Oxytocin is an anabolic bone hormone

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We report that oxytocin (OT), a primitive neurohypophyseal hormone, hitherto thought solely to modulate lactation and social bonding, is a direct regulator of bone mass. Deletion of OT or the OT receptor (Oxtr) in male or female mice causes osteoporosis resulting from reduced bone formation. Consistent with low bone formation, OT stimulates the differentiation of osteoblasts to a mineralizing phenotype by causing the up-regulation of BMP-2, which in turn controls *Schnurri-2* and *3*, *Osterix*, and *ATF-4* expression. In contrast, OT has dual effects on the osteoclast. It stimulates osteoclast formation both directly, by activating NF-κB and MAP kinase signaling, and indirectly through the up-regulation of RANK-L. On the other hand, OT inhibits bone resorption by mature osteoclasts by triggering cytosolic Ca^{2+} release and NO synthesis. Together, the complementary genetic and pharmacologic approaches reveal OT as a novel anabolic regulator of bone mass, with potential implications for osteoporosis therapy.

osteoblast | osteoclast | osteoporosis | pituitary hormones | bone density

Oxytocin (OT), a hypothalamic nanopeptide secreted into the circulation from the posterior pituitary, is indispensable for lactation. It acts on a G protein-coupled receptor (Oxtr), the expression of which in reproductive tissues is regulated by sex steroids and OT. In humans and rodents, plasma OT levels are elevated maximally during suckling (1, 2).

Mice lacking OT or its receptor (Oxtr) are unable to lactate, despite unperturbed breast tissue and milk formation (3, 4). Most notably, newborn pups die shortly after birth in the absence of a foster mother postpartum. This effect of OT is exerted peripherally, as the i.p. administration of recombinant OT to $OT^{-/-}$ mice rescues milk ejection, allowing the newborn to feed normally. In contrast to the milk ejection defect, no deficits in copulation, gestation, fecundity, or parturition have been noted in either $OT^{-/-}$ or $Oxtr^{-/-}$ mice, suggesting that these mice are typically eugonadal (5). Furthermore, compound mutants with both the Oxtr and the prostaglandin F2α receptor deleted exhibit no defects in parturition, indicating significant redundancy in the birth process per se (5). However, in view of the established pharmacology of circulating OT on the uterine myometrium, the possibility of a physiological action of OT during childbirth cannot be excluded, even without a loss-of-function phenotype.

Two other key actions of OT warrant mention: effects on social behavior and on the regulation of food intake. Male $OT^{-/-}$ and $Oxtr^{-/-}$ mice show deficits in social recognition, without altered cognition or olfactory learning. That this social amnesia is a central rather than a peripheral action of OT is supported by the observation that recombinant OT injected directly into the amygdala rescues the defect (6). Compared with males, female OT or Oxtr null mice display anxiety and exaggerated stress responses, which are likewise mediated through central OT-ergic neurones (7). OT also is involved in the regulation of food (particularly carbohydrate) intake (8). The loss of OT's anorexogenic effect leads to overfeeding, increased carbohydrate intake, and increased body weight in OT and Oxtr null mice (5). But the mice are not rendered diabetic, and serum glucose homeostasis remains unaltered (9). Thus, whereas the effects of OT on lactation and parturition are hormonal, actions that mediate appetite and social bonding are exerted centrally. The precise neural networks underlying OT's central effects remain unclear; nonetheless, one component of this network might be the interactions between leptin- and OT-ergic neurones in the hypothalamus (10).

Considering that calcium is mobilized from the maternal skeleton during late pregnancy and lactation, we speculated that the same hormone that regulates parturition and lactation also might also control skeletal homeostasis. Thus, we explored whether the deletion of OT or Oxtr in mice affects bone mass and bone remodeling. However, in view of OT's known central actions, we attempted to determine directly whether intracerebroventricular (ICV) OT affects bone remodeling. We conducted further gain-of-function studies that focused on the effects of OT on osteoclast and osteoblast formation. Finally, we probed the signaling cascades that mediate OT action on bone cells.

We report a direct and dominant action of peripheral OT on the skeleton that is mediated mainly through its stimulation of osteoblast formation, with variable effects on osteoclasts. We suggest that OT, as a circulating peptide, is indispensable for basal skeletal homeostasis in both sexes and may play an additional role in the initial mobilization and subsequent restoration of the maternal skeleton during periods of calcium stress in pregnancy and lactation. We speculate that because of its skeletal anabolic action, recombinant OT or its analogs might have potential utility in therapy for human osteoporosis.

