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Site-specific differences in osteoblast phenotype, mechanical loading response and estrogen receptor-related gene expression

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

3
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14 **Abbreviated title: Differences in osteoblastic ER gene expression**

15 **Key words or phrases:** Osteoblast, skull, femur, osteoporosis, estrogen, Rerg,

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27 **ABSTRACT**

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

32

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

39

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

49

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

53

54 **INTRODUCTION**

55 Osteoporosis occurs as the result of an imbalance in bone remodelling predominately in the
56 appendicular and vertebral skeleton such that bone resorption exceeds bone formation and is most
57 obvious in post-menopausal women with relative estrogen deficiency, in men with reduced androgen
58 levels, and with decreased levels of physical activity¹. Paradoxically, the skull bones retain structural
59 integrity despite low levels of mechanical loading, and furthermore, they are essentially unaffected by
60 osteoporosis^{2,3}. That not all bones are equally susceptible to the condition implies inherent differences
61 in the resident bone cells, potentially dependent on their position in the body.

62 The reasons for these differences in susceptibility to metabolic and disuse osteoporosis are not fully
63 understood, and could be dependent differences between intramembranous (calvarial) versus
64 endochondral (limb) primary ossification processes. Contrary to this, primary ossification of the lateral
65 aspect of the clavicle is intramembranous, yet it is this aspect that is more prone to osteoporosis
66 compared with the medial endochondral-derived aspect⁴. What is common, however, is that the medial
67 aspect of the clavicle and skull bones contain neural crest-derived cells⁵. Previous studies have also
68 shown differences in proliferation, osteogenic differentiation and response to growth factors between
69 neural crest dual intramembranous-endochondral-derived orofacial and mesodermal endochondral-
70 derived appendicular bones^{6,7}. Thus differences may not be solely determined by the mechanism of
71 bone formation or turnover per se, but also possibly by differential *regulation* of bone formation in
72 distinct sites – in this case an intramembranous-derived calvarial bone with a neural crest component
73 compared with an endochondral-derived bone with no neural crest component.

74 As well distinct formation processes, numerous studies have also shown physical and functional
75 differences between osteoclasts derived from appendicular and calvarial sites both *in vitro* and *in vivo*
76 which could impact on the susceptibility of individual bones to osteoporosis⁸⁻¹¹. In addition both
77 matrix composition^{12,13} and osteocyte morphology¹⁴ have been shown to differ between calvaria and
78 long bones. These differences may be related to features of site-related osteoblastic heterogeneity
79 including sensitivity to PTH¹⁵ as well as induction of osteoclastogenesis and levels of signalling
80 pathway genes involved in osteoclast formation¹⁶.

81 It has become increasingly clear that defined mature cell phenotypes may in fact show much greater
82 diversity than perhaps has been traditionally conceived and understood^{17,18}. In a previous study from
83 our group we investigated differences in global gene expression patterns between adult rodent long
84 bones and skull bones and matched pairs of isolated osteoblasts derived from femurs and calvaria *in*
85 *vitro*. In the isolated bone cells we found 246 differentially expressed genes between osteoblasts from
86 these sources¹⁹. Prominent amongst differentially expressed genes were genes associated with cell
87 embryonic origin such as homeobox containing genes (*Hoxa*, *Hoxb*, *Hoxc*, *Hoxd*, *Shox*) and other
88 transcription factors which are thought to act specifically on embryologically distinct bone formation.
89 These include *Msx-2*, *Dlx-5* and *Cart1* whose disruption in knock out mice specifically affects cranial
90 bone formation and *Tbx-3* which specifically affects limb bone formation¹⁹. Furthermore, in
91 experiments described by Leucht and co-workers, mandibular *Hoxa* -ve and femoral *Hoxa*+ve
92 osteogenic stem cells were tested for their ability to contribute to healing in mandibular and femoral
93 bone defects *in vivo*. They demonstrated that *Hoxa* +ve cells were unable to contribute to healing in
94 the mandibular site, but *Hoxa* -ve cells contributed to wound healing in the femoral site²⁰. Taken
95 together the data suggest the hypothesis that regionally specific osteoblasts are phenotypically distinct,
96 due to cell autonomous mechanisms. That osteoblasts have “positional memory” such that the
97 localised information they express during embryogenesis persists into the adult organism results in
98 regionally specified differences in osteoblast phenotypes, and further, that these differences translate
99 to functional physiological responses controlling bone homeostasis. Here we studied the phenotypic
100 differences in adult-derived calvarial and femoral osteoblast responses to the induction of
101 osteogenesis, mechanical loading, estrogen, growth factor and cytokine stimulation. Adult derived
102 rodent calvarial cells have reduced proliferation, mineralisation, and growth factor responsiveness
103 when compared to juvenile osteoblasts²¹. Therefore it appears that these altered characteristics make
104 them more suitable to investigate in this context.

