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Blocking FSH action attenuates osteoclastogenesis

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ABSTRACT

A direct effect of FSH on bone turnover *via* stimulation of osteoclast formation has been reported. Here we show that monoclonal or polyclonal antibodies to FSH inhibit osteoclast formation induced by FSH to an extent similar to that noted in FSH receptor (FSHR) knockout cells. Furthermore, we document the amplification of FSHR cDNA from well-characterized human CD14+ osteoclast precursors and osteoclasts, and the direct sequencing of the PCR products to definitively establish the expression of FSHRs. At these sites, the FSHR was expressed predominantly as an isoform that omits exon 9, a linker between the FSH-binding region and a long, invariant signaling domain of the receptor. These data provide compelling evidence for expression of a FSH receptor isoform in osteoclasts and their precursors.

1. Introduction

FSH has been shown to stimulate osteoclast formation through its action on a $G_{i2\alpha}$-coupled FSH receptor (FSHR) [1]. FSHR stimulation reduces cAMP levels, and activates MAP kinase and NF-κB signaling to produce its osteoclastogenic effect. Others confirmed existence of FSHRs on human precursor monocytes, in which instance, FSH stimulates the production of the osteoclastogenic cytokine interleukin-1 (IL-1) [2]. Likewise, we showed that FSH enhances the production of another osteoclastogenic cytokine tumor necrosis factor-α (TNF-α) from cells of the monocyte/macrophage lineage [3,4]. Thus, a direct action on osteoclast precursors and indirect stimulation through osteoclastogenic cytokines may together contribute to the effects of FSH in simulating bone resorption. Notably, FSH injections increased alveolar bone loss in ovariectomized rats [5].

We surmise that FSH bypasses the ovary to act on bone independently of estrogen in what is now considered as a pituitary-bone axis. We have proposed that rising FSH levels contribute to the rapid rates of bone loss during the late peri-menopause; these declines have been attributed traditionally solely to low estrogen levels. If this is the case, not only is the pathophysiology of post-menopausal osteoporosis more complex than previously understood, but a rising FSH could potentially be a target for therapeutic intervention.

Indeed, a role for FSHRs in humans has been demonstrated by the fact that women with an activating FSHR polymorphism have a low bone mass and high resorption rates [6]. Additional consistent findings, reviewed elsewhere [7], include observations that amenorrheic women with high FSH levels (>35 mIU/L) suffer from greater bone loss than those with a relatively lower serum FSH (8 mIU/L) [8]. Despite this, some studies have been unable to demonstrate the expression of FSHRs on human and murine osteoclasts and osteoclast-like cells by PCR [9–11].

Here, we used a highly specific human anti-FSHβ monoclonal antibody, as well as a polyclonal murine anti-FSHβ antibody, to block the effects of FSH on osteoclastogenesis *in vitro*. This provides functional evidence for FSH action on osteoclastogenesis. We also show that FSHR-deficient osteoclast precursors form fewer osteoclasts *in vitro* compared with precursors from wild type littermates. Further, focusing on human osteoclasts, nested PCR primers, and DNA sequencing of PCR products, we provide definitive evidence for an alternatively spliced FSHR, which is slightly shorter than the ovarian isoform. Overall, these studies add to the body of evidence favoring a role for FSH in skeletal regulation.

2. Materials and methods

Human peripheral blood monocytes were isolated by centrifugation on a density gradient to isolate cells of specific gravity <1.077. CD14+ cells were isolated by anti-CD14 immuno-magnetic

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A

Anti-FSH	Monoclonal Anti-FSHβ			Polyclonal Anti-mouse FSHβ		
	0	0	1 μg/ml	0	0	0.01 mg/ml
FSH	0	30	30 ng/ml	0	30	30 ng/ml
OCs	59 ±2.65	68 ±3.21	53 ±2.38	58 ±3.58	68 ±2.96	56 ±2.12
p vs Control		*0.036*	*0.061*		*0.025*	*0.351*
30 ng/ml FSH			*0.001*			*0.003*