Results

Mice lacking OT or Oxtr displayed profound osteoporosis. Histomorphometry and μ-CT revealed marked reductions in trabecular volume [bone volume per trabecular volume (BV/TV)] in both $OT^{-/-}$ and $Oxtr^{-/-}$ mice, despite an expected increase in weight [supporting information (SI) Table S1] (5). There were no significant sex differences (Fig. 1 *A–D*). These findings confirm that the action of OT is indispensable for maintaining optimal bone mass in both sexes. Importantly, haploinsufficiency of OT or the Oxtr reduced BV/TV in the face

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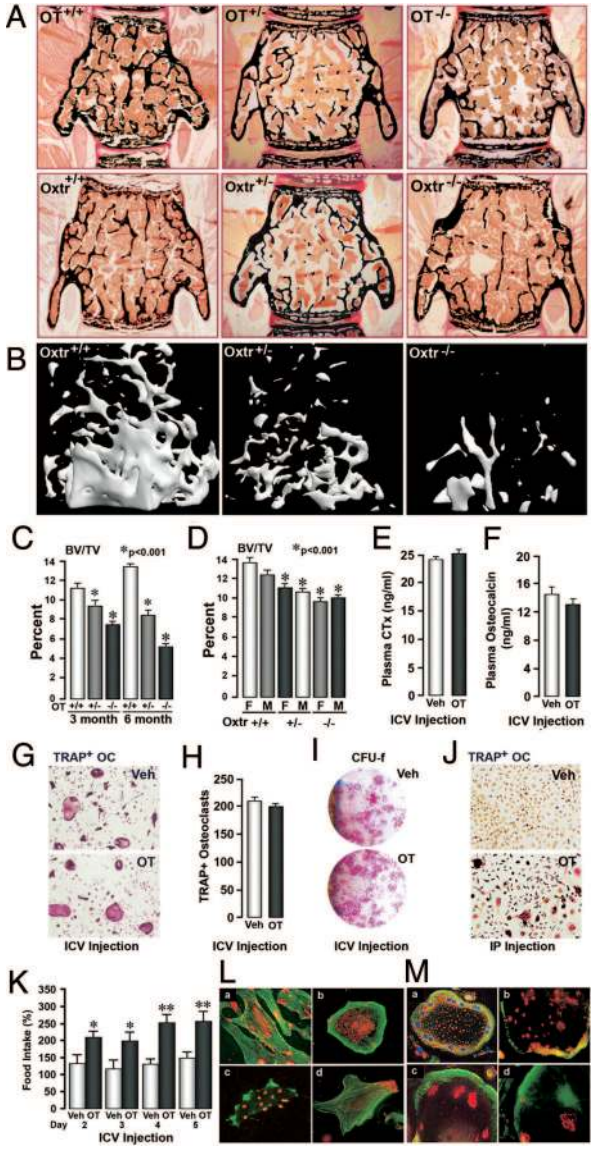

Fig. 1. Direct skeletal action of OT. Loss-of-function studies demonstrated severe trabecular bone loss in 3- or 6-month-old OT- and Oxtr-null mice and heterozygotes compared with wild-type littermates. Von Kossa-stained sections of the 5th lumbar vertebrae (*A*; representative) revealed significant reductions in trabecular bone volume (BV/TV, %) at both 3 and 6 months (*C*), but with no sex-specific differences at 6 months (*D*). This profound trabecular bone loss was confirmed in Oxtr-deficient mice by μ-CT of the femoral epiphysis (*B*; representative). Statistics: Student *t* test, *P* values as indicated comparing OT- and Oxtr-deficient mice against wild-type littermates ($n = 8$ mice/group). Indirect skeletal effects of OT through hypothalamic OT-ergic neurones were examined by ICV injection of OT (25 ng/day) via Alzet pumps for 5 days. We found no significant differences in plasma C-telopeptide (*E*) or OC (*F*) level; moreover, TRAP-positive osteoclasts (*G* and *H*) or alkaline phosphatase-positive CFU-f (*I*) did not enhance in ex vivo bone marrow cell cultures of ICV-injected mice. The finding that food intake was stimulated by ICV injections (*K*; percentage of control at day 1) indicates that OT does exert a central action, but that this action does not affect the skeleton. In contrast, 2 i.p. injections of OT (4 μg/mouse) given to 2-month-old mice 12 h apart resulted in a significant increase in TRAP-positive osteoclast formation (*J*), further supporting a peripheral, rather than a central, action of OT. Statistics: Student *t* test; *P* values shown; $n = 8$ mice per group; 2 experiments pooled. The peripheral action of OT was consistent with the presence of Oxtr on both mouse and human osteoblasts (*L*) and osteoclasts (*M*). The cells were first labeled with fluorescent phalloidin (green) to delineate the actin filaments, and thereafter with an anti-Oxtr antibody (red). The experiment was repeated with 3 different batches of human or mouse cells; representative cells are shown. The bottom panels represent Oxtr internalization, seen as intracellular staining, after a 12-hour exposure to OT (10 nM), further confirming functional Oxtr specificity.

of unperturbed lactation and no increase in weight (Table S1), attesting to OT's exquisitely sensitive actions on the skeleton.

We sought to determine whether OT acts directly on bone cells or indirectly via a central neural mechanism. Hypothalamic leptinergic neurons directly regulate bone formation through sympathetic relay directed to osteoblasts via Adrβ_2 receptors (11). Furthermore, hypothalamic OT-ergic neurons regulate social bonding and food intake (5–8, 12). However, we found that ICV OT infusion for 5 days neither affected serum markers of osteoblast [osteocalcin (OC)] and osteoclast (C-telopeptide) function nor influenced ex vivo osteoblast or osteoclast formation (Fig. 1 *E–I*). At the same doses, OT stimulated food intake on all days (Fig. 1*K*), suggesting an action on the central nervous system that does not regulate bone metabolism. Our findings also largely exclude a role for leptinergic/sympathetic relay as a mediator of OT's effects on bone formation and bone mass (13).

