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Oxytocin deficiency impairs maternal skeletal remodeling

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ABSTRACT

We have reported that the posterior pituitary hormone, oxytocin (OT), known for its effects in inducing parturition, lactation and social bonding, is also a skeletal hormone. Here, we demonstrate that OT plays a key role in enabling maternal skeletal mobilization during pregnancy by enhancing the formation of bone resorbing osteoclasts. Osteoclast formation *ex vivo* is thus diminished in pregnant mothers with genetic OT-deficiency. $OT^{-/-}$ pups at day E20 also show a defect in trabecular bone. μCT measurements reveal normal bone volume, but increased trabecular numbers, suggesting that trabeculae in $OT^{-/-}$ pups are hypomineralized. We suggest that OT facilitates intergenerational transfer of calcium ions from a pregnant mother to the pups.

Introduction

Originating as a highly conserved nanopeptide for electrolyte homeostasis in primitive vertebrates over 400 million years ago [1], OT has acquired a primary role in mammalian lactation [2]. While the peptide also facilitates parturition, it is not indispensible for this function [2]. OT null mice deliver normally, but are unable to nurse their pups absent a milk ejection reflex. This defect is fully reversible upon peripheral OT injection, attesting to a hormonal rather than a central mechanism [2]. The central effects of OT, mediated by hypothalamic neurons, include the regulation of social behavior, such as sexual and maternal behavior, affiliation and social memory, as well as, in males, penile erection and ejaculation [3–6]. The central actions of OT can be elicited by intracerebroventricular injection, and the global deletion of OT in mice causes social amnesia and aggressiveness [7]. Thus, OT acts by two distinct mechanisms: a hormonal action obligatory to milk let down when circulating levels are high [8], and a central neurogenic pathway that regulates behavior.

The rise of OT during late pregnancy and lactation coincides with rapid fetal skeletogenesis and post-natal bone modeling, both requiring an elaborate supply of calcium ions for mineralization. In fact, about 30 of a total of 1000 grams maternal calcium is mobilized during late pregnancy [9]. This calcium arises from elevated maternal bone resorption relative to declining bone formation during the last trimester [10]. Following childbirth, a further 80 g of calcium is secreted into milk during lactation; this calcium, in contrast, comes from an absolute increase in bone resorption [9]. Pregnant and lactating women thus suffer the most rapid bone loss of their lifetime, up to 10% over 6 months, mainly at trabecular sites [11]. The recovery of bone formation, through a yet uncharacterized mechanism, lends to a near-restoration of bone mass to virgin levels at weaning [10]. If bone formation is insufficient, the end result is "pregnancy- and lactation-associated osteoporosis".

We hypothesized that a pituitary hormone that controls parturition and lactation might also regulate intergenerational calcium transfer, and hence, maternal bone mass. In an exciting set of observations [12], we showed that mice lacking OT or its receptor (Oxtr) display severe low-turnover osteoporosis due to defective bone formation. That this defect is recapitulated in haploinsufficient $OT^{+/-}$ mice suggests that the skeletal effects of OT are dominant, unlike the lactation defect, seen only in OT null mice [2,12]. The injection of OT into wild type mice expectedly increases bone mass by inducing a state of high bone turnover, in which an increase in bone formation predominates [12].

Our evidence suggesting that OT is an anabolic bone hormone, with additional effects on resorption [12], led us to hypothesize that OT mediates the high remodeling of pregnancy, at least in

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part. We were also prompted to speculate that fetal skeletal development was OT-sensitive. Here, we report that maternal OT-deficiency results in attenuated osteoclast and osteoblast formation *ex vivo*, and the resulting OT-deficiency in the fetus impairs trabecular development, and likely, trabecular mineralization.

Materials and methods

Ex vivo assays. To study osteoclast formation, bone marrow cells from mice were cultured for 1 day with M-CSF (30 ng/ml). Non-adherent cells were then collected, purified by Ficoll-PackPlus

