RAPID COMMUNICATION

The novel estrogen receptor G-protein-coupled receptor 30 is expressed in human bone

Terhi J Heino, Andrei S Chagin and Lars Sävendahl

Pediatric Endocrinology Unit, Department of Woman and Child Health, Karolinska Institutet, Astrid Lindgren Children's Hospital, Q2:08, SE-171 76 Stockholm, Sweden

(Correspondence should be addressed to T J Heino; Email: terhi.j.heino@utu.fi)

Abstract

Estrogens have significant impact on bone mineral metabolism. Besides the classical estrogen receptors (ER α and ER β), a trans-membrane G-protein-coupled receptor (GPR30) has been demonstrated to mediate estrogenic effects. We aimed to study whether GPR30 is expressed in bone cells and if so, whether the level of expression is developmentally regulated. Metaphyseal bone biopsies were collected from the tibia in 14 boys and 6 girls, all at different stages of puberty. GPR30 protein expression was studied by immunohistochemistry in paraffin-embedded sections. GPR30-positive osteocytes and osteoblasts were quantified and linear regression analysis was applied. Cytoplasmic GPR30 expression was detected in osteoblasts, osteocytes, and osteoclasts. Osteocytes were more frequently

positive for GPR30 than osteoblasts ($58 \pm 4\%$ vs $46 \pm 3\%$ positive cells respectively, $P < 0.05$). Detailed analysis demonstrated that GPR30 positivity declined during pubertal development in osteocytes ($R = -0.56$, $P < 0.01$) but not in osteoblasts ($R = -0.31$, $P > 0.05$). No sex difference was observed in the numbers of GPR30-positive osteoblasts or osteocytes. Furthermore, GPR30 expression did not correlate with chronological or bone age. In conclusion, the novel ER GPR30 is expressed in osteoblasts, osteocytes, and osteoclasts suggesting that non-genomic estrogen signaling via GPR30 may exist in bone. However, the functional role of GPR30 in bone tissue remains to be elucidated.

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Introduction

Estrogen (17 β -estradiol) is an important regulator of bone metabolism, which is illustrated by the low bone mass phenotype in a man carrying a mutation in estrogen receptor- α (ER α ; Smith *et al.* 1994) and in individuals with a deficiency in aromatase activity (Morishima *et al.* 1995). Furthermore, girls and boys with hypogonadism are known to develop osteopenia, and even though sex steroid replacement in adulthood can increase bone mineral density (BMD) in these individuals, they do not reach adult BMD values (Vanderschueren *et al.* 2005).

A large proportion of the total bone mass is accumulated during pubertal development in both genders (Bonjour *et al.* 1994). It has been proposed that bone mass accrual is mediated by the direct action of estrogens on classical ERs. ER α is expressed in osteoblasts and osteocytes *in vitro* and *in vivo* (Eriksen *et al.* 1988, Komm *et al.* 1988, Braidman *et al.* 1995, Kusec *et al.* 1998, Zaman *et al.* 2006). Mature osteoclasts have been reported to express ER α (Oursler *et al.* 1991, 1994), while others have found expression in osteoclast precursors but not in mature cells (Kusec *et al.* 1998, Batra *et al.* 2003). Another

ER β , has been shown to be expressed in osteoblasts, osteocytes, and osteoclasts (Vidal *et al.* 1999, Braidman *et al.* 2001).

Previous *in vitro* studies in osteoblasts and osteocytes have demonstrated that estrogen can stimulate non-genotropic responses via the Src/Shc/ERK pathway (Kousteni *et al.* 2001). However, the *in vivo* role of non-genomic estrogen signaling in bone growth and development is unknown. The description of G-protein-dependent estrogen signaling and membrane localization of estrogen-binding sites has led to the speculation about a trans-membrane ER (Prossnitz *et al.* 2007). In the late 1990s, a putative receptor was cloned by four different groups (Owman *et al.* 1996, Carmeci *et al.* 1997, Takada *et al.* 1997, O'Dowd *et al.* 1998). G-protein-coupled receptor 30 (GPR30) mRNA has been demonstrated to be expressed in several tissues, including central nervous system (O'Dowd *et al.* 1998, Brailoiu *et al.* 2007), liver (Owman *et al.* 1996, O'Dowd *et al.* 1998), and ovary (Wang *et al.* 2007). Recently, two groups have independently shown that GPR30 acts as a membranous ER and mediates non-genomic estrogen signaling (Revankar *et al.* 2005, Thomas *et al.* 2005).