In contrast, OT injected i.p. dramatically increased the number of tartrate-resistant acidic phosphatase (TRAP)-positive osteoclasts in ex vivo marrow cultures (Fig. 1*J*). Importantly, the direct i.p. injection of OT into mice increased bone mineral density (BMD) as well as osteoblast (CFU-f) formation at 5 weeks (Fig. S1), confirming a peripheral skeletal action of OT, a molecule that otherwise does not cross the blood–brain barrier. Finally, it is highly unlikely that OT's skeletal action is mediated via prolactin, and although serum prolactin levels might decline in OT deficiency, this decline will (and should not) account for an osteoporotic phenotype considering the known proresorptive actions of prolactin.

Providing further confirmation of a peripheral action of OT, Fig. 1*L* and *M* clearly show Oxtr immunolabeling on osteoblasts and osteoclasts, respectively. The bottom panels show that Oxtr localizes to the cytoplasm on exposure to recombinant OT, providing evidence not only for ligand-mediated receptor internalization, but also for ligand specificity. Overall, the data confirm original reports for Oxtr on bone cells (14, 15) and reaffirm a direct action of OT on osteoblasts and osteoclasts.

To examine whether the observed osteopenia in OT$^{-/-}$ mice was due to reduced bone formation, increased resorption, or both, we performed histomorphometry after 2 injections of calcein (15 mg/kg) given 7 days apart. We found a significant reduction in mean apposition rate (MAR) in OT$^{-/-}$ mice, with only few observable double labels (Fig. 2 *A* and *B*). We then studied the effect of OT deletion on osteoblast differentiation ex vivo by culturing calvarial osteoblasts. Von Kossa staining, used to examine mineral deposition in 21-day cultures, revealed a striking reduction in mineralization by OT$^{-/-}$ calvarial osteoblasts (Fig. 2*C*). Exposure to recombinant human OT during the same 21-day culture partially rescued the mineralization defect (Fig. 2*C*).

The reduction in osteoblast colonies seen in OT$^{-/-}$ mice could be caused by attenuated proliferation, inhibited differentiation, or both. We found that calvarial osteoblast proliferation was decreased in OT$^{-/-}$ mice, whereas in wild-type cells, recombinant OT enhanced osteoblast precursor proliferation in a concentration-dependent manner (Fig. 2 *D* and *E*). To examine the effects of OT on osteoblast differentiation, we measured the per-cell expression of genes indicative of osteoblast maturation. We found that OT treatment increased osteopontin (OPN) and OC mRNA expression in calvarial osteoblasts (Fig. 2*F*). Similarly, OT$^{-/-}$ osteoblasts had reduced OPN and OC mRNA levels (Fig. 2*G*). Thus, although OT increases both osteoblast proliferation and differentiation, genetic OT ablation, expectedly, has the opposite effects.

We next explored the mechanism by which OT causes increased osteoblast differentiation. Western blot analysis revealed that the 2 critical transcription factors for osteoblast formation, Runx2 and Osterix (Osx), were differentially regulated in OT$^{-/-}$ mice. Runx2 protein was increased and Osx