105 Cellular responses to estrogen stimulation are dependent on expression of Estrogen receptors (ER)
106 which may alter markedly according to the stage of cell differentiation. Estrogen receptors are nuclear
107 receptors which consist of 2 isoforms, ER α and ER β , which have distinct expression patterns and may
108 have distinct functions¹. In addition their function may be regulated particularly by specific co-

109 regulators either as receptor co-activators such as the SRCs, and receptor co-repressors such as the N-
110 CoRs and *regr*¹.

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

118

119

120

121

122 MATERIALS AND METHODS

123 Cell culture

124 All animal protocols were in accordance with the UK Home Office Scientific Procedures Act (1986).
125 Male Wistar rats (210 g, 10 weeks old) were purchased from Charles River, housed, and fed *ad*
126 *libitum* in accordance with local Queen Mary University of London, School of Medicine and Dentistry
127 rules and sacrificed by cervical dislocation. Calvarial and femoral bones were aseptically removed,
128 cleared of soft tissues and cut into pieces. The bone chips were transferred evenly into 6-well culture
129 plates containing DMEM supplemented with 10% fetal bovine serum with glutamine (2 mM) and 1%
130 P/S and incubated at 37°C in 5% CO₂ incubator, left undisturbed for 48-72 hours until an ‘osteoid
131 seam’ was noticed. When adherent cells of seam formation were observed, the bone chips were
132 collected and bone cells isolated by enzymatic digestion. Osteoblasts were cultured in α -MEM without
133 ribo/deoxyribonucleosides, L-glutamine (2 mM), penicillin (50 U/mL), streptomycin (50 μ g/mL)
134 and 10% fetal bovine serum (FBS) (all from Sigma–Aldrich) and media was replenished every 3-4
135 days. To stimulate osteogenesis cells were treated with medium supplemented with 0.1 μ M
136 dexamethasone, 0.05mM Ascorbic Acid (AA) and 5mM β -glycerophosphate (all from Sigma–
137 Aldrich). To assess osteogenic differentiation, mRNA expression of markers of differentiation (i.e.
138 *Runx-2*, *ALPL*, *osteopontin* and *osteocalcin*) was determined by quantitative (q)RT-PCR and
139 accumulation of calcium deposits was visualised and quantified by staining with Alizarin Red dye.
140 Briefly, cells were fixed (15 min. with 4% formaldehyde in PBS), stained for 10 min with Alizarin
141 Red S (1:100 dilution in H₂O) and washed in 50% ethanol and air-dried. Cell cultures were stimulated
142 with the following recombinant growth factors and cytokines FGF-2 (10ng/ml) (Peprotech, London,
143 UK), PDGF-BB (10ng/ml) (Peprotech), BMP2 (100ng/ml) and Wnt3a (50ng/ml) (both from R&D
144 systems), as well as β -estradiol (Sigma-Aldrich).

145

146 Raman spectroscopy

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

149 785-nm diode laser (100mW sample power). The laser beam was focused through a water immersion
150 x60/1.2NA objective lens (working distance: 0.27mm) while the Raman signal was acquired using a
151 600-lines/mm diffraction grating centered between 857 and 1231 cm^{-1} and 2s CCD exposure time. The
152 spectra were recorded at a resolution of $\sim 1\text{-}2 \text{ cm}^{-1}$.