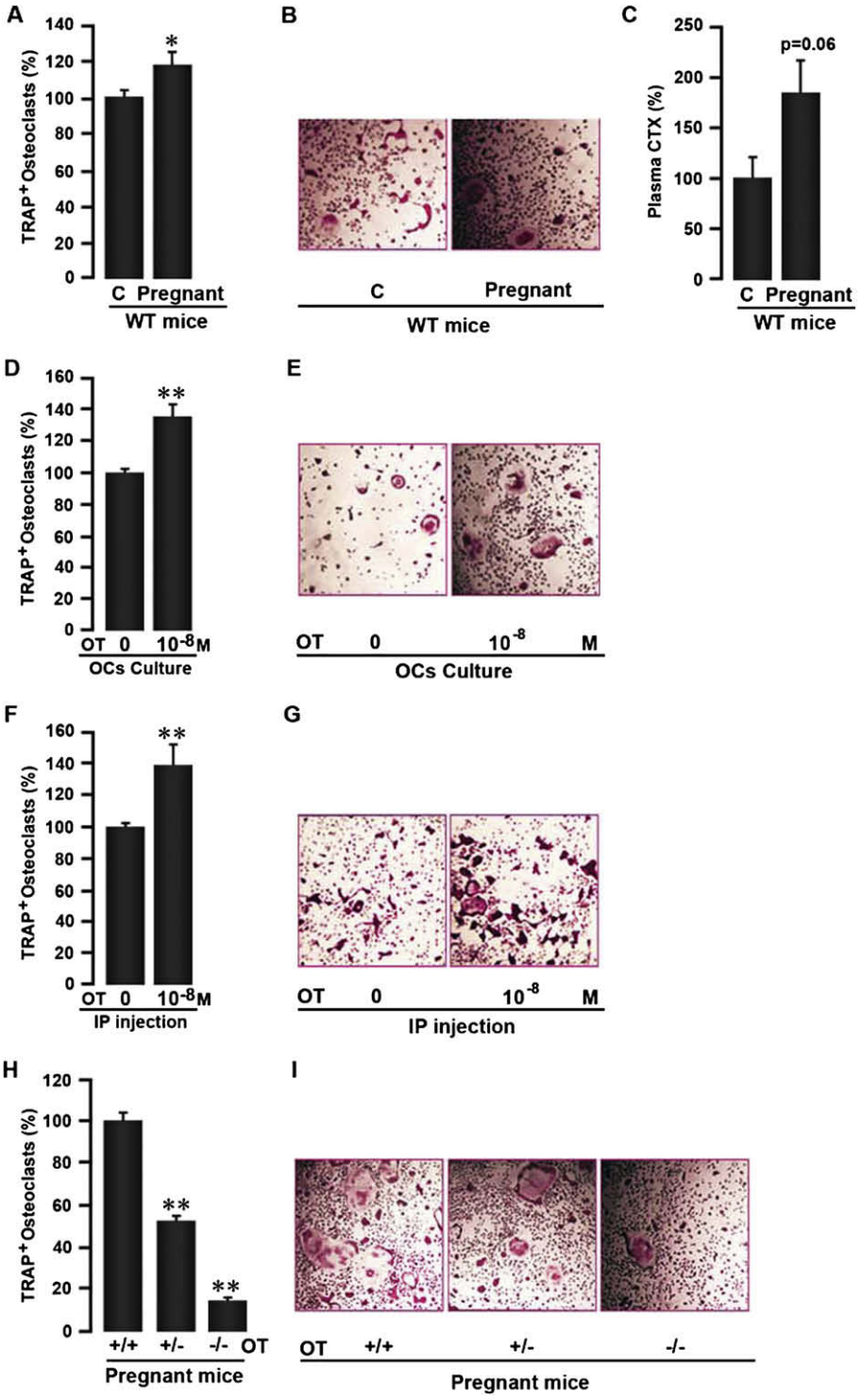

Fig. 1. Oxytocin (OT) deficiency attenuates the increases in osteoclast formation *ex vivo* during late pregnancy. Mean tartrate-resistant acid phosphatase- (TRAP-) positive multinucleated osteoclasts in bone marrow cell cultures from 18-day pregnant females plotted as percent of control, non pregnant females (A) or shown as representative images (B). This increase corresponds to an elevation in plasma C-telopeptide (CTX) levels, which approached significance ($p = 0.06$) (C). The enhanced osteoclast formation was mimicked by exposure to OT in culture (10^{-8} M, D and E), as well as by intraperitoneal (IP) OT injection, 25 μg/mouse, thrice a week, for 5 weeks (F and G). This increase was attenuated in pregnant mice deficient in OT, namely in the heterozygote (+/−) and homozygote (−/−) groups (H and I). Statistics: mean + SEM; comparisons against control mice in each group, and wild type littermates in panels H and I; Student's *t*-test, $^{**}p < 0.01$, $n = 3$–5 mice per group, 8 wells per experiment.

