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Site-Specific Differences in Osteoblast Phenotype, Mechanical Loading Response and Estrogen Receptor-Related Gene Expression

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ABSTRACT

The osteoporosis-resistant nature of skull bones implies inherent differences exist between their cellular responses and those of other osteoporosis-susceptible skeletal sites. Phenotypic differences in calvarial and femoral osteoblastic responses to induction of osteogenesis, mechanical loading, estrogen, growth factor and cytokine stimulation were investigated.

Primary rat calvarial and femoral adult male osteoblasts were cultured and osteoblastic mineralisation and maturation determined using Alizarin Red staining and expression of osteogenic marker genes assessed. Expression of known mechanically-responsive genes was compared between sites following loading of scaffold-seeded cells in a bioreactor. Cell proliferation and differentiation following growth factor and estrogen stimulation were also compared. Finally expression of estrogen receptors and associated genes during osteogenic differentiation were investigated.

Calvarial osteoblasts exhibited delayed maturation (45d. vs 21d.) and produced less mineralised matrix than femoral osteoblasts when osteogenically induced. PDGF-BB and FGF2 both caused a selective increase in proliferation and decrease in osteoblastic differentiation of femoral osteoblasts. Mechanical stimulation resulted in the induction of the expression of *Ccl2 and Anx2a* selectively in femoral osteoblasts, but remained unchanged in calvarial cells. Estrogen receptor beta expression was selectively upregulated 2-fold in calvarial osteoblasts. Most interestingly, the estrogen responsive transcriptional repressor RERG was constitutively expressed at 1000-fold greater levels in calvarial compared with femoral osteoblasts. RERG expression in calvarial osteoblasts was down regulated during osteogenic induction whereas upregulation occurred in femoral osteoblasts.

Bone cells of the skull are inherently different to those of the femur, and respond differentially to a range of stimuli. These site-specific differences may have important relevance in the development of strategies to tackle metabolic bone disorders.

INTRODUCTION

Osteoporosis occurs as the result of an imbalance in bone remodelling predominately in the appendicular and vertebral skeleton such that bone resorption exceeds bone formation and is most obvious in post-menopausal women with relative estrogen deficiency, in men with reduced androgen levels, and with decreased levels of physical activity[1]. Paradoxically, the skull bones retain structural integrity despite low levels of mechanical loading, and furthermore, they are essentially unaffected by osteoporosis[2,3]. That not all bones are equally susceptible to the condition implies inherent differences in the resident bone cells, potentially dependent on their position in the body.

The reasons for these differences in susceptibility to metabolic and disuse osteoporosis are not fully understood, and could be dependent differences between intramembranous (calvarial) versus endochondral (limb) primary ossification processes. Contrary to this, primary ossification of the lateral aspect of the clavicle is intramembranous, yet it is this aspect that is more prone to osteoporosis compared with the medial endochondral-derived aspect[4]. What is common, however, is that the medial aspect of the clavicle and skull bones contain neural crest-derived cells[5]. Previous studies have also shown differences in proliferation, osteogenic differentiation and response to growth factors between neural crest dual intramembranous-endochondral-derived orofacial and mesodermal endochondral-derived appendicular bones[6,7]. Thus differences may not be solely determined by the mechanism of bone formation or turnover per se, but also possibly by differential *regulation* of bone formation in distinct sites – in this case an intramembranous-derived calvarial bone with a neural crest component compared with an endochondral-derived bone with no neural crest component.

As well distinct formation processes, numerous studies have also shown physical and functional differences between osteoclasts derived from appendicular and calvarial sites both *in vitro and in vivo* which could impact on the susceptibility of individual bones to osteoporosis [8-11]. In addition both matrix composition[12,13] and osteocyte morphology[14] have been shown to differ between calvaria and long bones. These differences may be related to features of site-related osteoblastic heterogeneity including sensitivity to PTH[15] as well as induction of osteoclastogenesis and levels of signalling pathway genes involved in osteoclast formation[16].