153

154 **Mechanical stimulation**

155 Mechanical stimulation of femoral and calvarial osteoblasts was conducted as previously described²².
156 Cells were seeded on to the top of one side of calcium phosphate monetite scaffolds at a density of
157 1.5×10^5 in 70 μl of media and allowed to attach for 30 min. The scaffold was then turned and the same
158 number of cells were seeded on to the top of the other side and left undisturbed for a further 30 min for
159 the cells to attach. Normal growth media was then carefully added to the culture plate and the
160 cell/scaffold was incubated at 37°C in a humidified 5% CO_2 :95% air atmosphere for 48 h prior to
161 stimulation. Mechanical loading was performed using a BOSE bioreactor (ElectroForce BioDynamic
162 test instrument; Bose) equipped with 200N load cell. Seeded scaffolds were positioned between two
163 loading plates inside the bioreactor chamber and loaded in the diametral compression mode by a
164 pulsating compressive force of $5.5 \pm 4.5 \text{ N}$ at a frequency of 0.1 Hz²². The corresponding head
165 displacement was 0.5–50 μm . The test was performed in load control and the evolution of the head
166 displacement versus the pulsating compressive force was recorded by the bioreactor software
167 (WinTest[®] controls). The stress distribution in the disk was analytically evaluated by the Timoshenko
168 model²³.

169

170 **qRT-PCR analysis**

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

177 previously¹⁹. For TaqMan based analysis primers and probes were purchased from Invitrogen and for
178 Sybr green qPCR the primers and sequences are listed in Table 1. EIF4A2 was used as a housekeeping
179 control.

180

181 **Cell viability assay**

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

192

193 **Alkaline phosphatase (ALP) activity assay**

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

199

200 **siRNA transfection and ERE reporter assay**

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

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

207

208

209 **Data Analysis**

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

214

215 **RESULTS**

216 **Responses of femoral and calvarial cells to stimulation of differentiation**

217 Rawlinson and co-workers previously showed that osteoblasts derived from calvaria and femur exhibit
218 a distinct local pattern of gene expression¹⁹. Here we investigated whether these cells are also distinct
219 in their phenotypic responses to induction of osteogenesis. Cells were treated with differentiation
220 media and changes in osteogenic gene expression and matrix mineralisation were analysed.

221 Expression of *Runx2*, *osteopontin (Spp1)*, *ALPL* and *osteocalcin* gradually increased during
222 stimulation in both femoral and calvarial cells (Fig 1A). No significant differences were observed in
223 the levels of induction of *Runx2*, *ALPL* or *Spp1* expression between femoral and calvarial cells during
224 stimulation compared to unstimulated cells at any time point. However, osteocalcin expression was
225 increased in femoral compared to calvarial cells (more than 1600 fold higher) at late stages of culture
226 (21D) (Fig 1A). As shown in Figure 1A right panel, when each of the 3 cell lines were examined
227 individually we observed a dramatically higher level of osteocalcin expression in femoral cells
228 compared to calvarial cells. However, due to variation in differentiation capacity between each line,
229 combination of all three lines failed to show a statistical difference even with up-regulation of 1600
230 fold. Similarly, rapid and potent matrix mineralisation was observed in femoral cells (as early as 10

231 days post induction (data not shown)), whereas only negligible mineralisation was evident even after
232 45 days of induction in calvarial cultures (Fig 1B). Furthermore, micro-Raman analysis of mineralised
233 nodules, suggested cell dependent differences in mineral matrix composition associated with femoral
234 compared to calvarial cells (Fig 1C). Raman spectra of native bone and mature mineralized nodules is
235 dominated by the PO_4^{3-} peak at 959cm^{-1} correspond to the mineral component of bone, hydroxyapatite
236 ²⁴. This peak was clearly evident in femoral but not in calvarial cells, indicating lack of mineral
237 composition in calvarial cell cultures.

238

239 **Responses of femoral and calvarial cells to mechanical loading.**

240 To investigate whether femoral and calvarial cells respond differently to mechanical stimulation,
241 candidate early response genes i.e. *Fosb*, *Junb*, *cFos*, *Fos11*, *Ccl2* and *Anx2a*, which have been
242 previously shown by our group and others to be stimulated in response to mechanical loading, were
243 analysed in calvarial and femoral osteoblasts following a pulsating compressive force of 5.5 ± 4.5 N
244 (0.2% strain) for a period of 2 hours as described previously ^{22,25}. As shown in Figure 2, mechanical
245 stimulation resulted in the induction of the expression of *Ccl2* and *Anx2a* in femoral osteoblasts, but
246 remained unchanged in calvarial cells in the same experiments. A similar trend was evident for *Fos11*
247 and *cFos* but statistical significance was not reached due to the previously mentioned variation in
248 responses by different primary cell lines. Expression of *Fosb* and *Junb* remained unchanged in both
249 femoral and calvarial cells (data not shown).