(Amersham Pharmacia Biotech Inc., Arlington Height, IL), and incubated with RANK-L (50 ng/ml) and M-CSF (30 ng/ml) for 4–6 days. This was followed by staining for tartarate-resistant acid phosphatase (TRAP) using a kit (Sigma) *per* manufacturer. The number of TRAP-positive cells was counted. To validate results from the osteoclast formation assay, parallel measurements of C-telopeptide, a bone resorption marker, were made by ELISA using a kit (RAT LAPS, Nordic Bioscience Diagnostics, Hovedgade, Denmark).

To study osteoblast formation, adherent stromal cells (after removal of the hematopoetic stem cell fraction from bone marrow) were cultured in the presence of ascorbic acid-2-phosphate (1 mM) to initiate differentiation. At around 10 days, multi-cellular fibroblastoid colonies (or CFU-fs) appeared; we stained these colonies for alkaline phosphatase using a kit (Sigma) *per* manufacturer, and counted alkaline phosphatase-positive CFU-fs. In separate cultures, the colonies were allowed to mature to form mineralizing, colony-forming units-osteoblastoid (CFU-ob) that were *von Kossa* stained and counted at 21 days.

All results were expressed as mean ± SEM and displayed as percent of control (or wild type) cultures. Comparisons were made between groups using the Student's *t*-test.

Gross skeletal phenotyping. Pups were obtained at day E20 from $OT^{+/-}$ mothers and stained with alizarin red and alcian blue to visualize gross abnormalities in bone and cartilage, respectively. The carcasses were cleaned and fixed overnight in ethanol (95% v/v) and then stained with alcian blue (Sigma A-3157; in acetic acid 20% v/v, and ethanol 80% v/v). Thereafter, the skeletons were transferred back into ethanol (95% v/v) for 3 h and incubated in KOH (2% w/v), and then in a solution of alizarin red S (Sigma A5533) in KOH (1% w/v) and glycerol (20% v/v). The skeletons were finally stored n a 1:1 mixture of glycerol and ethanol (95% v/v) for visualization of gross abnormalities.

μCT measurements of trabecular structure. A total 16 femurs were isolated from four $OT^{-/-}$ and $OT^{+/+}$ pups. The bones were subjected to μCT analysis using a CT40 scanner (SCANCO USA Inc., Southeastern, PA) at 45 kV and 177 μA. Bone structure and quality were analyzed by the proprietary software (SCANCO USA Inc). The threshold value for all analyses was set at 177. Indices analyzed included bone volume/total volume (BV/TV), density of connectivity of trabecular bone (Conn.D), trabecular number per mm^3 (DT-Tb.N), separation of trabecular bone or marrow thickness (DT-Tb.Sp), and trabecular bone thickness (DT-Tb.Th). Average values of the pair of femurs were first calculated, and statistical comparisons made using the Student's *t*-test.

Results and discussion

Fig. 1A and B show enhanced TRAP-positive osteoclast formation in bone marrow cultures, as means and representative images, respectively, from mice pregnant for 18 days. The increase in osteoclastogenesis corresponds with a near-doubling of the plasma levels of C-telopeptide, a marker of bone resorption, shown in Fig. 1C. That osteoclast formation and serum C-telopeptide levels correlate validates the use of *ex vivo* bone marrow cell cultures for our experiments. This is important as plasma C-telopeptide levels arise from bone resorption and provide a secondary functional end-point, while osteoclastogenesis assays measure the formation of the cells themselves. In addition, we have found previously that OT has dual actions on the osteoclast [12]: it stimulates osteoclastogenesis, but inhibits bone resorption by mature osteoclasts. Thus, we consider it valuable to use changes in *ex vivo* osteoclastogenesis as valid surrogates for measuring OT responsiveness.

The pregnancy-induced increases in osteoclastogenesis were mimicked by the addition of OT (10^{-8} M) to bone marrow cell cultures (Fig. 1D and E), as well as by intraperitoneal injections of OT (25 μg/mouse) thrice weekly for 5 weeks (Fig. 1F and G). This is consistent with similar increases noted earlier by us in response to two OT injections given 12 h apart [12], indicating a very rapid cellular response seen well before changes in serum remodeling markers ensue.