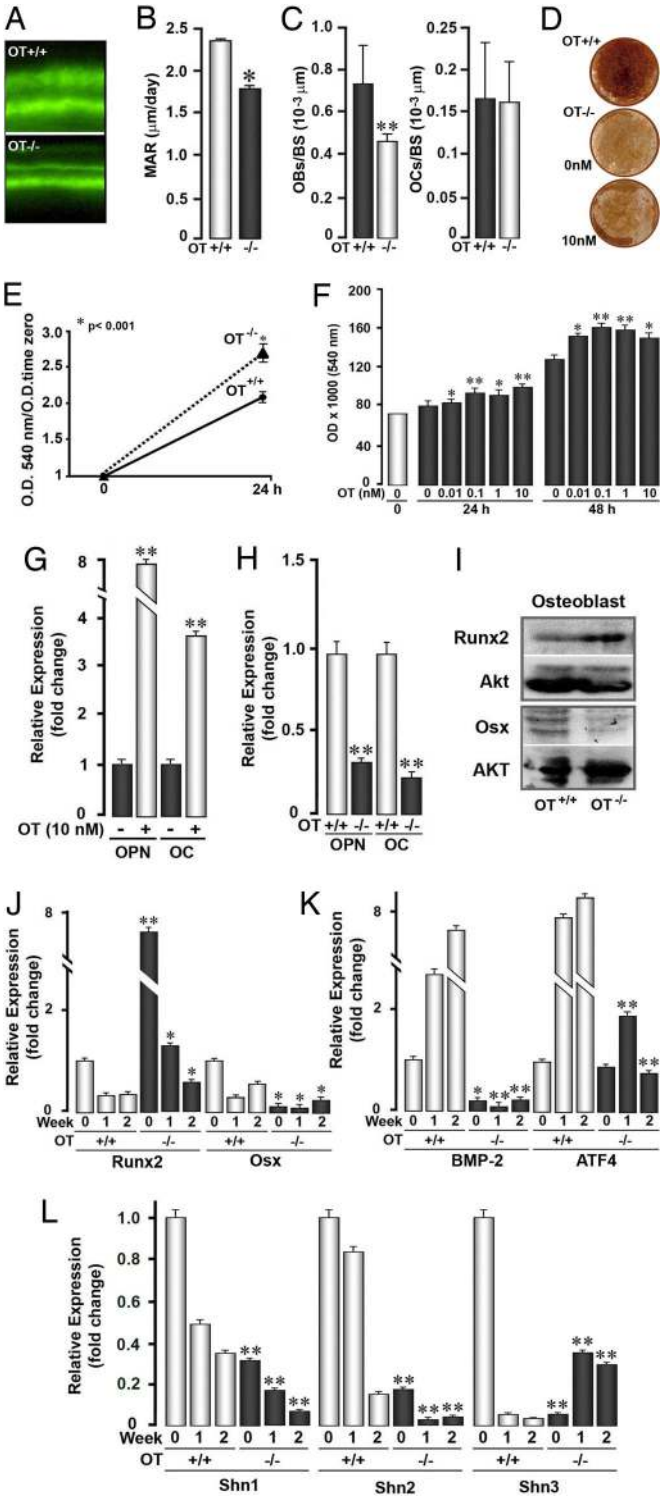

Fig. 2. OT stimulates osteoblastic bone formation. OT deficiency reduced MAR, an index of bone formation in calcein-labeled calvaria from 7-week-old mice (*A* and *B*), as well as mineralization (*C*) and proliferation (*D*) in ex vivo bone marrow cell cultures, evident on von Kossa staining and the MTT assay, respectively. OT (10 nM) partly rescued the ex vivo mineralization defect (*C*). Statistics: Student *t* test comparing $OT^{-/-}$ with wild-type mice; $n = 4$ mice per group; *, $P < 0.05$. In contrast, OT (at stated doses) stimulated the proliferation (*E*) and differentiation (*F*) of wild-type murine osteoblast precursors in the MTT assay and qPCR for the osteoblast markers OPN and OC, respectively. OC and OPN mRNA was expectedly low in ex vivo murine $OT^{-/-}$ osteoblast cultures compared with wild-type cultures (*G*). This result was associated with reduced Osx protein [*H*; Western blot analysis, at time 0 (confluence)] and mRNA [*I*; qPCR, at time 0 (confluence) and at 1 and 2 weeks postdifferentiation induction], but with elevated Runx2 protein and mRNA in $OT^{-/-}$ osteoblasts. Likewise, BMP-2 and ATF4 (*J*), as well as Schnurri (Shn) isoforms 1, 2, and 3 (*K* and *L*), were reduced dramatically at 0 weeks in $OT^{-/-}$ osteoblasts compared with wild-type controls, with the exception of Shn3 at 1 and 2 weeks. Statistics: Student *t* test comparing $OT^{-/-}$ with wild-type mice at every time point; *, $P < 0.05$; **, $P < 0.01$ (in triplicate).

protein was decreased in $OT^{-/-}$ cells (Fig. 2*H*). Enhanced *Runx2* and reduced *Osx* mRNA expression were confirmed by quantitative PCR (qPCR) in osteoblasts that were cultured to confluence for 2 weeks and then allowed to differentiate (Fig. 2*I*). The pattern of *Runx2* expression was similar in wild-type and $OT^{-/-}$ mice, with both exhibiting decreased levels as cells mature. But whereas during differentiation, wild-type cells displayed decreases in *Osx* mRNA, $OT^{-/-}$ osteoblasts exhibited persistently low basal *Osx* levels.

We next measured *BMP-2* expression after 1 and 2 weeks of culture, and a lack of increase in $OT^{-/-}$ mice (Fig. 2*J*). The transcription factors of the ZAS family of zinc-finger proteins, Schnurri (Shn) 1–3, lie downstream of BMP-2 (16). Shn3 is known to physically associate with Runx2 to promote its degradation; thus, its absence in mice is known to result in retained Runx2 protein and increased bone formation (17). Therefore, we reasoned that the down-regulation of *Shn3* in $OT^{-/-}$ cells might increase *Runx2* protein levels by preventing degradation; this result indeed was the case (Fig. 2 *H* and *K*).