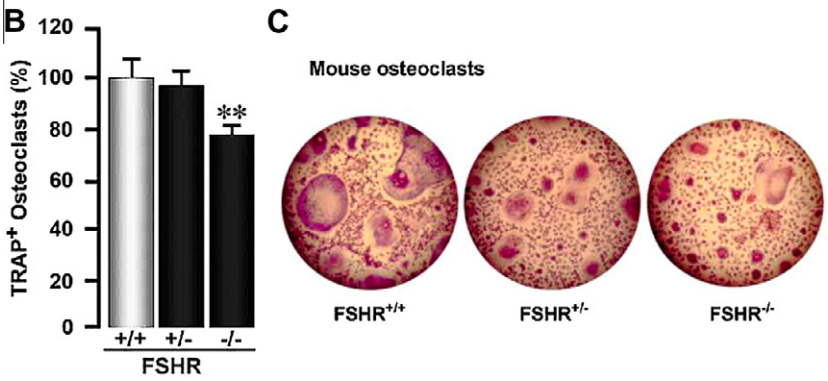

Fig. 1. FSH increases osteoclastic differentiation from mouse marrow cells, but the effect is abrogated by neutralizing antibodies. (A) Blocking antibodies for FSH reverse the effect of FSH on osteoclast formation. Bone marrow cells from 6 month old mice were isolated and osteoclast differentiation was induced with murine RANKL and murine CSF-1 [1]. This increased osteoclast formation by ~20%; duplicate experiments are shown. In the first experiment monoclonal anti-FSHβ was added in excess and this eliminated eliminating the effect of FSH ($p = 0.001$). In the second experiment polyclonal anti-FSHβ also returned osteoclast formation to background ($p = 0.003$). (B) The effect of FSHR deletion on osteoclast formation is shown. Multinucleated TRAP-expressing osteoclasts 5-days after RANK-L treatment were attenuated in $FSHR^{-/-}$ cultures compared with $FSHR^{+/-}$ or wild type ($p < 0.001$ $FSHR^{-/-}$ relative to WT littermates). The size of the effect was similar to that seen when FSH is added during osteoclast formation in wild type cells (A). (C) Photomicrographs of multinucleated TRAP-expressing cells in wild type, $FSHR^{+/-}$, and $FSHR^{-/-}$ marrow cell cultures with 30 ng/ml of FSH during differentiation in RANKL and CSF-1. The knockout cells produce fewer multinucleated cells.

selection with verification of purity by flow cytometry [15]. Recombinant human CSF-1 and RANKL were used to induce osteoclast differentiation *in vitro* [15]. Procedures were approved by the Institutional Review Board. Murine osteoclasts were produced as described [1]. Procedures were approved by the Institutional Animal Care and Use Committees. TRAP-positive osteoclast number was determined [1,3].

Messenger RNA was isolated and first strand cDNA synthesis was performed using gene-specific primers for FSHR targets, or random hexamers for other targets. In replicating this work it should be noted that since the FSHR is a low abundance target in monocyte-derived cells, the reactions are not reliable unless gene-specific antisense primers are used. First strand cDNA was synthesized using MMLV reverse transcriptase (Superscript; Invitrogen). PCR reactions were initiated by adding 2.5 mM Mg, 100 nM of primers, and first strand mixture containing 1–2 μg of RNA. After 10 min at 95 °C, cycles of 30 s at 95 °C, and 1 min at 54–59 °C (as indicated) were run on mastercycler Gradient PCR (Epindorf, Hippauge, NY), for 40 cycles.

Oligonucleotide primers for GAPDH were as reported [3]. For FSHR isoforms, reference sequences shown and primers are from: Human gene: FSHR; Genbank RefSeq NM_000145.3. Primer set 1: Forward primer, inside exon 8, 5′-AGC CTC TGG ACC AGT CAT TCT-3′; reverse primer, inside exon 10, 5′-CTC TGC TGT AGC TGG ACT CAT-3′. These produce products of 140 bp for the FSHR variant missing exon 9, and 320 bp from FSHR including exon 9. Primer set 2 (specific for the form excluding exon 9): Forward primer, extending across the exon 8–10 boundary, 5′-TGG ACC AGT CAT TCT CTC TGA-3′; Reverse primer (the same as in primer set 1) 5′-CTC TGC TGT AGC TGG ACT CAT-3′; Product size is 134 bp. A second primer, internal to the set 2 forward primer by three bases, was forward primer (2B) 5′-ACC AGT CAT TCT CTC TGA GCT-3′. This nested primer produces a product of 131 bp and was used for verification and to eliminate nonspecific products.