Overall, these findings not only confirmed an osteoclastogenic action of OT *in vitro* and *in vivo*, but also questioned whether the enhanced osteoclast formation was due to elevated OT levels during late pregnancy [8]. We therefore measured osteoclast formation *ex vivo* in pregnant mice genetically devoid of OT (Jackson Laboratory, Bar Harbor, ME). Of note is that homozygote $OT^{-/-}$ mice have impaired lactation and poor social bonding, but are fully fertile and without a defect in fecundity [2]. In contrast, haploinsufficient, heterozygote $OT^{+/-}$ mice are known to lactate and deliver normally, and have no discernable basal reproductive phenotype [2]. However, Fig. 1H and I show profound decreases in osteoclast formation in both genotypes. In particular, pregnant $OT^{-/-}$ mice showed a ~80% reduction in osteoclastogenesis compared with wild type pregnant littermates.

Impressively, that these decrements in osteoclastogenesis were evident even after 5 days of incubation of bone marrow cells with RANK-L and M-CSF suggested that the chronic absence of OT had induced a defect that did not reverse in culture. Together the results demonstrate that the osteoclastogenesis triggered in pregnant mice is, at least in part, dependent upon an intact OT axis. It is therefore likely that the increased serum levels of OT during pregnancy [8] drive the initial osteoclastogenesis that tends to mobilize maternal calcium towards fetal skeletogenesis. This response, we have shown, is attenuated in mice deficient in OT.

Considering that OT stimulates osteoblastogenesis as an anabolic hormone [12], we next examined whether osteoblast formation *ex vivo* was enhanced in pregnant wild type mice, and whether this response was attenuated in OT-deficiency. We studied the formation of both early, alkaline phosphatase-positive CFU-f, and late, mineralizing *von Kossa*-positive CFU-ob colonies. We found, as expected, that pregnancy strongly stimulated CFU-f formation (Fig. 2A and B). This was mimicked by intraperitoneal OT injection, thrice weekly, as before, indicating that OT might, at least in part, mediate this increase (Fig. 2C and D).

However, complete OT-deficiency in $OT^{-/-}$ mice only modestly decreased CFU-f formation with respect to wild type pregnant controls, whereas haploinsufficiency had no effect in decreasing CFU-f (Fig. 2E and F). In contrast, mineralizing CFU-ob formation was significantly decreased in $OT^{+/-}$ mice, but surprisingly so, not as much in the homozygotes (Fig. 2G and H). Together, the findings show a modest effect of OT depletion on osteoblast formation during pregnancy. This is not unexpected as recovery of bone formation occurs mainly during lactation to approach virgin levels at weaning [9].

The uncoupling of bone remodeling with net skeletal loss during lactation has been attributed to hypoestrogenemia [13]. However, menopausal estrogen deficiency causes irreversible bone loss [14], while the bone loss of pregnancy and lactation is mostly reversible in both rodents and humans [15]. Elegant studies by Wysolmerski and colleagues suggest that PTHrP, a hormone indispensible for mammary gland development, is also pro-resorptive during lactation [13,16]. The evidence is solid: when PTHrP is deleted conditionally in mammary cells, not only do serum PTHrP levels drop as expected, lactation-induced hyper-resorption is attenuated, thus reducing bone loss [16].

It is hard to imagine, though, that a single or even two mechanisms are solely responsible for a process as fundamental to procreation and skeletal morphogenesis as maternal hyperresorption. We now implicate OT, which is also a mammary gland-specific peptide [2], in the regulation of intergenerational calcium transfer by stimulating maternal osteoclastogenesis dur-