In contrast to *Shn3* deficiency, *Shn2* deficiency in mice is known to reduce osteoblastic bone formation by suppressing *Osx* and *ATF4* expression and terminal mineralization (18). Thus, we speculated that if *Shn2* also is reduced in parallel with *Shn3*, then *Osx* and *ATF4* expression will both decline, even in the face of elevated Runx2 protein expression. Fig. 2*K* shows that *Shn2* expression was reduced in parallel with *Shn3* expression in $OT^{-/-}$ cells, providing a single molecular explanation for the parallel elevations in *Runx2* and reductions in *Osx* and *ATF4* compared with wild-type littermates (Fig. 2 *I* and *J*). The latter reductions were consistent with a defect in mineralization seen in $OT^{-/-}$ cultures (Fig. 2*C*); however, the increase in *Runx2* mRNA expression (Fig. 2*I*) in $OT^{-/-}$ osteoblasts cannot be explained by reduced *Shn3*, because Shn3 participates in protein degradation, not in the modulation of mRNA expression. Finally, *Shn1* expression also was reduced in $OT^{-/-}$ cells (Fig. 2*K*); however, the effects of *Shn1* on the skeleton remain unknown in the absence of a *Shn1* null mouse (16).

Because of coupling between osteoblastic bone formation and osteoclastic bone resorption, enhanced osteoblastogenesis usually is accompanied by increased osteoclastogenesis and vice versa. To explore whether an osteoclastic defect accompanies the reduced bone formation, we incubated osteoclast precursors from $OT^{-/-}$ mice for 12 days with the pro-osteoclastogenic cytokines M-CSF and RANK-L. We found significant reductions in TRAP-positive osteoclast formation (Fig. 3*A*), precursor proliferation (Fig. 3*B*), and the expression of *c-fms*, *RANK*, and downstream NF-κB subunits and inhibitors (Fig. 3*C*). Expectedly, recombinant OT caused increased TRAP-positive osteoclast formation through the enhancement of both proliferation and differentiation (Fig. 3 *D–F*).

The attenuated osteoclast formation seen in $OT^{-/-}$ mice could arise from decreased RANK-L production, increased osteoprotegerin (OPG) production, or reduced osteoclast precursor numbers. To explore the possibility that RANK-L and/or OPG contributed to the decreased osteoclastogenesis in $OT^{-/-}$ mice, we measured RANK and OPG in bone marrow stromal cells. We found low RANK-L and, surprisingly, low OPG mRNA in $OT^{-/-}$ osteoblasts (Fig. 3*G*). But when OT was applied to wild-type marrow stromal cells, we found an increase in