It has become increasingly clear that defined mature cell phenotypes may in fact show much greater diversity than perhaps has been traditionally conceived and understood [17,18]. In a previous study from our group we investigated differences in global gene expression patterns between adult rodent long bones and skull bones and matched pairs of isolated osteoblasts derived from femurs and calvaria *in vitro*. In the isolated bone cells we found 246 differentially expressed genes between osteoblasts from these sources [19]. Prominent amongst differentially expressed genes were genes associated with cell embryonic origin such as homeobox containing genes (*Hoxa*, *Hoxb*, *Hoxc*, *Hoxd*, *Shox*) and other transcription factors which are thought to act specifically on embryologically distinct bone formation. These include *Msx-2*, *Dlx-5* and *Cart1* whose disruption in knock out mice specifically affects cranial bone formation and *Tbx-3* which specifically affects limb bone formation[19]. Furthermore, in experiments described by Leucht and co-workers, mandibular Hoxa –ve and femoral Hoxa+ve osteogenic stem cells were tested for their ability to contribute to healing in mandibular and femoral bone defects *in vivo.* They demonstrated that *Hoxa* +ve cells were unable to contribute to healing in the mandibular site, but Hoxa –ve cells contributed to wound healing in the femoral site [20]. Taken together the data suggest the hypothesis that regionally specific osteoblasts are phenotypically distinct, due to cell autonomous mechanisms. That osteoblasts have "positional memory" such that the localised information they express during embryogenesis persists into the adult organism results in regionally specified differences in osteoblast phenotypes, and further, that these differences translate to functional physiological responses controlling bone homeostasis. Here we studied the phenotypic differences in adult-derived calvarial and femoral osteoblast responses to the induction of osteogenesis, mechanical loading, estrogen, growth factor and cytokine stimulation. Adult derived rodent calvarial cells have reduced proliferation, mineralisation, and growth factor responsiveness when compared to juvenile osteoblasts[21]. Therefore it appears that these altered characteristics make them more suitable to investigate in this context.

Cellular responses to estrogen stimulation are dependent on expression of Estrogen receptors (ER) which may alter markedly according to the stage of cell differentiation. Estrogen receptors are nuclear receptors which consist of 2 isoforms, ERα and ERß, which have distinct expression patterns and may have distinct functions[1]. In addition their function may be regulated particularly by specific co-

regulators either as receptor co-activators such as the SRCs, and receptor co-repressors such as the N-CoRs and rerg[1].

Despite these previous findings more systematic studies are required to elucidate the differences between mature skull and limb bone derived osteoblasts from to further recognise potential strategies to treat osteoporosis. Therefore, the aim of the study here was to investigate the hypothesis that there are intrinsic differences in expression of genes involved in the estrogen signal cascade between adult-derived calvarial and femoral osteoblasts. It is proposed that the results of these experiments may contribute to identification of potential signalling targets responsible for distinct behaviour of positionally distinct osteoblast populations.

MATERIALS AND METHODS

Cell culture

All animal protocols were in accordance with the UK Home Office Scientific Procedures Act (1986). Male Wistar rats (210 g, 10 weeks old) were purchased from Charles River, housed, and fed *ad libitum* in accordance with local Queen Mary University of London, School of Medicine and Dentistry rules and sacrificed by cervical dislocation. Calvarial and femoral bones were aseptically removed, cleared of soft tissues and cut into pieces. The bone chips were transferred evenly into 6-well culture plates containing DMEM supplemented with 10% fetal bovine serum with glutamine (2 mM) and 1% P/S and incubated at 37^{o}C in 5% CO2 incubator, left undisturbed for 48-72 hours until an 'osteoid seam' was noticed. When adherent cells of seam formation were observed, the bone chips were collected and bone cells isolated by enzymatic digestion. Osteoblasts were cultured in α-MEM without ribo/deoxyribonucleosides, L-glutamine (2 mM), penicillin (50 U/mL), streptomycin (50 µg/mL) and 10% fetal bovine serum (FBS) (all from Sigma–Aldrich) and media was replenished every 3-4 days. To stimulate osteogenesis cells were treated with medium supplemented with 0.1µM dexamethasone, 0.05mM Ascorbic Acid (AA) and 5mM β-glycerophosphate (all from Sigma–Aldrich). To assess osteogenic differentiation, mRNA expression of markers of differentiation (i.e. *Runx-2*, *ALPL*, *osteopontin and osteocalcin*) was determined by quantitative (q)RT-PCR and accumulation of calcium deposits was visualised and quantified by staining with Alizarin Red dye. Briefly, cells were fixed (15 min. with 4% formaldehyde in PBS), stained for 10 min with Alizarin Red S (1:100 dilution in H_2O) and washed in 50% ethanol and air-dried. Cell cultures were stimulated with the following recombinant growth factors and cytokines FGF-2 (10ng/ml) (Peprotech, London, UK), PDGF-BB (10ng/ml) (Peprotech), BMP2 (100ng/ml) and Wnt3a (50ng/ml) (both from R&D systems), as well as β-estradiol (Sigma-Aldrich).

Raman spectroscopy

A Renishaw 'inVia' Raman microscope (Renishaw plc, Wotton-under-Edge, UK) was used in this study. The spectra of femoral and calvarial osteoblasts after 21 days in culture were analyzed using a

785-nm diode laser (100mW sample power). The laser beam was focused through a water immersion x60/1.2NA objective lens (working distance: 0.27mm) while the Raman signal was acquired using a 600-lines/mm diffraction grating centered between 857 and 1231 cm^{-1} and 2s CCD exposure time. The spectra were recorded at a resolution of ~1-2 cm^{-1}.

