

Osteoblast regulation via ligand-activated nuclear trafficking of the oxytocin receptor

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We report that oxytocin (Oxt) receptors (Oxtrs), on stimulation by the ligand Oxt, translocate into the nucleus of osteoblasts, implicating this process in the action of Oxt on osteoblast maturation. Sequential immunocytochemistry of intact cells or isolated nucleoplasts stripped of the outer nuclear membrane showed progressive nuclear localization of the Oxtr; this nuclear translocation was confirmed by monitoring the movement of Oxtr-EGFP as well as by immunogold labeling. Nuclear Oxtr localization was conclusively shown by Western immunoblotting and MS of nuclear lysate proteins. We found that the passage of Oxtrs into the nucleus was facilitated by successive interactions with β -arrestins (Arrbs), the small GTPase Rab5, importin- β (Kpnb1), and transportin-1 (Tnpo1). siRNA-mediated knockdown of *Arrb1*, *Arrb2*, or *Tnpo1* abrogated Oxt-induced expression of the osteoblast differentiation genes *osterix* (*Sp7*), *Atf4*, *bone sialoprotein* (*Ibsp*), and *osteocalcin* (*Bglap*) without affecting Erk phosphorylation. Likewise and again, without affecting pErk, inhibiting Arrb recruitment by mutating Ser rich clusters of the nuclear localization signal to Ala abolished nuclear import and Oxtr-induced gene expression. These studies define a previously unidentified mechanism for Oxtr action on bone and open possibilities for direct transcriptional modulation by nuclear G protein-coupled receptors.

nuclear translocation | G protein-coupled receptor | transcriptional regulation

The oxytocin (Oxt) receptor (Oxtr), a member of the rhodopsin-type (class I) family of G protein-coupled receptors (GPCRs), responds to the neurohypophyseal hormone Oxt to stimulate lactation and social bonding (1, 2). More recently, we and others have shown that Oxt also stimulates bone formation directly by interacting with an osteoblastic Oxtr (3, 4). The genetic deletion of Oxt or Oxtr in mice thus decreases osteoblast differentiation and bone formation, causing osteopenia (5, 6). We believe that this action supports maternal skeletal remineralization after the intergenerational transfer of calcium during pregnancy and lactation (5). However, the precise mechanism through which Oxtr activation translates into increased bone formation remains unclear.

Oxt binding to Oxtrs is known to activate both G protein $G_{\alpha i}$ and $G_{\alpha q/11}$ -mediated phospholipase C pathways (7). However, in common with other GPCRs, prolonged or repetitive agonist stimulation by Oxt results in Oxtr internalization through β -arrestin (Arrb). GPCR internalization can activate signaling pathways quite distinct from those activated by the same receptors resident at the cell surface. Hence, desensitization of primary G protein-dependent signaling can be followed by a second wave of Arrb-mediated signaling (8). β -Arrestins can also recruit signaling proteins that connect GPCRs to various cytoplasmic effector pathways, such as mitogen-activated protein kinase (MAPK) and protein kinase B/phosphatidylinositol-4,5-

bisphosphate 3-kinase (Akt/PI3K) (9, 10). Such mechanisms can elicit delayed genomic responses.

After being internalized, GPCRs are either recycled back to the plasma membrane to resensitize the cell to ligand action or transported to lysosomes for degradation. Although G proteins have been found in the Golgi body, endoplasmic reticulum, and cytoskeleton (11–13), GPCRs can localize to the nucleus or nuclear membrane (14–16). For example, on treatment with sphingosine-1-phosphate (S1P), the S1P receptor subtype 1 can translocate to endothelial cell nuclei to activate transcription (17). Likewise, chemokine receptor 2 (CCR2), expressed in monocyte, has been shown to interact directly with transportin-1 (Tnpo1) to undergo nuclear localization (18). Cysteine (C)-X-C Receptor 4 (CXCR4) similarly responds to stromal cell-derived factor-1 α (SDF-1 α) to undergo internalization and Tnpo1-mediated nuclear targeting in prostate cancer cells (19).

Human osteosarcoma and breast cancer cell lines display both cell membrane and nuclear Oxtrs (20). However, the functional significance of the nuclear localization of the Oxtr or indeed, any GPCR continues to unravel. Here, we have combined proteomic, molecular, and cell biological approaches to confirm the translocation of the Oxtr to the nucleus in osteoblasts after Oxt binding and to study the putative function of this pathway in Oxt-induced bone formation. We find that Oxtr nuclear localization is Arrb- and Tnpo1-dependent. Thus, siRNA knockdown of Arrb or Tnpo1 inhibits not only nuclear localization but also, the proosteoblastic action of Oxt, interestingly without affecting Erk phosphorylation. These findings represent a hitherto unrecognized pathway for Oxt action.

Significance

We have shown previously that oxytocin (Oxt), other than regulating lactation and social bonding, is a potent stimulator of bone formation by the osteoblast. Here, we present evidence that this action is exerted through the nuclear localization of the Oxt receptor (Oxtr). Our findings prompt additional studies into the contribution of nuclear Oxtr signaling in regulating lactation and social bonding.