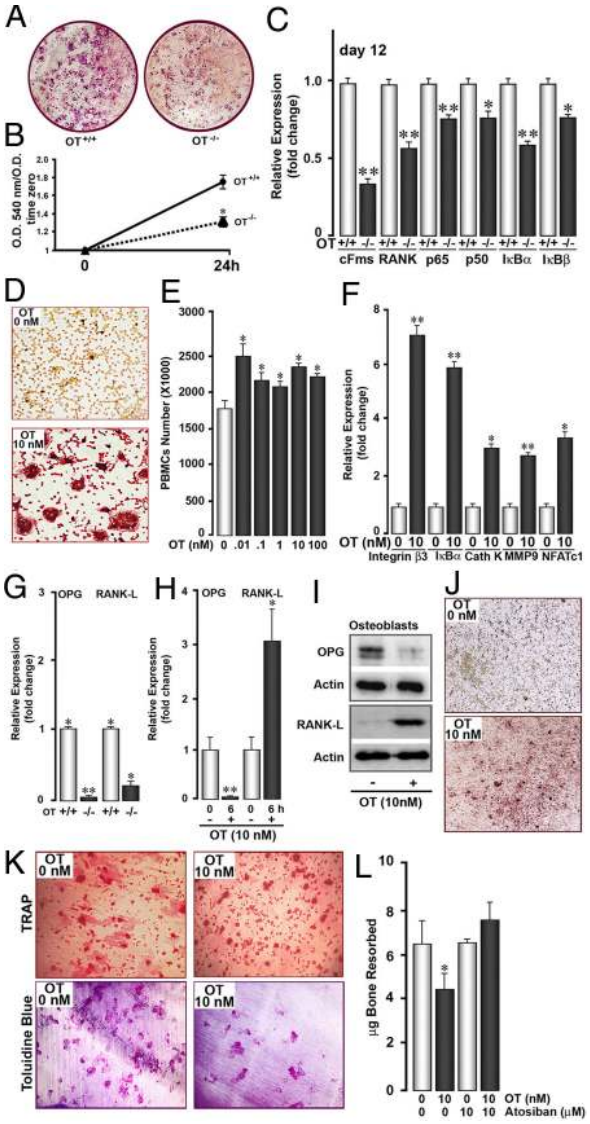

Fig. 3. OT acutely inhibits resorption in the face of increased osteoclastogenesis. OT deficiency reduced TRAP-positive osteoclast formation at day 6 (*A*), as well as precursor proliferation (*B*; MTT assay) and markers of osteoclast differentiation (*C*; as shown, qPCR) at day 12 of ex vivo murine bone marrow cell culture. As expected, recombinant OT (≤100 nM) stimulated TRAP-positive osteoclasts (*D*) and enhanced both precursor proliferation [*E*; human peripheral blood mononuclear cells (PBMCs), MTT assay] and differentiation, as evidenced by elevated osteoclast marker mRNA (*F*; as stated, qPCR). Reciprocal effects of OT deficiency (*G*) and recombinant OT (*H*) on RANK-L and OPG mRNA (qPCR) and protein (*I*; Western blot analysis) were seen in osteoblasts. RANK-L expression was stimulated and OPG expression was inhibited by OT, suggesting that OT-induced osteoclastogenesis may be exerted in part through an altered RANK-L/OPG ratio. We explored the relevance of enhanced RANK-L through a transwell coculture experiment, in which osteoclast precursors were on the filter and osteoblasts were on the plate. In the absence of added RANK-L (i.e., with M-CSF alone; 50 ng/mL), TRAP-positive osteoclasts were formed (*J*) only in the presence of added OT, suggesting the release of diffusible soluble RANK-L in response to OT. Whereas osteoclastogenesis was stimulated by OT in precursors plated on dentine (*K*, *Upper*), bone resorption, as assessed by toluidine-blue staining for pits, was sharply reduced within 48 h (*K*, *Lower*). The acute inhibition of resorption was consistent with a ≈30% decline in the resorption of ^{3}H-proline-labeled bone fragments, which was reversed by a specific OT antagonist, atosiban (*L*; doses as stated), as well as with a reduction in supernatant cross-laps (see Fig. 4*I*). Statistics: Student *t* test comparing $OT^{-/-}$ with wild-type mice or vehicle (zero dose) with OT (various doses); qPCR and MTT in triplicate; bone resorption, 4 slices per treatment (representative); ^{3}H-proline resorption assay in quadruplicate; cultures in triplicate (representative); *, $P < 0.05$; **, $P < 0.01$.

RANK-L and, as expected, decreases in OPG mRNA and protein (Fig. 3 *H* and *I*).

To examine the functional significance of increased RANK-L production in OT-induced osteoclastogenesis, we cocultured osteoblasts and preosteoclasts in a transwell plate. The bottom of the plate contained calvarial osteoblasts, and the filter membrane was loaded with bone marrow osteoclast precursors. The cultures were maintained in the presence of M-CSF alone, without RANK-L, and with or without OT for 12 days, after which the number of osteoclasts was determined. If OT does in fact increase RANK-L production, then soluble RANK-L would diffuse through the transwell membrane, thereby stimulating osteoclast formation in the absence of added RANK-L. Fig. 3*J* shows substantial osteoclast formation in OT-treated wells and, as expected, a lack of osteoclastogenesis in control wells. Together, these results provide compelling evidence that OT favors osteoclastogenesis by increasing soluble RANK-L and decreasing OPG, suggesting both direct and indirect actions of OT on osteoclastogenesis.

Whereas OT triggers osteoclastogenesis, published data indicate that when administered acutely, OT decreases serum calcium in rats (19). Because this action is mimicked by calcitonin (20), we speculated that despite increasing osteoclast formation, OT might inhibit the resorptive function of mature osteoclasts. Consequently, we explored the ability of osteoclasts plated on dentine substrate to form resorption "pits." Although TRAP-positive osteoclasts increased with OT (Fig. 3*K Upper*), as expected, toluidine-blue-positive pits were diminished (Fig. 3*K Lower*). We further confirmed this antiresorptive action by incubating tritiated proline-labeled bone particles with mature osteoclasts for 48 h and separately, and measuring supernatant levels of cross-laps (a resorption marker) in osteoclast–dentine cultures. A ≈30% reduction in bone resorption was found with OT (Figs. 3*L* and 4*I*). The inhibition was fully reversed by atosiban, a specific OTR antagonist, confirming OT specificity (Fig. 3*L*).

We examined the specific pathways triggered by OT in osteoclasts, focusing on MAPK, NF-κB, and Ca^{2+} signaling. OT elicited rapid phosphorylation of Erk1/2 within 5 min and of Iκ-B and Akt within 15 min in osteoclast precursors (Fig. 4 *A–C*). Activation of all 3 osteoclastogenic pathways by OT likely underlies OT's stimulatory action on osteoclastogenesis and is reminiscent of pro-osteoclastogenic signals triggered by FSH (21).

To gain insight into the mechanism underlying OT-induced inhibition of bone resorption, we examined the effect of OT on Ca^{2+} signaling, because an elevated intracellular Ca^{2+} level is invariably associated with diminished osteoclast function (22). We performed single-cell measurements of cytosolic Ca^{2+} in fura-2-loaded mature osteoclasts. To separate cytosolic Ca^{2+} release from extracellular Ca^{2+} influx, we used thapsigargin and EGTA, respectively (23). OT triggered rapid elevations in cytosolic Ca^{2+}, which were abolished by depleting intracellular Ca^{2+} stores with thapsigargin (Fig. 4*D Middle*). In contrast, the chelation of extracellular Ca^{2+} by EGTA did not alter the Ca^{2+} signal (Fig. 4*D Bottom*). Our findings attribute OT-induced Ca^{2+} signals to intracellular Ca^{2+} release rather than to Ca^{2+} influx.