Mechanical stimulation

Mechanical stimulation of femoral and calvarial osteoblasts was conducted as previously described [22]. Cells were seeded on to the top of one side of calcium phosphate monetite scaffolds at a density of 1.5×10^5 in 70 µl of media and allowed to attach for 30 min. The scaffold was then turned and the same number of cells were seeded on to the top of the other side and left undisturbed for a further 30 min for the cells to attach. Normal growth media was then carefully added to the culture plate and the cell/scaffold was incubated at 37°C in a humidified 5% CO_2:95% air atmosphere for 48 h prior to stimulation. Mechanical loading was performed using a BOSE bioreactor (ElectroForce BioDynamic test instrument; Bose) equipped with 200N load cell. Seeded scaffolds were positioned between two loading plates inside the bioreactor chamber and loaded in the diametral compression mode by a pulsating compressive force of 5.5±4.5 N at a frequency of 0.1 Hz [22]. The corresponding head displacement was 0.5–50 µm. The test was performed in load control and the evolution of the head displacement versus the pulsating compressive force was recorded by the bioreactor software (WinTest® controls). The stress distribution in the disk was analytically evaluated by the Timoshenko model [23].

qRT-PCR analysis

Total RNA was extracted using TRI reagent (Ambion, Warrington, UK) and Phase Lock Gel Heavy tubes (5 prime, VWR, Leicestershire, UK) according to the manufacturer's instructions. RNA purity and quantity was assessed by Nanodrop (Fisher Scientific) (A_{260}/A_{280} 1.8-2 was considered suitable for further analysis), possible contaminating DNA was removed and cDNA prepared from 1 µg RNA using QuantiTect Reverse Transcription Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions. qRT-PCR was performed on a Mx3000P real time PCR as described

previously [19]. For TaqMan based analysis primers and probes were purchased from Invitrogen and for Sybr green qPCR the primers and sequences are listed in Table 1. EIF4A2 was used as a housekeeping control.

Cell viability assay

Cell doubling and viability (DNA synthesis) was assessed by measuring 5-ethynyl-2′ -deoxyuridine (EdU) DNA incorporation using the Click-iT EdU Alexa Fluor 647 cell proliferation assay kit (Invitrogen) and by MTS (CellTiter 96 AQ_{ueous} solution cell proliferation assay; Promega, Southampton, UK) calorimetric assay following manufacturer instruction respectively. For EdU DNA incorporation assay, cells were treated with EdU at 10 μg/ml for 48 hrs, harvested by trypsinization, washed in PBS/1% BSA and fixed with Click-iT fixative. The cells were then permeabilized using saponin-based permeabilization reagent, treated with the Click-iT EdU reaction cocktail in the dark and washed with saponin-based permeabilization reagent. The number of EdU-positive cells was determined using a FACS-Canto II flow cytometer, and data analysis was performed using DIVA software (Becton Dickinson Biosciences, San Jose, CA).

Alkaline phosphatase (ALP) activity assay

Alkaline phosphatase (ALP) activity was determined by an assay based on the hydrolysis of *p*-nitrophenylphosphate to *p*-nitrophenol (Sigma-Aldrich). Cells were washed with PBS, and 100 μl substrate solution was added to each well. After 20 minutes in the dark, the reaction was stopped and absorbance (405 nm) was read on a spectrophotometer. ALP activity was normalized to cell number by MTS assay (Promega).

siRNA transfection and ERE reporter assay

siRNA against *Rerg*, scrambled siRNA and Cignal Reporter assay (Qiagen, West Sussex, UK) were performed according to the manufacturer's instructions. Femoral or calvarial osteoblasts were seeded at 4×10^4 cells/cm and co-transfected with 150ng of reporter construct and siRNA (final concentration

of 10nM) in 100 µl of Opti-MEM serum free media containing 2 µl of Attractene transfection reagent. After 48hrs incubation, luciferase activity was determined using the Dual-Luciferase Reporter assay system (Promega), according to the manufacturer's instructions.

Data Analysis

Statistical comparisons between means were made by one-way ANOVA (SPSS 17, SPSS) and post hoc analyses using the Tukey test to evaluate the differences among the mean values between groups. If comparisons were made only between two groups two tailed Student's t test (SPSS 16, SPSS) was used. A P-value of less than 0.05 was considered statistically significant.