Some functions of estrogen-mediated GPR30 signaling have been suggested previously. These include the non-genomic

actions such as MAP kinase activation in breast cancer cells (Filardo *et al.* 2000), as well as anti-apoptotic (Kanda & Watanabe 2003) and cell growth stimulatory (Kanda & Watanabe 2004) effects on keratinocytes. Recently, GPR30 was demonstrated to mediate the proliferative effects of estrogens in endometrial (Vivacqua *et al.* 2006a), thyroid (Vivacqua *et al.* 2006b), and ovarian cancer cells (Albanito *et al.* 2007), suggesting a role in the development of pathological conditions. Interestingly, GPR30 is differentially regulated by gonadotropins in normal ovarian cells (Wang *et al.* 2007), indicating that GPR30 may also be important in the regulation of normal estrogen physiology. The cellular and developmental differences in GPR30 expression have been recently demonstrated in human adrenal tissues (Baquedano *et al.* 2007) and growth plate (Chagin & Savendahl 2007), indicating that GPR30 may mediate local estrogen-dependent effects on cell proliferation and differentiation.

In this study, our aim was to establish whether GPR30 is expressed in human bone cells and whether there is any correlation between the expression level and the pubertal stage.

Materials and Methods

Patients and tissues

Tissue samples were obtained from 14 boys and 6 girls who were subjected to epiphyseal surgery to arrest longitudinal bone growth. The patients suffered from leg length difference or extreme tall stature (Table 1). Biopsies containing metaphyseal bone were taken from the tibial and femoral growth plates

employing a bone marrow biopsy needle (8 gauge, Gallini Medical Products and Services, Mirandola, Italy) and immediately fixed in 4% formaldehyde, decalcified, and embedded in paraffin as described previously (Nilsson *et al.* 2003). The pubertal staging of the patients was performed by a trained pediatric endocrinologist. This study was pre-approved by the local ethics committee at the Karolinska University Hospital, Stockholm, Sweden and informed consent was obtained from each patient and both parents.

Immunohistochemistry

After deparaffinization, immunohistochemical staining of GPR30 in metaphyseal bone sections was performed. Intrinsic peroxidase activity and non-specific binding were blocked by incubation with 3% H₂O₂ for 10 min, followed by a blocking with 3% horse serum for 1 h. Antigen retrieval was performed in citrate buffer, pH 6.0, for 15 min at +95 °C. The samples were incubated with rabbit anti-serum against human GPR30 (1:1500, a kind gift from Dr E Prossnitz, University of New Mexico, Albuquerque, NM, USA) overnight at +4 °C. After washing, secondary biotin-conjugated anti-rabbit antibody (1:1000, Jackson ImmunoResearch Europe Ltd, Suffolk, UK) was added and incubated for 1 h. Bound antibodies were detected with avidin-conjugated peroxidase (1 h incubation) and 3,3'-diaminobenzidine (3 min; Vector Laboratories, Burlingame, CA, USA). Sections were counterstained with Alcian blue for 2 min. Pre-immune rabbit serum was used as a negative control. Specificity of the anti-GPR30 antibody

Table 1 Background characteristics of study subjects and levels of G-protein-coupled receptor 30 (GPR30) expression in osteoblasts and osteocytes

	Sex	Age (year: months)	Tanner pubertal stage	GPR30 in osteoblasts (% pos. cells)	GPR30 in osteocytes (% pos. cells)	Diagnosis
Patient no.						
1	F	9:6	B1	52.5	79.9	Leg length difference
2	F	12:7	B1–2	65.4	78.9	Leg length difference
3	F	12:8	B2	48.4	58.3	Leg length difference
4	F	12:6	B2	45.1	53.7	Constitutional tall stature
5	F	14:2	B2–3	42.3	60.6	Leg length difference
6	F	13:8	B3	46.8	46.2	Leg length difference and CHL
7	M	12:3	G1	44.1	77.2	Marfan's syndrome
8	M	12:3	G3	51.9	60.3	Leg length difference and FF
9	M	14:3	G3	44.6	74.8	Leg length difference
10	M	14:10	G3	55.2	72.0	Constitutional tall stature
11	M	15:4	G3–4	40.4	64.8	Leg length difference
12	M	14:7	G3–4	69.0	74.8	Leg length difference
13	M	16:0	G4	25.6	18.7	Leg length difference and FF
14	M	13:5	G4	14.6	13.1	Leg length difference
15	M	14:7	G4	41.3	59.1	Leg length difference and FTFF
16	M	16:4	G4	52.1	70.6	Constitutional tall stature
17	M	14:3	G4–5	63.3	73.1	Leg length difference and FF
18	M	14:0	G4–5	39.6	51.3	47, XYY syndrome
19	M	14:1	G4–5	64.8	44.9	Leg length difference
20	M	12:2	G5	11.4	29.5	Klinefelter's syndrome

% pos. cells, percentage of positive cells; CHL, congenital hip luxation; FF, femur fracture; FTFF, femur, tibia, and fibula fractures.