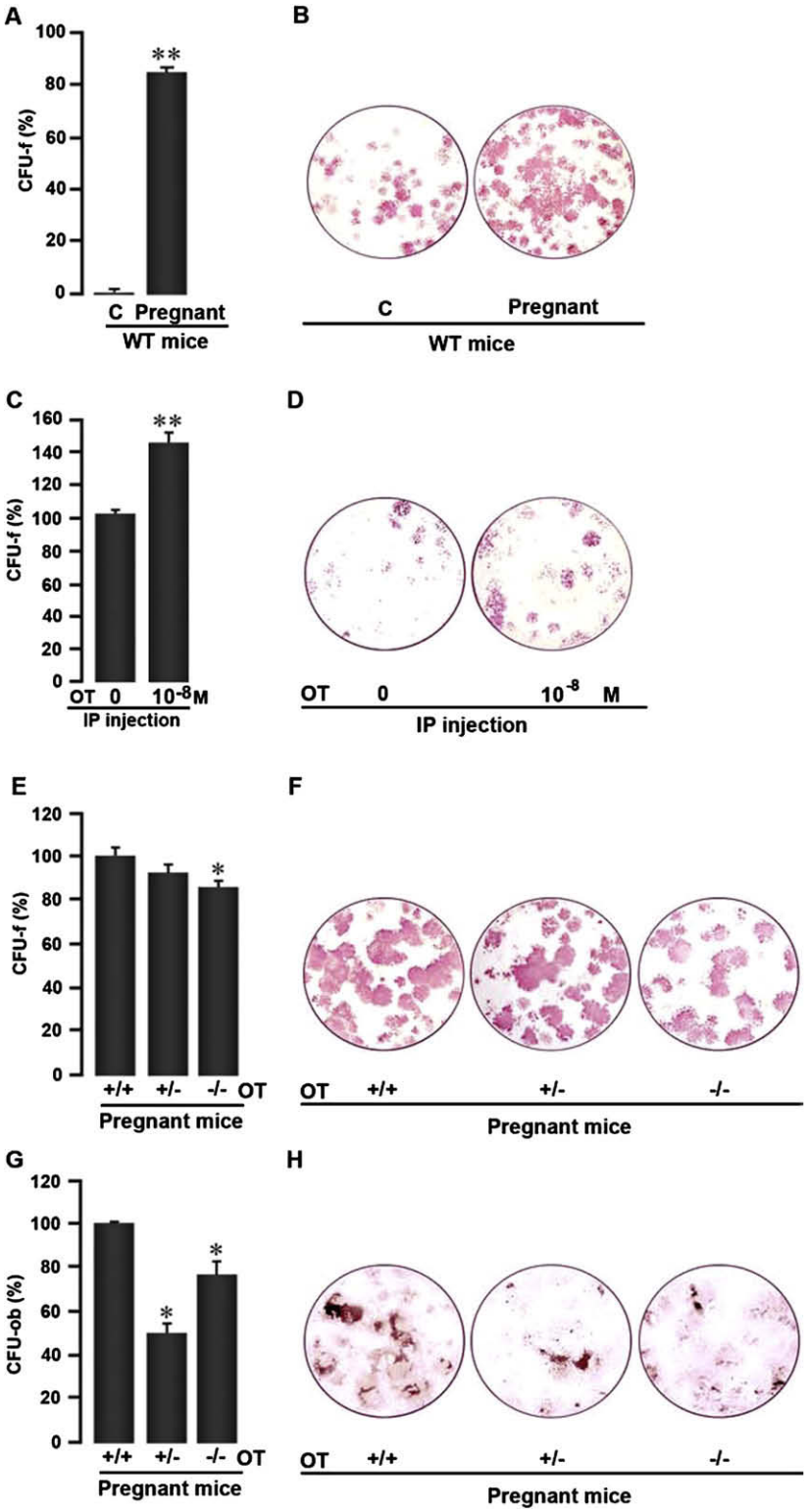

Fig. 2. Modest effects of oxytocin (OT) deficiency on osteoblast formation *ex vivo* during late pregnancy. Mean colony counts and representative wells showing either colony-forming units-fibroblastoid (CFU-f) or colony-forming units-osteoblastoid (CFU-ob) colonies under different experimental conditions, namely in control *versus* 18-day pregnant female mice (A and B); mice injected with vehicle *versus* OT (25 μg/mouse, thrice weekly, for 5 weeks) (C and D); and mice deficient in OT [heterozygote (+/−), homozygote (−/−) and wild type (+/+) littermates] (E, F, G and H). Statistics: means + SEM, as percent of control; comparisons against control or wild type mice by Student's *t*-test; $^{*}p < 0.05$, $^{**}p < 0.01$, $n = 3$–5 mice per group, 3 wells per experiment.

ing pregnancy, and perhaps during lactation, at which times serum OT levels rise significantly [8].