RESULTS

Responses of femoral and calvarial cells to stimulation of differentiation

Rawlinson and co-workers previously showed that osteoblasts derived from calvaria and femur exhibit a distinct local pattern of gene expression[19]. Here we investigated whether these cells are also distinct in their phenotypic responses to induction of osteogenesis. Cells were treated with differentiation media and changes in osteogenic gene expression and matrix mineralisation were analysed. Expression of *Runx2*, *osteopontin* (*Spp1*), *ALPL* and *osteocalcin* gradually increased during stimulation in both femoral and calvarial cells (Fig 1A). No significant differences were observed in the levels of induction of *Runx2, ALPL* or *Spp1* expression between femoral and calvarial cells during stimulation compared to unstimulated cells at any time point. However, osteocalcin expression was increased in femoral compared to calvarial cells (more than 1600 fold higher) at late stages of culture (21D) (Fig 1A). As shown in Figure 1A right panel, when each of the 3 cell lines were examined individually we observed a dramatically higher level of osteocalcin expression in femoral cells compared to calvarial cells. However, due to variation in differentiation capacity between each line, combination of all three lines failed to show a statistical difference even with up-regulation of 1600 fold. Similarly, rapid and potent matrix mineralisation was observed in femoral cells (as early as 10

days post induction (data not shown)), whereas only negligible mineralisation was evident even after 45 days of induction in calvarial cultures (Fig 1B). Furthermore, micro-Raman analysis of mineralised nodules, suggested cell dependent differences in mineral matrix composition associated with femoral compared to calvarial cells (Fig 1C). Raman spectra of native bone and mature mineralized nodules is dominated by the PO_4^{3-} peak at 959cm^{-1} correspond to the mineral component of bone, hydroxyapatite [24]. This peak was clearly evident in femoral but not in calvarial cells, indicating lack of mineral composition in calvarial cell cultures.

Responses of femoral and calvarial cells to mechanical loading.

To investigate whether femoral and calvarial cells respond differently to mechanical stimulation, candidate early response genes i.e. *Fosb, Junb, cFos, Fosl1, Ccl2* and *Anx2a*, which have been previously shown by our group and others to be stimulated in response to mechanical loading, were analysed in calvarial and femoral osteoblasts following a pulsating compressive force of 5.5±4.5 N (0.2% strain) for a period of 2 hours as described previously [22,25]. As shown in Figure 2, mechanical stimulation resulted in the induction of the expression of *Ccl2 and Anx2a* in femoral osteoblasts, but remained unchanged in calvarial cells in the same experiments. A similar trend was evident for *Fosl1* and *cFos* but statistical significance was not reached due to the previously mentioned variation in responses by different primary cell lines. Expression of *Fosb* and *Junb* remained unchanged in both femoral and calvarial cells (data not shown).

Differences in responses of femoral and calvarial cells to induction of proliferation and ALP activity by estrogen and growth factors.

To investigate whether femoral and calvarial cells respond differently to major extracellular stimuli, cells were treated with estrogen (17β-estradiol), Wnt3a, BMP2, FGF-2 or PDGF-BB and the effects on proliferation and ALP activity were assessed. Estrogen had no significant effect on either femoral or calvarial cells (Fig 3A). Similarly, we were unable to observe any stimulatory effect in presence of

BMP2 and Wnt3a (Fig 3B). Disparities between our findings and others could be due to culture conditions. Most studies were conducted in presence of osteogenic media or other stimulus in combination with BMP2 or Wnt3a, while we treated the cells in absence of any other factors. On the other hand both FGF-2 and PDGF-BB significantly stimulated calvarial cell proliferation over controls, while only a minor effect was observed on femoral cell proliferation (Fig 3B). FGF-2 was significantly inhibitory for ALP activity in both femoral and calvarial cells (Fig 3B). Since no significant effect was observed following treatment with estrogen, we further investigated the role of estrogen signalling on function of femoral and calvarial cells by blocking the estrogen receptors (ER) signalling using a selective antagonist of ER (ICI 182,780) during proliferation and induction of osteogenesis of both osteoblast cultures. Similar to stimulation studies using estrogen, no significant effect was observed on proliferation in response of either femoral or calvarial cells when ER were inhibited by ICI 182,780 and also ICI did not have any effect on FGF-2 or PDGF-BB induced cell proliferation or ALP activity (Fig 3C). The differentiation capacity of the either femoral or calvarial osteoblasts also remained unchanged in presence of ICI 182,780, showing a similar gene expression profile when stimulated by either differentiation media alone or with BMP2 (Fig 3D). Similarly, ICI 182,780 had no effect on ALP activity of femoral and calvarial cells either in normal growth media or when stimulated in presence of Wnt3a, BMP2, FGF-2 or PDGF-BB (Fig 3C).