(Revankar *et al.* 2005) and its applicability for immunohistochemistry of calcified tissues has been confirmed earlier (Chagin & Savendahl 2007).

In addition, the fluorescent detection of bound antibodies was applied in some sections, where double staining for GPR30 and the osteoclastic marker tartrate-resistant acid phosphatase (TRACP) was performed. Secondary FITC-conjugated anti-rabbit antibody (1:100, Jackson ImmunoResearch Europe Ltd) was used for the detection of GPR30-bound antibody and the binding of primary monoclonal anti-human TRACP antibody (1:800, a kind gift from Dr Jussi Halleen, University of Turku, Turku, Finland) was detected with an Alexa633-conjugated mouse antibody (1:300, Molecular Probes Invitrogen). Nuclei were stained with 4',6-diamidino-2-phenylindole (Vector Laboratories). Some sections were stained for TRACP using a histochemical kit (386-A, Sigma Diagnostics).

Image analysis

Digital images of stained sections were collected by a Nikon Eclipse E800 microscope and an Olympus DP70 digital camera. Three equivalent images of trabecular bone at the primary spongiosa of metaphysis (1.5–2 mm below growth plate) were captured from each sample and used for cell quantification. Osteoblasts were identified as cuboidal cells on trabecular surfaces, while osteocytes were identified as smaller cells within the lacunae inside the bone matrix. The total number of osteocytes (both empty lacunae and lacunae occupied by cells) and the number of GPR30-positive osteocytes were counted manually. For osteoblast quantitation, the total trabecular surface covered with osteoblastic cells and the surface covered by GPR30-positive osteoblasts were measured using the Image-Pro Plus software (Media Cybernetics Inc., Bethesda, MD, USA). Cartilaginous areas and areas where bone had detached from the slides were excluded from the analysis. The analysis was performed by a blinded observer. The results are presented as the percentages of cells stained positive for GPR30.

Statistical analysis

Data were expressed as mean $\pm$ S.E.M. and statistical differences were calculated by Kruskal–Wallis ANOVA on ranks. Regression analysis was used to evaluate developmental trends. Pubertal stage, chronological age, or bone age was used as the independent variable, and the percentage of GPR30-positive cells as the dependent variable. A *P* value of <0.05 was considered significant.

Results

Immunodetection of GPR30 in human bone

The specificity of the GPR30 antibody used in this study has earlier been well documented, both in human cell lines (Revankar *et al.* 2005) and tissue sections (Chagin &

Savendahl 2007). With an identical immunohistochemical protocol (Chagin & Savendahl 2007), we detected a high level of GPR30 expression in human bone cells. More detailed analysis demonstrated that osteocytes and osteoblasts (Fig. 1A and D) as well as multinucleated osteoclasts (Fig. 1A, B and F) expressed GPR30. GPR30 immunostaining was cytoplasmic in all cells (Fig. 1D and F). Histochemical TRACP staining on comparable sections confirmed the presence of osteoclasts (data not shown). We further confirmed the presence of GPR30 in osteoclasts by performing double staining with TRACP (Fig. 1G) and observed a co-localization of these two proteins (Fig. 1H). No GPR30 staining was observed in negative controls (Fig. 1C and E).

Quantitative analysis of GPR30 expression revealed that osteocytes were more often positive than osteoblasts ($58 \pm 4\%$ vs $46 \pm 3\%$ respectively, $P < 0.05$). Furthermore, the newly embedded osteoid-osteocytes appeared to be more frequently positive for GPR30 than osteocytes deeper in the bone matrix (data not shown). When analyzing all samples, we did not observe any gender differences in GPR30 expression. In boys, the percentages of positive osteocytes and osteoblasts were $56 \pm 6\%$ and $44 \pm 5\%$ respectively, and in girls $63 \pm 6\%$ and $50 \pm 3\%$ respectively. Even though we observed GPR30 expression in osteoclasts, we could not perform any statistical analysis due to small numbers of these cells.

Expression of GPR30 during pubertal development

When evaluating developmental trends, we observed that the number of GPR30-positive osteocytes varied between different pubertal stages. More cells were positive at earlier than at later pubertal stages. Detailed analysis demonstrated that the number of GPR30-positive osteocytes declined during pubertal development but significantly correlated with the pubertal stage only in girls ($R = -0.89$, $P < 0.05$, Fig. 2A), although there was a trend toward a decline in boys as well ($R = -0.51$, $P > 0.05$, Fig. 2A).