3. Results

We previously showed that $FSH\beta^{+/-}$ mice, with reduced FSHβ, and $FSHR^{-/-}$ mice, devoid of the FSHR, are resistant to bone loss despite severe hypoestrogenemia [1]. Although we had first attempted to explain this attenuated bone loss (and, in instances, bone gain) solely through reduced FSH signaling, the accompanying hyper-androgenemia was shown to be, in part, responsible for the noted preservation of bone mass [12]. This is nonetheless subject to debate as high serum testosterone levels in mice lacking aromatase gene, in which FSH levels are high, continue to lose bone. Thus, we elected to use blocking antibodies to determine whether we can specifically block FSH-induced osteoclastogenesis.

We used a highly specific blocking monoclonal antibody against human FSHβ (IgG1) (MedixMab cat. #6602, BiosPacific, Emeryville, CA). This antibody has a high affinity of 3×10^{-10} M for FSHβ, without measurable binding with LH, hCG, or TSH by RIA [13]. In parallel, we generated a peptide-based polyclonal antibody in goat against a 14 amino acid FSHR-binding sequence of FSHβ [14]. This antibody has been shown by others to block FSH action on estradiol production in mice [14].

Bone marrow cells were isolated from 6 month-old mice, and osteoclast differentiation was induced with 40 ng/ml of murine RANKL and 10 ng/ml of murine CSF-1. As expected [1], the addition of FSH, 30 ng/ml, increased osteoclast formation (Fig. 1A, $p = 0.025$ and 0.036 in duplicate experiments). In one experiment monoclonal anti-FSHβ was added in excess (1 μg/ml), completely eliminating the effect of the FSH ($p = 0.001$). In the second experiment,