250

251 **Differences in responses of femoral and calvarial cells to induction of proliferation and ALP** 252 **activity by estrogen and growth factors.**

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

257 BMP2 and Wnt3a (Fig 3B). Disparities between our findings and others could be due to culture
258 conditions. Most studies were conducted in presence of osteogenic media or other stimulus in
259 combination with BMP2 or Wnt3a, while we treated the cells in absence of any other factors. On the
260 other hand both FGF-2 and PDGF-BB significantly stimulated calvarial cell proliferation over
261 controls, while only a minor effect was observed on femoral cell proliferation (Fig 3B). FGF-2 was
262 significantly inhibitory for ALP activity in both femoral and calvarial cells (Fig 3B). Since no
263 significant effect was observed following treatment with estrogen, we further investigated the role of
264 estrogen signalling on function of femoral and calvarial cells by blocking the estrogen receptors (ER)
265 signalling using a selective antagonist of ER (ICI 182,780) during proliferation and induction of
266 osteogenesis of both osteoblast cultures. Similar to stimulation studies using estrogen, no significant
267 effect was observed on proliferation in response of either femoral or calvarial cells when ER were
268 inhibited by ICI 182,780 and also ICI did not have any effect on FGF-2 or PDGF-BB induced cell
269 proliferation or ALP activity (Fig 3C). The differentiation capacity of the either femoral or calvarial
270 osteoblasts also remained unchanged in presence of ICI 182,780, showing a similar gene expression
271 profile when stimulated by either differentiation media alone or with BMP2 (Fig 3D). Similarly, ICI
272 182,780 had no effect on ALP activity of femoral and calvarial cells either in normal growth media or
273 when stimulated in presence of Wnt3a, BMP2, FGF-2 or PDGF-BB (Fig 3C).

274

275 **Expression of ER α and ER β estrogen receptors and estrogen receptor co-activators SRC-1, -2**
276 **and -3, and receptor co-repressors during differentiation of femoral and calvarial cells.**

277 Since both agonists (17 β -estradiol) and antagonists (ICI 182,780) failed to elicit a response in femoral
278 and calvarial cells we investigated possible intracellular regulation of ER. ER have been shown to
279 have ligand-independent activity, involving receptor co-activators and receptor co-repressors. We
280 therefore studied expression of ER α and ER β estrogen receptors, estrogen receptor co-activators Ncoa-
281 1, -2 and -3, and receptor co-repressors Ncor 1-2 and repressor of estrogen receptor activity (REA)
282 during osteogenic maturation of the cells (Fig 4). REA expression was significantly higher in femoral

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

291

292 **Role of *Rerg* in femoral and calvarial cells.**

293 We also investigated the Ras-related and estrogen-regulated growth-inhibitor (*Rerg*) which has been
294 suggested as an estrogen responsive gene²⁶. *Rerg* was differentially expressed in calvarial cells
295 compared with femoral cells with basal *Rerg* expression being 1200 fold greater in calvarial than
296 femoral osteoblasts (Fig 5A-B). However, upon osteogenic induction *Rerg* expression was
297 dramatically increased in femoral cells reaching a maximum of 64-fold when compared with
298 unstimulated cells in every line examined (Fig 5C). Expression of *Rerg* in calvarial osteoblasts was
299 reduced following osteogenic stimulation although this was not statistically significant (Fig 5C).
300 Therefore, to determine possible role for *Rerg* in regulation of estrogen signalling between femoral
301 and calvarial cells, we analysed the effect of siRNA knock down of *Rerg* on transcriptional activity
302 down-stream of estrogen signalling using an ERE luciferase reporter assay. Interestingly, *Rerg* knock
303 down resulted in significant reduction in ERE luciferase activity in calvarial but not in femoral
304 osteoblasts (Fig 6B). These results suggest *Rerg* may play a site-specific role in regulating estrogen
305 signalling in calvarial osteoblasts selectively.