When separately analyzing patients whose only diagnosis was leg length difference (thus excluding patients with constitutional tall stature and Marfan's, Klinefelter's, and XYY syndromes), a similar decline in the number of GPR30-positive osteocytes was observed during pubertal development ($R = -0.61$, $P < 0.05$).

In osteoblasts, the level of GPR30 positivity did not correlate with pubertal stage either in boys ($R = -0.19$, $P > 0.05$ Fig. 2B) or in girls ($R = -0.59$, $P > 0.05$, Fig. 2B). Similarly, for both boys and girls whose only diagnosis was leg length difference, the number of GPR30-positive osteoblasts did not correlate with pubertal stage ($R = -0.19$, $P > 0.05$).

Total numbers of bone cells per area were similar in all patients (data not shown), thus confirming that our finding was not dependent on an overall decline in osteocyte or osteoblast density during pubertal development. Furthermore, when the GPR30-positive osteoblasts and osteocytes were plotted against chronological age or bone age, no correlation was observed (data not shown).

Figure 1 Expression of GPR30 in the human bone. Representative images of bone sections stained for GPR30 (A–E, patient no. 1 in Table 1) and for GPR30 and TRACP (F–H, patient no. 13 in Table 1) as described in Materials and Methods. (A) Positive staining for GPR30 in osteocytes (arrowheads), osteoblasts (block arrows), and an osteoclast (arrow). (B) Positive staining for GPR30 in multiple osteoclasts (arrows). (C) Negative control. (A–C) Scale bar = 100 µm. (D) Osteoblasts (arrows) and osteocytes (arrowheads) show cytoplasmic staining of GPR30. (E) Negative control. (D and E) Scale bar = 10 µm. (F–H) A multinucleated osteoclast showing cytoplasmic staining of (F) GPR30, (G) TRACP, and (H) a complete co-localization of GPR30 and TRACP (yellow color). (F–H) Scale bar = 20 µm. Blue, nuclei; green, GPR30; red, TRACP.

Discussion

This study is the first report of GPR30 expression in human bone cells. We observed an intensive GPR30 staining in all three cell types of bone, i.e. osteoblasts, osteocytes, and osteoclasts. The staining was cytoplasmic, which is in accordance with recent reports showing that functional GPR30 has intracellular localization (Filardo *et al.* 2006, Smith *et al.* 2007), most likely on the endoplasmic reticulum (Revankar *et al.* 2007). Since the ligands for GPR30 (i.e. estrogens) are membrane permeable,

the intracellular localization is certainly consistent with its function. The fact that osteocytes were more frequently positive for GPR30 than osteoblasts suggests that GPR30 expression is differentially regulated during cell differentiation. Supporting this, the highest level of GPR30 expression was recently observed in terminally differentiated chondrocytes in the human growth plate (Chagin & Savendahl 2007).

Our detailed analysis of the GPR30 staining indicates that the number of GPR30-positive osteocytes negatively correlates with pubertal progression. However, a major limitation of our

Figure 2 Decline in GPR30-positive (A) osteocytes and (B) osteoblasts during pubertal progression. The relationship between the percentages of GPR30-positive (A) osteocytes and (B) osteoblasts in pubertal stage (Tanner) were analyzed by linear regression. The correlation coefficients (R), P values (P), and regression lines for girls (solid) and boys (dashed) are indicated.

study is the relatively low number of patients. In our analysis, the lack of girls in late puberty (Tanner stages 4 and 5) and only one male in early puberty (Tanner stage 1) does not allow us to draw any further conclusions on the significance of the observed decline in GPR30 expression during pubertal progression. In addition, since there is a lack of functional data on any effects of GPR30 in bone tissue, it cannot be concluded that such a decline in expression would have any physiological significance.

In line with the previously reported distribution patterns of ERs (van der Eerden *et al.* 2004), we did not observe any gender difference in the numbers of GPR30-positive bone cells. This suggests that neither classical nor non-genomic estrogen signaling is dependent on gender-specific receptor expression. Moreover, no correlation was observed between the numbers of GPR30 expressing cells and chronological age or bone age, indicating that the level of GPR30 expression might better reflect hormonal status than chronological age or bone age.

In summary, the present study is the first demonstration of GPR30 protein expression in bone cells. Our data clearly

show that osteoblasts, osteocytes, and osteoclasts all express GPR30 suggesting that non-genomic estrogen signaling via GPR30 may exist in bone. However, more mechanistic and functional studies are needed to confirm this.

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