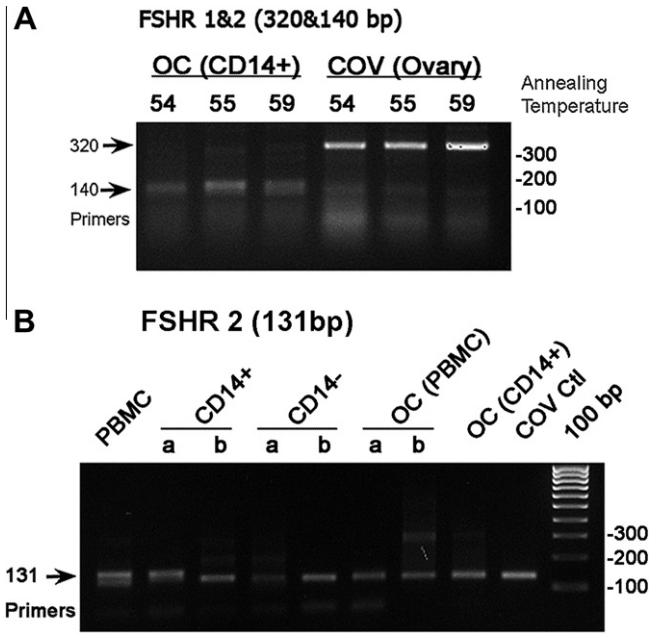

Fig. 2. An alternatively spliced FSH receptor transcript is expressed in human osteoclasts, CD14+ monocytes, and CD14-depleted monocytic cells. (A) FSHR isoform 1 is expressed in the ovary, but a truncated form is the main form found in monocytes and osteoclasts. Primer set 1 (Methods), amplifying across exon 9, shows the full length FSHR fragment from distal exon 8 to early exon 10, and the smaller isoform missing exon 9. The smaller, type 2 isoform, 140 bp, is barely visible in ovary, but is the major product in osteoclasts made from CD14 cells with 14 day incubation in RANKL and CSF-1. Exon 9 is a short extracellular exon just distal to the FSH-binding sequence and proximal to the invariant transmembrane signaling region, exon 10. Results from three reactions in a temperature gradient PCR are shown. Further reactions were annealed at 54 °C. (B) FSHR isoform 2 with exon 9 omitted. Primer set 2, the forward primer of which extends across the exon 8–10 boundary, was used, and the products of 30 cycles of amplification were then re-amplified with an internal nested primer set (131 bp) for a further 20 cycles. Transcript of this isoform was seen in fractions of peripheral blood mononuclear cells (unselected, CD14-selected, CD14-depleted, and osteoclasts from CD14+ cells), and ovarian control (COV) cells. Presence of the smaller isoform, at low levels, in ovarian cells was previously described (see text).

polyclonal anti-FSHβ, 10 μg/ml, similarly returned osteoclast formation to background ($p = 0.003$). We also compared osteoclast development in $FSHR^{-/-}$ and $FSHR^{+/-}$ mice to wild type littermates in the presence of 30 ng/ml of FSH. Formation of multinucleated TRAP-positive osteoclasts 5-days after RANK-L treatment was significantly attenuated in $FSHR^{-/-}$ cultures compared with $FSHR^{+/-}$ or wild type cultures (Fig. 1B, $p < 0.001$ $FSHR^{-/-}$ relative to wild type). The magnitude of the effect was similar to the effect of FSH addition on osteoclast formation in wild type cells. Photomicrographs illustrating the effect of FSHR on the formation of multinucleated TRAP-expressing cells in the wild type, $FSHR^{+/-}$, and $FSHR^{-/-}$ marrow is shown in Fig. 1C. Thus, osteoclast formation decreased with decrements in FSH signaling using either an antibody or through the genetic deletion of the FSHR.

Whereas we and others have demonstrated that functional FSHRs are present on osteoclasts [1–4], some studies have failed to identify such receptors by PCR [9–11]. The major ovarian FSHR transcript has 10 exons, nine of which are short and encode the extracellular domain, while a large tenth exon encodes the conserved transmembrane domain. We showed in earlier studies that the FSHR isoform that is predominantly expressed in osteoclasts has a slightly smaller size than the ovarian form, consistent with deletion of an exon, probably exon 9 [3]. To address this hypothesis in sufficient detail and to produce definitive results, we made new preparations from peripheral blood mononuclear cells (PBMCs), and using a purified (98%+) CD14+ fraction [15]. Both PBMC and CD14+ cells were induced to form osteoclasts by incubation for up to 14 days with RANK-L, as in murine preparations, except that human-specific cytokines were used. RNA isolated from human ovarian cancer cells (COV-413) was obtained for use as a positive control. Several PCR primer sets were used to amplify FSHR, and the meaningful results are shown in Figs. 2 and 3.

Fig. 2A shows results using a primer pair that amplifies regions from exon 8 through exon 10 with a predicted size of 320 bp (primer set 1 in Methods). This gave a strong band of 320 bp in ovarian COV cells (type 1 FSHR) at an annealing temperature of 54 °C and up to 59 °C; this 320 bp product displayed very weak bands in osteoclasts prepared from CD14+ cells. However, in these cells, a much stronger signal amplified consistently at 54–59 °C; the size of the product was 140 bp, which suggests the absence of exon 9. At this size, a faint band also was produced using the ovarian cDNA; this is consistent with the description of truncated isoforms as minor FSHR variants in mammals [16].

Fig. 2B shows results of PCR amplification using primers specific for FSHR isoform 2 with exon 9 omitted (the 5′ primer with 18 nucleotides in exon 8 and the last three nucleotides from exon 10) followed by re-amplification using an internal nested primer set (amplicon size 131 bp) to reduce noise (primer sets 2 and 2B, Methods). The key to this amplification is that, because the 5′ primer extends across the exon 8–10 boundary, it cannot amplify cDNA that contains exon 9. The products of these two sequential amplification procedures showed that the FSHR type 2 isoform was present in PMBCs, CD14+ cells, osteoclasts prepared from CD14+ cells, and ovarian control (COV) cells. A weak band was seen in CD14-negative cells, the fall-through from magnetic bead isolation of CD14+ cells [15]. This might represent type 2 FSHR in other white blood cells, but some CD14+ cells might not be captured by the magnetic beads.