306

307 **Discussion**

308 It is well documented that bones of the skull tend to be resistant to osteoporosis with previous studies
309 investigating differences in characteristics between susceptible long bones and resistant skull bones
310 ^{12,27,28}. However, the idea that this may be due to differences in the action of osteoblasts from these
311 sites has not been extensively investigated. In our previous study we demonstrated that the genes
312 associated with bone mass and mineral density are differentially expressed in functionally distinct
313 skeletal sites ¹⁹. Here, we report that osteoblasts derived from skull and long bones are also
314 functionally distinct. We observed that osteoblasts derived from mature calvariae have a significantly
315 lower ability for accumulation of mineralised matrix compared with femoral osteoblasts when exposed
316 to osteogenic induction medium containing dexamethasone, ascorbic acid and β -glycerophosphate.
317 They were unable to produce any mineralisation by 21 days and only negligible amounts by day 45
318 and is consistent with previously observed findings for adult-derived calvarial osteoblasts. Raman
319 spectroscopy analysis of mineralised nodules further revealed that only mineral deposited by femoral
320 osteoblasts produced a peak near 960 cm^{-1} which corresponds to the mineral component of bone
321 hydroxyapatite ²⁹. To find out the reason behind this observation we carried out mRNA expression
322 analysis for genes involved in osteogenesis. As with previous studies indicating the involvement of
323 *Runx2* in both femoral and calvarial cells, a similar expression pattern was observed in both cell types
324 here³⁰. However, osteocalcin expression was significantly induced in femoral compared to calvarial
325 cells at late stage of 21 days. Osteocalcin is suggested to be involved in bone mineralisation and
326 formation of hydroxyapatite and therefore a higher level of osteocalcin expression seen in femoral
327 osteoblasts could be associated with potent mineralisation ability of these cells ³¹. Our observations are
328 also in line with the previous study showing differences in protein composition of flat and long bones,
329 suggesting functional differences in formation, resorption, and mechanical properties of these bone
330 types¹². In contrast, a recent study has shown no differences between adult mouse-derived calvarial
331 and femoral osteoblastic proliferation rates, mineralisation capacity and levels of osteogenic gene
332 expression¹⁶. Among other characteristics that separate these bones is the difference in response to
333 mechanical loading. It is known that maintenance of BMD in long bones is dependent on mechanical
334 loading and osteoblasts derived from these sites are responsive to mechanical loading, while BMD in

335 the skull remains high in absence of loading and calvarial cells are shown to be insensitive to
336 mechanical loading^{32,33}. However, a contrasting observation has been reported showing that
337 compressive forces upregulate the expression of osteogenic genes *Bmp2*, *Runx2* and *Smad5* and
338 promote osteogenesis of calvarial osteoblasts³⁴. This study was however conducted on newborn
339 calvarial cells which could probably account for the disparities seen between data. Here we confirm
340 the idea supporting the lack of response by calvarial cells and show that only osteoblasts derived from
341 femur are sensitive to mechanical loading, showing elevated expression of early genes associated with
342 mechanical stimulation²⁵.

343 The calvarial bone is known to be insensitive to post-menopausal hormonal changes and osteoporosis.
344 Osteoblasts *in vitro* are known to be estrogen responsive and classically, stimulation with 17 β -
345 estradiol has been shown to upregulate osteoblast proliferation and expression of differentiation
346 markers³⁵⁻³⁸. However more recent experiments have demonstrated a more complex set of distinct
347 responses to estrogen stimulation (both stimulation and inhibition) which are dependent on expression
348 of ER and which may alter markedly according to the stage of cell differentiation^{39,40}. Almeida and
349 co-workers demonstrated that the effects of estrogen signalling are due to activity in osteoblast
350 progenitors but not in mature osteoblasts or osteocytes⁴⁰. In line with this we observed no stimulatory
351 effect on mature osteoblasts from either calvarial or femoral bones by estrogen or any inhibitory effect
352 in presence of ER antagonist (ICI 182,780).

353 Interestingly a distinctive response in proliferation and ALP activity was observed when cells were
354 treated with FGF-2 or PDGF-BB, suggesting possible proliferative involvement of these growth
355 factors in calvarial but not femoral osteoblasts. Since both agonist (estrogen) and antagonist (ICI
356 182,780) treatment failed to elicit a response in femoral and calvarial cells we investigated the possible
357 intracellular involvement of receptor co-activators and receptor co-repressors of ER. The most
358 significant difference was shown by *Rerg* which has been suggested to be an estrogen responsive gene.
359 Interestingly, in previous work we also found that *Rerg* was preferentially expressed in calvarial cells
360 in our microarrays¹⁹. As yet there is no information about the involvement of *Rerg* in regulating
361 osteoblastic responses to estrogen. Here, using qRT-PCR analysis we showed that basal *Rerg*
362 expression was 1200 fold greater in calvarial than femoral osteoblasts. Upon osteogenic induction