Expression of ERα and ERß estrogen receptors and estrogen receptor co-activators SRC-1, -2 and -3, and receptor co-repressors during differentiation of femoral and calvarial cells.

Since both agonists (17β-estradiol) and antagonists (ICI 182,780) failed to elicit a response in femoral and calvarial cells we investigated possible intracellular regulation of ER. ER have been shown to have ligand-independent activity, involving receptor co-activators and receptor co-repressors. We therefore studied expression of ERα and ERß estrogen receptors, estrogen receptor co-activators Ncoa-1, -2 and -3, and receptor co-repressors Ncor 1-2 and repressor of estrogen receptor activity (REA) during osteogenic maturation of the cells (Fig 4). REA expression was significantly higher in femoral

compared to calvarial cells and remained unchanged during maturation. Expression of the other receptor co-repressors was elevated at day 21 of stimulation in femoral compared to calvarial during the maturation and mineralisation period (Fig 4A-B), whereas expression levels of co-activators cells remained relatively unchanged between femoral and calvarial cells (Fig 4B). Interestingly expression of ERα was low at mRNA level and remained at an undetectable level during the course of the study. ERß expression was higher in unstimulated femoral compared to calvarial osteoblasts (Fig. 5A). However, ERß expression was significantly reduced when osteogenesis was induced in both calvarial and femoral cells and remained low to the late stage of mineralisation (21 days) (Fig 5C).

Role of *Rerg* in femoral and calvarial cells.

We also investigated the Ras-related and estrogen-regulated growth-inhibitor (*Rerg*) which has been suggested as an estrogen responsive gene[26]. *Rerg* was differentially expressed in calvarial cells compared with femoral cells with basal *Rerg* expression being 1200 fold greater in calvarial than femoral osteoblasts (Fig 5A-B). However, upon osteogenic induction *Rerg* expression was dramatically increased in femoral cells reaching a maximum of 64-fold when compared with unstimulated cells in every line examined (Fig 5C). Expression of *Rerg* in calvarial osteoblasts was reduced following osteogenic stimulation although this was not statistically significant (Fig 5C). Therefore, to determine possible role for *Rerg* in regulation of estrogen signalling between femoral and calvarial cells, we analysed the effect of siRNA knock down of *Rerg* on transcriptional activity down-stream of estrogen signalling using an ERE luciferase reporter assay. Interestingly, *Rerg* knock down resulted in significant reduction in ERE luciferase activity in calvarial but not in femoral osteoblasts (Fig 6B). These results suggest *Rerg* may play a site-specific role in regulating estrogen signalling in calvarial osteoblasts selectively.

Discussion

It is well documented that bones of the skull tend to be resistant to osteoporosis with previous studies investigating differences in characteristics between susceptible long bones and resistant skull bones [12,27,28]. However, the idea that this may be due to differences in the action of osteoblasts from these sites has not been extensively investigated. In our previous study we demonstrated that the genes associated with bone mass and mineral density are differentially expressed in functionally distinct skeletal sites [19]. Here, we report that osteoblasts derived from skull and long bones are also functionally distinct. We observed that osteoblasts derived from mature calvariae have a significantly lower ability for accumulation of mineralised matrix compared with femoral osteoblasts when exposed to osteogenic induction medium containing dexamethasone, ascorbic acid and β-glycerophosphate. They were unable to produce any mineralisation by 21 days and only negligible amounts by day 45 and is consistent with previously observed findings for adult-derived calvarial osteoblasts. Raman spectroscopy analysis of mineralised nodules further revealed that only mineral deposited by femoral osteoblasts produced a peak near 960 cm^{-1} which corresponds to the mineral component of bone hydroxyapatite [29]. To find out the reason behind this observation we carried out mRNA expression analysis for genes involved in osteogenesis. As with previous studies indicating the involvement of *Runx2* in both femoral and calvarial cells, a similar expression pattern was observed in both cell types here[30]. However, osteocalcin expression was significantly induced in femoral compared to calvarial cells at late stage of 21 days. Osteocalcin is suggested to be involved in bone mineralisation and formation of hydroxyapatite and therefore a higher level of osteocalcin expression seen in femoral osteoblasts could be associated with potent mineralisation ability of these cells [31]. Our observations are also in line with the previous study showing differences in protein composition of flat and long bones, suggesting functional differences in formation, resorption, and mechanical properties of these bone types[12]. In contrast, a recent study has shown no differences between adult mouse-derived calvarial and femoral osteoblastic proliferation rates, mineralisation capacity and levels of osteogenic gene expression[16]. Among other characteristics that separate these bones is the difference in response to mechanical loading. It is known that maintenance of BMD in long bones is dependent on mechanical loading and osteoblasts derived from these sites are responsive to mechanical loading, while BMD in

the skull remains high in absence of loading and calvarial cells are shown to be insensitive to mechanical loading [32,33]. However, a contrasting observation has been reported showing that compressive forces upregulate the expression of osteogenic genes *Bmp2, Runx2* and *Smad5* and promote osteogenesis of calvarial osteoblasts [34]. This study was however conducted on newborn calvarial cells which could probably account for the disparities seen between data. Here we confirm the idea supporting the lack of response by calvarial cells and show that only osteoblasts derived from femur are sensitive to mechanical loading, showing elevated expression of early genes associated with mechanical stimulation [25].