Ca^{2+} signaling increases NO production as a mechanism to inhibit bone resorption (24, 25). Thus, we measured the expression and function of the Ca^{2+}-sensitive NOS isoform eNOS, as well as, more directly, NO production in mature osteoclasts. As expected, OT stimulated eNOS mRNA and protein expression at 6 h (Fig. 4 *E* and *F*). In addition, OT triggered a time-dependent increase in the production of nitrite, a surrogate for NO (Fig. 4 *G* and *H*). Importantly, whereas OT induced the reduction of supernatant cross-laps, this effect was fully reversed by the NOS inhibitor L-NAME (Fig. 4*I*), providing evidence of a role of NO in OT-induced resorption inhibition.

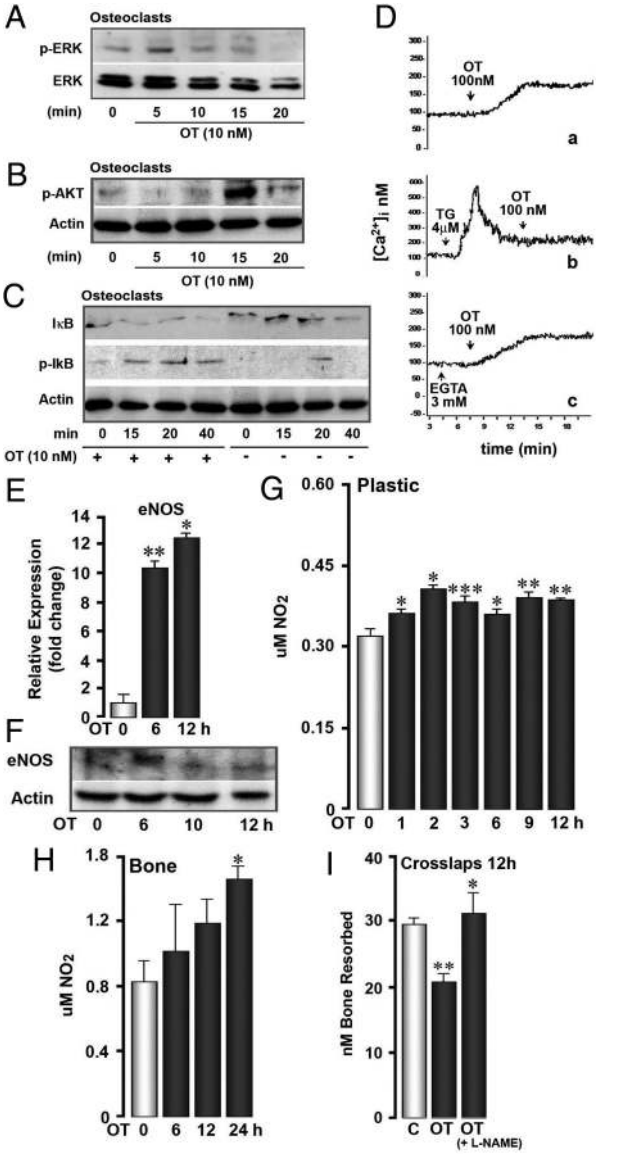

Fig. 4. OT triggers MAP kinase and NF-κB activation and Ca^{2+} release in osteoclasts. In Western blot analyses by using whole-cell lysates of osteoclasts, OT (10 nM) stimulated the phosphorylation of Erk within 5 min (*A*) and that of Akt (*B*) and IκBα (*C*) within 15 min. The respective loading controls were total Erk, total IκBα, and actin, all of which are pro-osteoclastogenic signals (21). In addition, cytosolic Ca^{2+} concentration (nM) was measured in single, isolated, fura-2-loaded, mature human osteoclasts treated with OT (10 nM) in the presence or absence of thapsigargin (4 μM) to block Ca^{2+} uptake or EGTA (3 mM) to chelate extracelular Ca^{2+}. (*D*) The rise of cytosolic Ca^{2+} (a, *Top*) was inhibited by thapsigargin (b, *Middle*) but not by extracellular Ca^{2+} chelation (c, *Bottom*), indicating that the OT-induced Ca^{2+} transient is attributed mainly to the release of intracellularly stored Ca^{2+}. OT also stimulated expression of the Ca^{2+}-sensitive isoform of NOS (eNOS) in a time-dependent manner, as shown by qPCR (*E*) and Western blot analysis (*F*; actin as loading control). The time course of the elevated eNOS expression was consistent with the time course of the increase in nitrite (NO_2) production when osteoclasts were cultured on plastic (*G*) or on bone (*H*). The functional role of NO in OT-induced resorption inhibition was assessed by using a specific eNOS blocker, L-NAME (*I*). Statistics: Student *t* test comparing zero dose with OT (various doses); qPCR in triplicate; cytosolic Ca^{2+} (representative); NO_2 and cross-lap assay in quadruplicate; *, $P < 0.05$; **, $P < 0.01$.