Fig. 3A(i) shows that re-amplification of the 320 bp DNA product gave a very strong signal in the ovary, as expected, but weaker products of the same size did occur in PMBCs and CD14+ cell fractions. Because this product was rare and might be an artifact of extreme amplification, we sequenced the 320 bp re-amplified band from CD14+ osteoclasts. This sequence is shown in Fig. 3A(ii). Reference sequence is black and product sequence in color; portions of the sequence within ~30 nucleotides of the primers (yellow highlight) were not resolved. This sequence includes full length exon 9; the read does extend across the exon 9–10 boundary. The exon 10 is the long, invariant signaling portion that contains the transmembrane domains. Exon 9, in contrast, is a short extracellular exon just proximal to that. The protein fragment derived from exons 1 through 8 binds FSH. We also sequenced the amplified cDNA from the ovarian cells (Fig. 2A). This sequence (not shown) was identical to the reference full length FSHR.

We also sequenced the PCR product from probe set 2, shown in Fig. 3B(i), specific for the FSHR isoform 2. Sequence using the primers (yellow highlight) across exons 8 and 10 for osteoclasts made from CD14+ cells and for CD14+ cells before differentiation is shown in Fig. 3B(ii). The reference sequence, in which exon 9 is absent, is shown in black with the read sequence in color. Sequence of the FSHR type 2 transcript from the ovarian cells was identical to the reference sequence. The sequences shown also included a few misread bases near the ends of the reads (underlined bases in color), a common artifact of the method. But, the long regions of matching sequence are definitive evidence that FSHR type 1 occurs. However, the FSHR type 2 isoform occurs mainly in osteoclasts and in CD14+ cells (Fig. 2A).

4. Discussion

The presence of different functional isoforms of glycoprotein hormone receptors is not surprising in the context of phylogenetic