The calvarial bone is known to be insensitive to post-menopausal hormonal changes and osteoporosis. Osteoblasts *in vitro* are known to be estrogen responsive and classically, stimulation with 17ß-estradiol has been shown to upregulate osteoblast proliferation and expression of differentiation markers[35-38]. However more recent experiments have demonstrated a more complex set of distinct responses to estrogen stimulation (both stimulation and inhibition) which are dependent on expression of ER and which may alter markedly according to the stage of cell differentiation [39,40]. Almeida and co-workers demonstrated that the effects of estrogen signalling are due to activity in osteoblast progenitors but not in mature osteoblasts or osteocytes [40]. In line with this we observed no stimulatory effect on mature osteoblasts from either calvarial or femoral bones by estrogen or any inhibitory effect in presence of ER antagonist (ICI 182,780).

Interestingly a distinctive response in proliferation and ALP activity was observed when cells were treated with FGF-2 or PDGF-BB, suggesting possible proliferative involvement of these growth factors in calvarial but not femoral osteoblasts. Since both agonist (estrogen) and antagonist (ICI 182,780) treatment failed to elicit a response in femoral and calvarial cells we investigated the possible intracellular involvement of receptor co-activators and receptor co-repressors of ER. The most significant difference was shown by *Rerg* which has been suggested to be an estrogen responsive gene. Interestingly, in previous work we also found that *Rerg* was preferentially expressed in calvarial cells in our microarrays[19]. As yet there is no information about the involvement of *Rerg* in regulating osteoblastic responses to estrogen. Here, using qRT-PCR analysis we showed that basal *Rerg* expression was 1200 fold greater in calvarial than femoral osteoblasts. Upon osteogenic induction

Rerg expression was however significantly increased in femoral cells reaching a maximum of 64-fold when compared with unstimulated cells. In contrast, osteogenic stimulation had an inhibitory effect on the expression of *Rerg* in calvarial osteoblasts, Depletion of *Rerg* results in significant reduction in ERE luciferase activity in calvarial but not in femoral osteoblasts. Taken together these data suggest a specific role for *Rerg* in regulation of estrogen signalling and function of calvarial, but not femoral osteoblasts.

In summary, the results demonstrate cell autonomous functional differences between calvarial and femoral osteoblasts *in vitro.* In particular the results demonstrate that femoral osteoblasts specifically express immediate-early response genes *Fosl1, Ccl2, Anx2a* and *cFos* following mechanical loading and this could in part responsible for site specific differences. A recent study has also shown distinct mechanosensitivities and architectural differences between osteocytes in calvarial and long bones[14]. Femoral cells also showed markedly elevated levels of estrogen receptor-ß (ERß). In contrast, calvarial cells specifically express the *Rerg* gene which is implicated in regulation of the estrogen response element. It is possible that *Rerg* is acting to maintain estrogen responses in the absence of ligand receptor binding in calvarial cells whereas femoral cells may be dependent on ERß ligand binding, although further work is required to investigate this. Overall these phenotypic functional differences are consistent with cell autonomous differences in regionally defined osteoblasts being responsible for variations in susceptibility to osteoporotic changes and suggest possible targeting of the estrogen signalling pathway as a future therapeutic opportunity.

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FIGURE LEGENDS

Figure 1. Femoral and calvarial osteoblast responses to osteogenic stimulation and mineralisation. Femoral and calvarial osteoblasts were cultured for indicated times in osteogenic medium. (A) mRNA expression of osteogenic markers *Runx2, Osteopontin (SSP1), ALPL* and *Osteocalcin* were quantified by quantitative-reverse transcription polymerase chain reaction (qRT-PCR) (n=3). EIF4A2 was used as a housekeeping control and expression of the gene of interest is shown as relative expression to cells at day 0. Osteocalcin expression in individual line is shown in the right panel. (B) The ability of each cell to form matrix mineralisation was determined following Alizarin Red staining (at least 3 experiments per individual cell line). (C) Raman spectra of the femoral (a-red line) and calvarial (b-black line) osteoblasts after 21 days in culture. The Raman spectrum of the femoral osteoblasts was characterised by the presence of a peak near 960cm^{-1} which correspond to the symmetric stretching vibration of P–O in PO_4^{3-} tetrahedra of hydroxyapatite (HA) crystals.