However, maternal bone loss would be unremitting in the absence of a mechanism that would restrict excessive osteoclastic resorption. We propose from our previous work that OT, which also inhibits resorption by mature osteoclasts [12], should provide one such mechanism for the self-regulation of OT-induced osteoclastogenesis and bone loss. In addition, the levels of another

osteoclast-inhibitory hormone, calcitonin, rise during pregnancy and lactation [17,18]. A high circulating calcitonin likely also prevents the excessive loss of calcium. However, while we provide genetic evidence for OT as a hormone that stimulates osteoclastogenesis during pregnancy, and likely during lactation, mechanisms restricting excessive resorption have yet to be explored.

In view of a plausible role for OT in maternal skeletal remodeling, we next chose to examine the skeletons of pups at E20. Fig. 3A shows $OT^{+/-}$, $OT^{-/-}$ and wild type pups derived from heterozygote mothers. These pups had apparently normal skeletons, specifically displaying no runting or bone/cartilage defects. Measurements of mainly trabecular bone at the epiphysis showed no difference in BV/TV (Fig. 3B and C). However, trabecular number was significantly increased, without any change in trabecular thickness (Fig. 3B). This resulted, as would be expected, in a significant reduction in trabecular spacing (Fig. 3B). The increase in trabecular number is reminiscent of reduced resorption in OT-deficiency; however, the expectation from this finding in isolation would be an osteopetrotic phenotype manifest by an increased BV/TV. Nonetheless, BV/TV was unchanged, even in the face of greater trabecular numbers. This, to us, implies that each trabeculum is less mineralized, or indeed, total