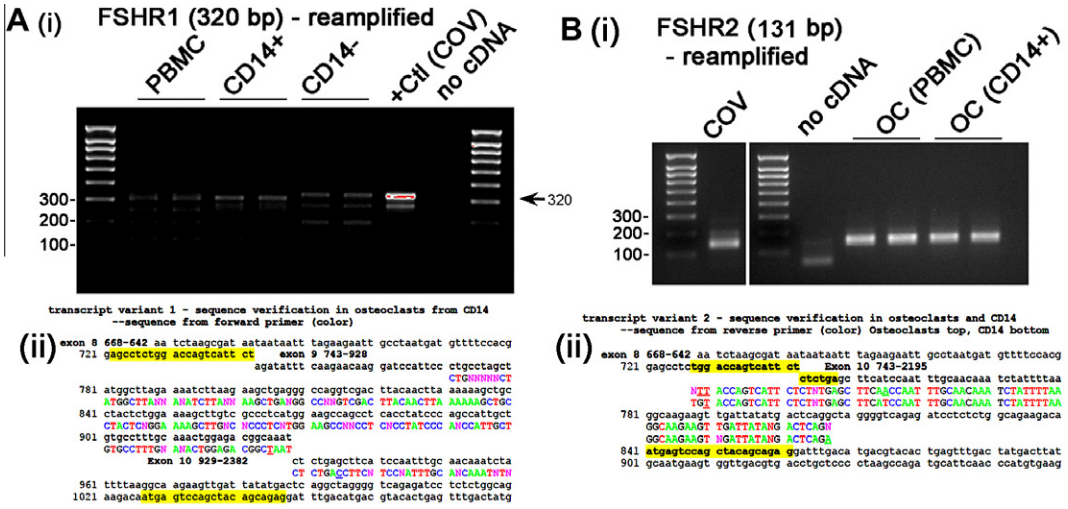

Fig. 3. Sequence confirmation of an alternatively spliced FSH receptor transcript. (A) (i) Full length FSHR transcript noted in osteoclasts. Bands at 320 bp from agarose gels (A) were excised, re-amplified 10 cycles, and (ii) direct sequencing of the PCR products was performed. Sequence of the FSHR from osteoclasts using the forward primer, extending across the exon 9–10 boundary, is shown in color below the reference genomic sequence. The ovarian product also matched the reference sequence (not shown). The osteoclast sequence had several unresolved bases (pink) and two mismatches (underlined), which are probably misreads due to low level signal, but might also be amplification errors. Bases in yellow are primer pair 1. (B) The FSHR isoform 2, missing exon 9, is expressed in osteoclasts and CD14 cells. Bands at 140 bp from agarose gels (i) were excised, re-amplified using primer set 2 for 10 cycles, and direct sequencing of the products was performed (ii). Sequence from the reverse primer, extending across the exon 8–10 boundary, is shown in color below the reference genomic sequence, for both osteoclasts (top color sequence) and CD14 cell (lower color sequence) amplicons. Bases in yellow are primer pair 2. The reference sequence was also obtained from control ovarian cells, and in the PCR product amplified from CD14 depleted cells (neither shown). As in (A), unresolved bases are shown in pink and misreads are underlined. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

studies of the FSHR. In fish, the gonadal expression of the receptors, LHR and FSHR, is established, and all higher orders of animals retain this. However, multiple differently processed forms of the FSHR occur as early as this gonadal specialization in fish [17]; this may reflect FSHR isoforms with differing functions, which we hypothesize is the basis for the difference in FSH response in osteoclasts [1]. There has been divergence in many respects, on the other hand: in fish the FSHR binds both FSH and LH, but the LHR recognizes only LH [18]. In contrast, while high-level FSHR expression in fish is restricted to gonads, low-level expression is was also found in spleen [19], in keeping with expression in immune-related cells such as the CD14+ monocyte.

The functional effects of the osteoclast receptor *in vivo* have been difficult to separate from the action of FSH on the ovary. FSH releases estrogen, and the actions of FSH and estrogen on the osteoclast are opposed. Thus, injection of FSH into mice with intact ovaries [12], or over-expression of FSH [20], even in hypogonadal mice, is unlikely to resolve the pro-resorptive action of FSH from effects due to sex steroids. Nonetheless, in ovariectomized rats, FSH injection aggravates bone loss [21]. The direct effects of FSH on the osteoclast will invariably be masked to a large extent by the anti-resorptive and anabolic actions of ovarian estrogen. Other than the menopause, one other physiological state, lactation, has tonically high FSH with low estrogen, which may in part explain why the seemingly paradoxical effect of FSH on bone mass exists and complicates the menopause, as discussed elsewhere [22].

Whether lowering FSH in hypogonadal states will be useful to prevent bone loss remains uncertain. Data suggesting that this might be useful include that amenorrheic women with lower FSH levels have less bone loss [23], and that the effectiveness of estrogen therapy varies with the degree of FSH suppression [24]. Nevertheless, pituitary hypogonadism causes bone loss. Leuprolide treatment, which reduces FSH, does not improve bone resorption indices when given experimentally with aromatase inhibitors for 11 weeks in post-menopausal women. Bone degradation markers are increased significantly in aromatase inhibitor-only controls; however, serum osteocalcin did not vary and bone density was not measured [25]. This demonstrates that extremely low estrogen exacerbates bone degradation in post-menopausal women, an important reaffirmation, but it does not exclude a role for FSH in human skeletal homeostasis. Rather than blocking FSH in acute hypogonadism, where the effect of very low estrogen is likely to be overwhelming, FSH inhibition during the late peri-menopause, particularly when estrogen levels are normal and FSH reaches extremely high levels, could potentially be therapeutically useful. A highly selective approach, such as the use of a blocking antibody, is thus envisaged.

In summary, our studies using antibodies to block the FSH stimulus of osteoclast formation, and the amplification of FSHR isoforms by nested PCR primer sets with sequencing of the PCR products, provide evidence that a splice variant of the FSHR missing exon 9 occurs in osteoclasts and CD14+ osteoclast precursors. An unknown issue remains whether the deletion of exon 9 in the FSHR affects specificity of the receptor for FSH, and whether other isoforms, for example, a splice variant lacking exon 6, exist in these cells.

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