Figure 2. Femoral and calvarial osteoblast responses to mechanical stimulation. Mechanical loading was performed using a BOSE bioreactor (ElectroForce BioDynamic test instrument; Bose) with a diametral compression mode by a pulsating compressive force of 5.5±4.5 N at a frequency of 0.1 Hz giving a corresponding head displacement of 0.5–50 μm. mRNA expression for *Fosl1, Ccl2, Anx2a and cFos* in femoral and calvarial osteoblasts subjected to mechanical strain was determined by qRT-PCR. Data is shown as mean ± SEM from at least five experiments. EIF4A2 was used as a housekeeping control and relative quantitative expression was calculated as a ratio of un-loaded cells that was assigned a value of 1. T test was used for statistical analysis between loaded and un-loaded cells ($p<0.05$*).

Figure 3. Differences in responses of femoral and calvarial cells to growth factors and estrogen signalling. (A) Effect of estrogen signalling was assessed on proliferation of calvarial and femoral osteoblasts in presence of 17β-estradiol, using EdU assay. Cells were serum deprived (1%) for 12 hrs

and subsequently cultured in presence of 17β-estradiol (E) (10 nM). The proliferation rate was determined by analyzing the proportion of cells that incorporated EdU following 48 hrs of incubation using flow cytometry. Data shown are representative of three separate experiments. All experiments involving 17β-estradiol stimulation were performed with charcoal stripped serum and phenol red-free medium. (B) Calvarial and femoral cells were cultured with or without Wnt3a (50ng/ml), BMP2 (100ng/ml), FGF-2 (10ng/ml) or PDGF-BB (10ng/ml) and cell proliferation and ALP activity was assessed after 7 days of culture (Mean ± SEM of three experimental groups). (C&D) Effect of inhibition of ER signalling on proliferation and differentiation of osteoblasts. Cells were treated with an ER antagonist (ICI 182,780) (500nM) (C) proliferation and ALP activity was assessed after 7 days of culture and (D) differentiation was assessed by analysing the expression of osteogenic markers *Runx2*, *Osteopontin (SSP1)* and *Osteocalcin* after 21 days of culture. EIF4A2 was used as a housekeeping control and relative quantitative expression was calculated as a ratio of un-treated cells that was assigned a value of 1. *p <0.01##, <0.001###* when compared with untreated control (C). *p<0.05*, <0.01**, <0.001**** when compared calvarial to femoral osteoblasts following the same treatment.

Figure 4. Expression of estrogen receptor co-activators and receptor co-repressors during induction of osteogenesis of femoral and calvarial osteoblasts. Expression of estrogen receptor co-activators (*Ncoa1-3*) and receptor co-repressors (*Ncor1-2* and *REA*) was quantified. (A) In femoral and calvarial of untreated cells. Expression of each gene was calculated and normalised relative to EIF4A2 (Mean ± SEM of three experiments, *p<0.001****). (B) During 21 days of differentiation in osteogenic culture. EIF4A2 was used as a housekeeping control and relative quantitative expression was calculated as a ratio of cells at day 0 that was assigned a value of 1.

Figure 5. *ESRβ* and *Rerg* expression during induction of osteogenesis of femoral and calvarial osteoblasts. (A) Expression of *ESRβ* and *Rerg* were quantified and normalised relative to EIF4A2 in untreated femoral and calvarial cells (Mean ± SEM of three experiments). (B) Expression of *Rerg* in

individual lines. (C) *ESRβ* and *Rerg* expression over 21 days of differentiation in osteogenic cultures. (Mean ± SEM of three experiments).

Figure 6. Effect of *Rerg* knockdown on estrogen signalling in femoral and calvarial osteoblasts. Cells were co-transfected with a Cingal dual-luciferase-based ERE reporter assay, Rerg siRNA and scramble control siRNA and the alteration in ERE promoter activity by *Rerg* knockdown was assessed after 48h. The activity was normalised to Renilla luciferase that acts as an internal control for transfection efficiencies and calculated as percentage of induction compared with cell transfected with control scramble siRNA. (Mean ± SEM of three experiments, *p<0.001****).

Table 1. List of primers and sequences for Sybr green based qRT-PCR.

