mesenchymal stem cell lineage commitment to adipocyte in osteoporosis bone marrow. Cell Death Dis 4:e600.

Litzenberger J, Kim J-B, Tummala P, Jacobs C. 2010a. $\beta_1$ Integrins Mediate Mechanosensitive Signaling Pathways in Osteocytes. Calcif Tissue Int 86:325-332.

Litzenberger JB, Kim JB, Tummala P, Jacobs CR. 2010b. β1 Integrins mediate mechanosensitive signaling pathways in Osteocytes. Calcif Tissue Int 86:325-332.

McNamara LM, Majeska RJ, Weinbaum S, Friedrich V, Schaffler MB. 2009. Attachment of Osteocyte Cell Processes to the Bone Matrix. Anat Rec:355–363.

Mullender MG, Vandermeer DD, Huiskes R, Lips P. 1996. Osteocyte Density Changes In Aging and Osteoporosis. Bone 18:109-113.

Nesbitt S, Nesbit A, Helfrich M, Horton M. 1993. Biochemical characterization of human osteoclast integrins. Osteoclasts express alpha v beta 3, alpha 2 beta 1, and alpha v beta 1 integrins. Journal of Biological Chemistry 268:16737-16745.

Nilsson S, Mäkelä S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M, Gustafsson J-Å. 2001. Mechanisms of Estrogen Action. Physiological Reviews 81:1535-1565.

Olivares-Navarrete R, Raz P, Zhao G, Chen J, Wieland M, Cochran DL, Chaudhri RA, Ornoy A, Boyan BD, Schwartz Z. 2008. Integrin α2β1 plays a critical role in osteoblast response to micron-scale surface structure and surface energy of titanium substrates. PNAS 105:15767-15772

Pavalko FM, Chen NX, H. TC, Burr DB, Atkinson S, Hsieh Y-F, Qiu J, Duncan RL. 1998. Fluid shear-induced mechanical signaling in MC3T3-E1 osteoblasts requires cytoskeleton-integrin interactions. Am J Physiol Cell Physiol:C1591-C1601.

Popov C, Radic T, Haasters F, Prall WC, Aszodi A, Gullberg D, Schieker M, Docheva D. 2011. Integrins α2β1 and α11β1 regulate the survival of mesenchymal stem cells on collagen I. Cell Death and Dis 2:e186.

Puklin-Faucher E, Sheetz MP. 2009. The mechanical integrin cycle. J Cell Sci 122:179-186.

Qiu S, Rao D, Palnitkar S, Parfitt A. 2002. Age and distance from the surface but not menopause reduce osteocyte density in human cancellous bone. Bone 31:313-318.

Randell KM, Honkanen RJ, Kroger H, Saarikoski S. 2002. Does hormone-replacement therapy prevent fractures in early postmenopausal women? Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 17:528-533.

Rodan SB, Rodan GA. 1997. Integrin function in osteoclasts. Journal of Endocrinology 154:S47-S56.

Saito T, Albelda S, Brighton C. 1994. Identification of integrin receptors on cultured human bone cells. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 12:384-394.

Sterck JG, Klein-Nulend J, Lips P, Burger EH. 1998. Response of normal and osteoporotic human bone cells to mechanical stress in vitro. American Journal of Physiology 274:E1113-1120.

Takada Y, Ye X, Simon S. 2007. The integrins. Genome Biology 8:215.

Thi MM, Suadicani SO, Schaffler MB, Weinbaum S, Spray DC. 2013. Mechanosensory responses of osteocytes to physiological forces occur along processes and not cell body and require αVβ3 integrin. Proceedings of the National Academy of Sciences 110:21012-21017.

Thompson DD, Simmons HA, Pirie CM, Ke HZ. 1995. FDA guidelines and animal models for osteoporosis. Bone 17:S125-S133.

Wang Y, McNamara LM, Schaffler MB, Weinbaum S. 2007. A model for the role of integrins in flow induced mechanotransduction in osteocytes. PNAS 104:15941-15946.

Whitfield JF. 2008. The solitary (primary) cilium--a mechanosensory toggle switch in bone and cartilage cells. Cellular signalling 20:1019-1024.

Yeh C-R, Chiu J-J, Lee C-I, Lee P-L, Shih Y-T, Sun J-S, Chien S, Cheng C-K. 2010. Estrogen augments shear stress–induced signaling and gene expression in osteoblast-like cells via estrogen receptor–mediated expression of β1-integrin. Journal of Bone and Mineral Research 25:627-639.

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

Young MF. 2003. Bone matrix proteins: their function, regulation, and relationship to osteoporosis. Osteoporos Int 14:35-42.

Zaman G, Cheng MZ, Jessop HL, White R, Lanyon LE. 2000. Mechanical strain activates estrogen response elements in bone cells. Bone 27:233-239.

Zimmerman D, Jin F, Leboy P, Hardy S, Damsky C. 2000. Impaired Bone Formation in Transgenic Mice Resulting from Altered Integrin Function in Osteoblasts. Developmental biology 220:2-15.

**Figure legends**

**Figure 1** Immunofluorescent staining for (a) $\beta_1$ (green) marrow positive cells (arrow), (b) $\beta_3$ integrin subunit marrow positive polykaryonic cell (arrow), and (c) no primary antibody negative control shows no staining on bone tissue. Nuclear counterstain: propidium iodide (red). Scale bar = 20 µm.

**Figure 2** Immunofluorescent staining for $\beta_1$ integrin subunit (green) in mono and polynucleated cells of the bone marrow and endosteal cells of the bone for SHAM (a, arrows) and OVX (c, arrows) groups, and in cortical bone cells of SHAM (b, arrows) and OVX (d, arrows) groups. Nuclear counterstain: propidium iodide (red). Scale bar = 20 µm. Bar chart (e) shows the percentage of $\beta_1$ positive cells per biological compartment and experimental group.

**Figure 3** Immunofluorescent staining for $\beta_3$ integrin subunit (green) in multi and polynuclear cells in SHAM (a, arrows) and OVX (c, arrows) bone marrow cells, in cells at the endosteum in SHAM (b, arrows) and OVX bone (d, arrow) and in osteocytes (d, zoom box). Nuclear counterstain: propidium iodide (red). Scale bar = 20 µm.

**Figure 4** Immunofluorescent staining for $\beta_3$ integrin subunit (green) in SHAM (a) and OVX (b) bone marrow. The zoom box demonstrates clustering of $\beta_3$ integrins at the interface between the adipocytes and other marrow cells. Nuclear counterstain: propidium iodide (red). Scale bar = 20 µm.

**Figure 1**(a) Percentage of $\beta_3$ positive cells per biological compartment and experimental group. Total cell count : marrow OVX cells: 8862, marrow SHAM cells: 8393, cortical OVX cells: 397, cortical SHAM cells: 348. Asterisk indicates significant difference, $p = 0.041$, (b) percentage of $\beta_3$ positive cells in the bone marrow and endosteum per cell type and experimental group. Total cell count for OVX bone lining cells: 1071, SHAM bone lining cells: 848, OVX adipocytes: 115 and SHAM adipocytes: 175.

**Figure 6**

Immunohistochemistry for TRAP in SHAM (a) and OVX (b) tissues. Immunohistochemistry was performed using peroxidase staining with ABC substrate. Arrows show TRAP positive osteoclasts. No primary antibody negative control showed no staining (c). Nuclear counterstain: Haematoxylin. Scale bar = 200 µm. (d) Graph of the number of osteoclasts counted per mm of endosteum. Asterisk indicates significant difference, $p = 0.0122$.