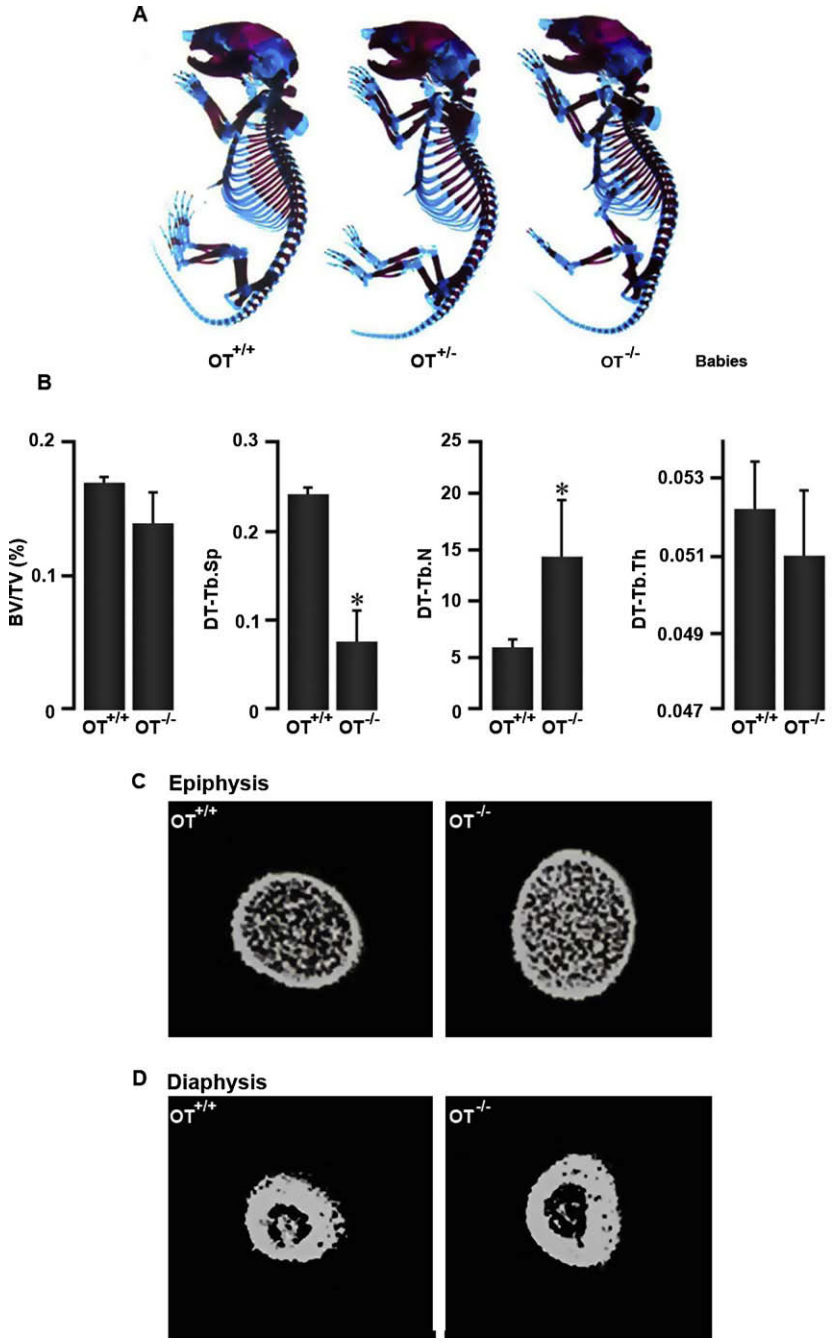

Fig. 3. Oxytocin (OT) deficiency impairs skeletogenesis *in utero*. (A) No overt defects or runting in alizarin red (bone) or alcian blue (cartilage) stained skeletons from oxytocin-deficient (heterozygote or homozygote) pups derived at E20 compared with those of wild type littermates from the same heterozygote mother. Micro-CT revealed significant decreases and increases, respectively, in trabecular spacing (DT-Tb.Sp) and trabecular number (DT-Tb.N), but without differences in bone volume (BV/TV) or trabecular thickness, as determined in epiphyseal trabecular bone (B). Representative images of epiphyseal and diaphyseal sections are shown in panels C and D, respectively. Means + SD shown for each parameter; comparisons between $OT^{-/-}$ pups and wild littermates ($OT^{+/+}$) by Student's *t*-test; $^{*}p < 0.05$, $n = 4$ mice per group.

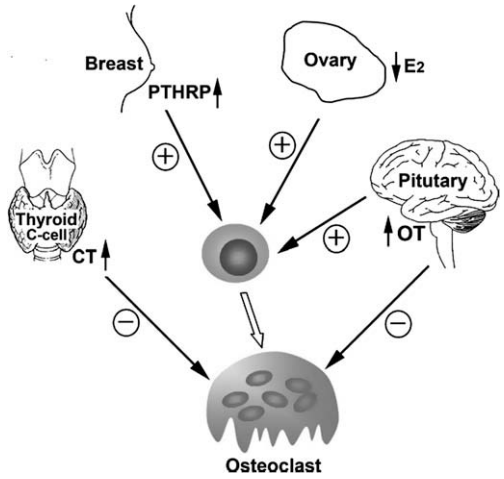

Fig. 4. Hypothetical schema for the control of osteoclast formation and resorptive function during pregnancy and lactation. The increased production of oxytocin (OT) from the posterior pituitary stimulates osteoclastogenesis by acting on the osteoclast precursor. During lactation, this is further accelerated by a transient decline in serum estrogen (E_2) and parathyroid hormone-related protein (PTHrP) secretion from mammary epithelial cells: all of these hormonal influences appear to play a role in maternal skeletal mobilization. However, to prevent excessive skeletal loss, OT inhibits bone resorption by mature osteoclasts, as does the hormone calcitonin (CT), produced in excess from the parafollicular C-cells of the thyroid.

mineral per unit volume is lower in $OT^{-/-}$ pups than in wild type controls. While we have not measured mineral *per se*, we can conclude safely that OT is essential for optimal skeletal maturation in the fetus, and that the absence of OT leads to increased numbers of trabeculae that appear hypomineralized. Finally, μCT measurements at the diaphysis showed no obvious differences between $OT^{-/-}$ pups and wild type littermates (Fig. 3D).

Fig. 4 schematizes our current understanding of the hormonal regulation of bone resorption during pregnancy and/or lactation. It is known that hypoestrogenemia stimulates osteoclastogenesis during lactation [13]. The breast-derived hormone, PTHrP does the same [13,16], as does OT, during late pregnancy, and likely during lactation. Our previous studies also implicate OT in stimulating bone formation [12], particularly when the maternal skeleton requires restoration to virgin levels at weaning. Calcitonin appears to aid this process by inhibiting resorption through its direct osteoclastic action, as does OT, as a means of self-regulation.

We are currently unclear which hormonal action predominates, or indeed, the precise sequence in which these and other, yet unidentified hormones and cytokines, orchestrate to regulate a process as fundamental as intergenerational calcium transfer. While we have just touched the surface, our study highlights the importance of further investigations using genetically-modified mice to probe into how the fetal skeleton is mineralized at the expense of the mother, and in particular, how the mother recovers from this heavy burden of calcium loss.

Acknowledgments

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