Genes	Forward (5' to 3')	Reverse (5' to 3')
Annexin A2	TCTGACTAACCGCAGCAATG	ACCAGACAAGGCCGACTTC
CCL2	CAAGAGAATCACCAGCAGCA	CTGGACCCATTCCTTATTGG
cFOS	GGGAGTGGTGAAGACCATGT	CGGATTCTCCGTTTCTCTTC
EIF4A2	TGTGCAACAAGTGTCCTTGG	ACCTTTCCTCCCAAATCGAC
ESRa	CACCAGGTGCCCTACTACCT	CGTCGATTGTCAGAATTGGA
Fosl1	AGAGCGGAACAAGCTAGCAG	CCGATTTCTCATCCTCCAAC
Junb	CAGTTACTCCCCAGCCTCTG	GCATGTGGGAGGTAGCTGAT

Figure. 1.

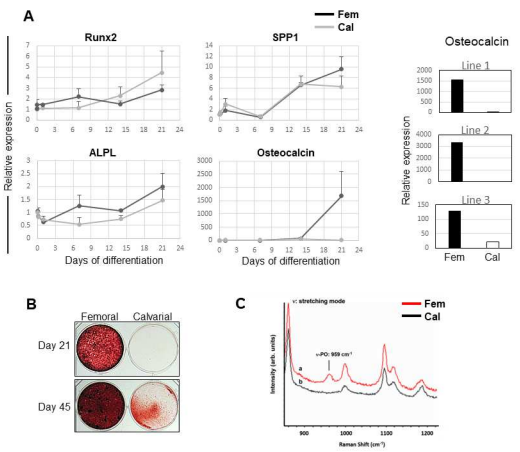
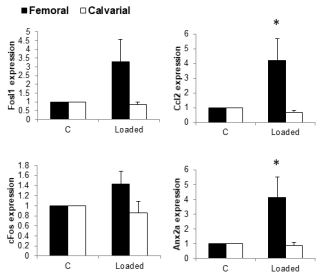


Figure. 2.



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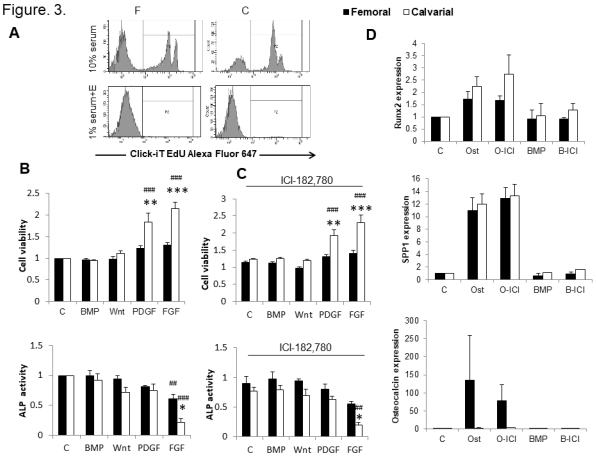

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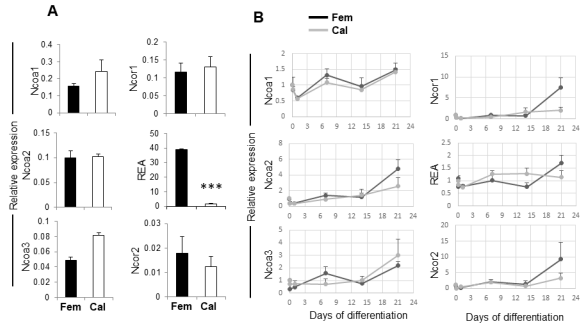


Figure. 5.

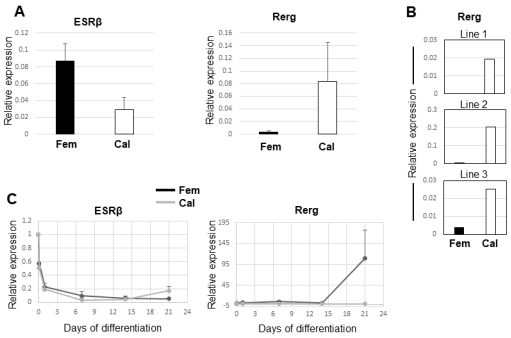
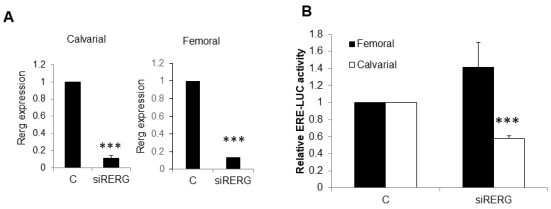


Figure. 6.



Highlights

- Adult-derived skull and limb osteoblasts exhibit phenotypic heterogeneity
- Skull osteoblasts have reduced mineralisation capacity and loading responsiveness
- Estrogen responsive gene *Rerg* selectively highly expressed in skull osteoblasts
- *Rerg* gene silencing reduced estrogen pathway signalling in skull osteoblasts only