In summary, OT increases osteoblastic bone formation, in decrements that likely account for the osteopenic phenotype of OT deficiency, with no discernable alterations in osteoclast numbers seen on histomorphometry (Fig. 2*B*). The latter finding is surprising, because OT stimulates osteoclastogenesis acutely but inhibits the resorptive activity of mature osteoclasts ex vivo. From this finding, we would expect decreasing numbers of osteoclast in $OT^{-/-}$ mice, particularly because osteoclastogenesis is diminished in ex vivo $OT^{-/-}$ cultures (Fig. 3*A*). That this expectation was not the case is likely due to redundancy arising in vivo from paracrine and autocrine mechanisms that keep osteoclast numbers constant despite chronic OT deficiency. Nonetheless, the inhibitory effect of OT on mature cells may in fact serve as a checkpoint for unrestricted bone resorption that otherwise may ensue from the stimulation of osteoclastogenesis. In addition, OT could be used as an anabolic stimulus in vivo to restore the maternal skeletal loss occurring during pregnancy and lactation. This anabolic action has been documented in both wild-type mice (Fig. S1) and ovariectomized mice (26).

Discussion

Our findings regarding the positive effects of OT on osteoblasts, together with the bone formation defect of OT and Oxtr deficiency, suggest that OT is of fundamental importance to skeletal physiology in both sexes. The finding that haploinsufficiency induces a bone phenotype without affecting lactation, hitherto considered the primary action of OT, suggests that bone is more sensitive to OT compared with the breast. Likewise, we found that haploinsufficiency of another pituitary hormone, FSH, increased bone mass without affecting the ovaries, the primary target organ of FSH (21). Moreover, haploinsufficiency of the receptor for the pituitary hormone TSH similarly caused a reduction in bone mass without affecting thyroid development or function (27). Thus, it seems that the newly identified pituitary–skeletal axis has a physiological sensitivity that surpasses, and is distinct from, that of the traditional pituitary–endocrine axis.

Indeed, pituitary hormone receptors have evolved from ancient chemical receptors widely distributed in evolution (28). TSH and FSH receptors, for example, date before the separation of vertebral ancestors from primitive animals. OT and the closely related vasopressin receptors are even more ancient, having emerged to regulate ion homeostasis in a sex-independent manner. Considering that the teleost skeleton predates the appearance of the breast, thyroid, and ovary, it would not be surprising if the hitherto unrecognized but exquisite sensitivity of the skeleton to pituitary hormones would exceed that of their respective target endocrine organs.

We speculate that in addition to maintaining bone mass in both sexes, OT might regulate maternal skeletal homeostasis during pregnancy and lactation, during which OT level rises (1, 2). The fetal skeleton is unlikely to be mineralized effectively in the absence of calcium mobilized from the maternal skeleton (29). Likewise, without adequate replenishment, the mother's skeleton would waste. We hypothesize that elevated OT levels during pregnancy and lactation not only enhance bone resorption by increasing the number of osteoclasts to make maternal calcium available to the fetus, but also prevent unrestricted bone removal by inhibiting the activity of mature osteoclasts and, in parallel, replenish the lost skeleton by enhancing osteoblastic bone formation.

Furthermore, like OT, parathyroid hormone-related protein (PTHrP), another breast-specific peptide, displays both proresorptive and anabolic actions. PTHrP secreted from the breast causes, at least in part, the bone loss that accompanies pregnancy and lactation (30). Thus, in mammary tissue–specific knockout mice, a reduction in serum PTHrP is associated with decreases in serum markers of resorption (31). Another primitive neuropeptide, calcitonin, also increases during both pregnancy and lactation, likely to counteract the stimulatory effects of OT (32). Finally, it also is possible that PTHrP and OT may interact with lower estrogen levels to cause the acute and rapid maternal bone

loss required for fetal skeletal morphogenesis and postnatal skeletal growth (33). If in certain instances, the lost bone is not replaced adequately, then severe osteoporosis ensues (34).

In conclusion, the presence of Oxtr on bone cells, the lack of evidence of a central neural mechanism for OT's skeletal action, and OT's anabolic effects all attest to a direct action of circulating OT on bone. The findings suggest that, like intermittent PTH, OT or another bone-active analog could be used as an anabolic therapy for postmenopausal osteoporosis. Indeed, we recently showed that TSH injected i.p. once every 2 weeks restored ovariectomy-induced bone loss (35). Further studies with OT should undoubtedly explore a truly anabolic human dosing schedule.

Materials and Methods

Skeletal Phenotyping. The generation of mice lacking OT or Oxtr on a C57BL/6 × 129SvEv background has been reported previously (3, 4). Histomorphometry and μ-CT were carried out as described previously (21, 35). Protocols were approved by the Institutional Animal Care and Use Committees of the Faculty of Medicine, University of Bari and Mount Sinai School of Medicine.

Cytosolic Free-Ca^{2+} Measurement. Cytosolic free-calcium concentration ($[Ca^{2+}]_i$) was measured in single human osteoclasts loaded with the intracellular Ca^{2+} indicator fura-2 (Sigma Aldrich).

Nitrite Production. NO was determined with a colorimetric NO assay kit (Fluka). In this assay, nitrate, resulting from autoxidation of NO from osteoclast culture media, is converted to nitrite by nitrate reductase (0.0005 IU) and NADH (0.2 mM). The concentration of nitrite is measured as a NO-stable end-product.

Statistics. Results are given as mean ± SEM. Statistical analyses were performed by using Student *t* tests (with Microsoft Excel) for significant differences at $P < 0.05$.

For additional details, see *SI Methods*.

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