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

369

370 In summary, the results demonstrate cell autonomous functional differences between calvarial and
371 femoral osteoblasts *in vitro*. In particular the results demonstrate that femoral osteoblasts specifically
372 express immediate-early response genes *Fosl1*, *Ccl2*, *Anx2a* and *cFos* following mechanical loading
373 and this could in part responsible for site specific differences. A recent study has also shown distinct
374 mechanosensitivities and architectural differences between osteocytes in calvarial and long
375 bones¹⁴. Femoral cells also showed markedly elevated levels of estrogen receptor- β (ER β). In
376 contrast, calvarial cells specifically express the *Rerg* gene which is implicated in regulation of the
377 estrogen response element. It is possible that *Rerg* is acting to maintain estrogen responses in the
378 absence of ligand receptor binding in calvarial cells whereas femoral cells may be dependent on ER β
379 ligand binding, although further work is required to investigate this. Overall these phenotypic
380 functional differences are consistent with cell autonomous differences in regionally defined
381 osteoblasts being responsible for variations in susceptibility to osteoporotic changes and suggest
382 possible targeting of the estrogen signalling pathway as a future therapeutic opportunity.

383

384

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ACCEPTED MANUSCRIPT

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- 521

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 (OSP1)*, *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 \pm 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 17 β -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 17 β -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 \pm 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 (OSP1)* 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 \pm 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. *ESR β* and *Rerg* expression during induction of osteogenesis of femoral and calvarial osteoblasts. (A) Expression of *ESR β* and *Rerg* were quantified and normalised relative to EIF4A2 in untreated femoral and calvarial cells (Mean \pm 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	CCGATTTCATCCTCCAAC
Junb	CAGTTACTCCCCAGCCTCTG	GCATGTGGGAGGTAGCTGAT

Figure 1.

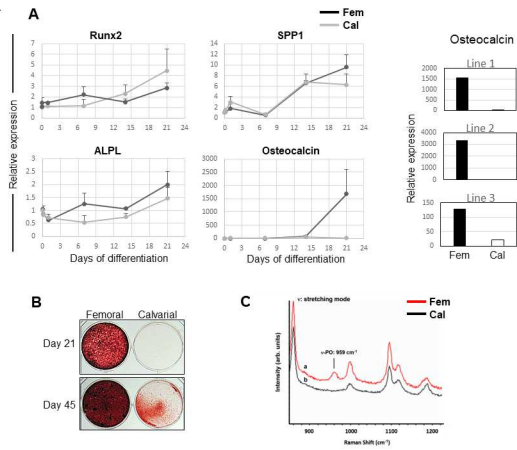


Figure 2.

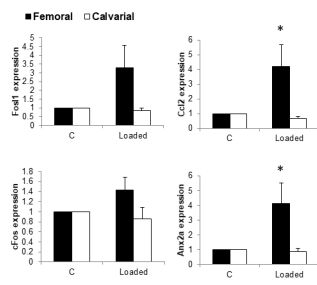


Figure 3.

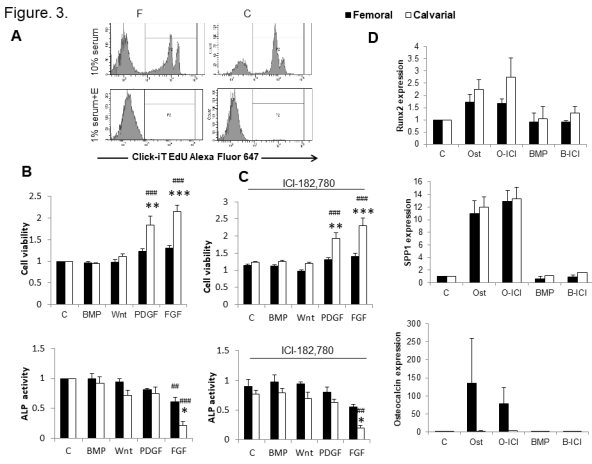
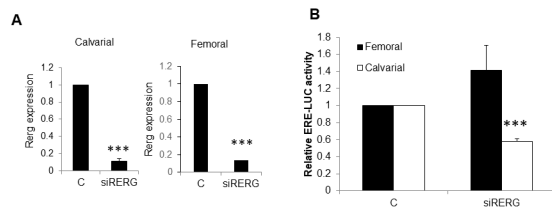


Figure 6.



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