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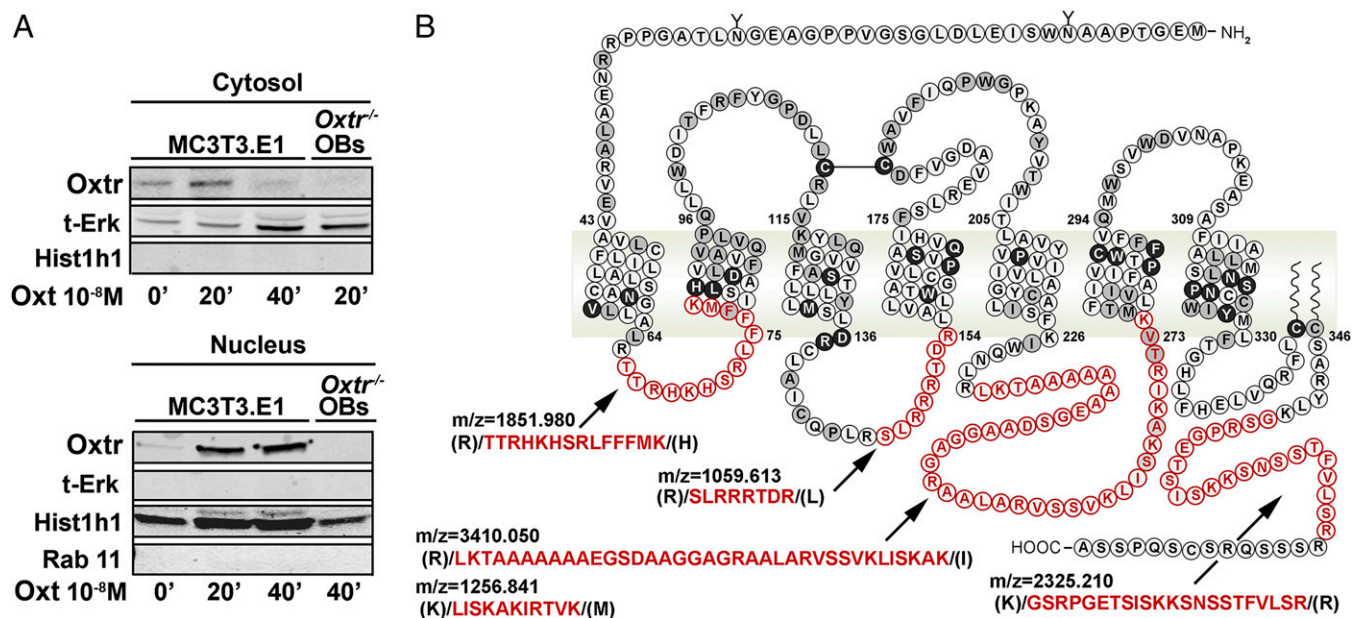


Fig. 2. Biochemical and proteomic detection of OxtRs in osteoblast nuclei. MC3T3.E1 osteoblasts and *OxtR*^{-/-} primary osteoblasts (OBs) (negative control) were treated with Oxt (10⁻⁸ M) for 20 and 40 min. (A) Western blots show OxtRs in the cytosolic and nuclear fractions at 40 min. Markers included t-Erk (cytosol), Rab11 (perinuclear), and Hist1h1 (nuclear). Proteins in the immunoprecipitate (with anti-OxtR antibody) were identified using MS-Fit software (<http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msfitstandard>), and all other proteins were recognized as nuclear proteins. (B) Analysis of the spectra (FindPept database) revealed five peptides corresponding to OxtR intracellular loops in the in-gel band (38 kDa) at *m/z* of 1,851.98 [(R)/TTRHKHSRLFFMK/(H); residues 66–79], 1,059.61 [(R)/SLRRRTDR/(L); residues 146–153], 3,410.05 [(R)/LKTAAAAAAEGSDAAGGAGRAALARVSSVKLISKAK/(I); residues 232–268], 1,256.84 [(K)/LISKAKIRTVK/(M); residues 263–273], and 2,325.21 [(K)/GSRPGETSISKNSNSTFVLSR/(R); residues 353–374]. ', minute.

of the OxtR-EGFP fusion protein was verified by examining its binding to an Oxt analog conjugated with Alexa-546 and the subsequent translocation of the ligand-receptor complex into the cytoplasm (Fig. 3A and *SI Materials and Methods*). The time-lapse confocal microphotographs show that the fluorescent agonist first appeared diffuse in the medium and eventually, colocalized with the OxtR-EGFP on the membrane surface, giving rise to a yellow vesicles. Within 2 min, these vesicles moved into cells, indicating internalization of ligand-receptor complex; 15 min after treatment, almost all of OxtR-EGFP was sequestered in cytoplasmic vesicles. This experiment established that the OxtR-EGFP fusion protein was functionally responsive to agonist stimulation and trafficked intracellularly (Fig. 3A).

Internalization and inactivation of GPCRs are classically mediated by the protein Arrb. After agonist addition, Arrb is recruited from the cytosol to the plasma membrane, where it binds to the OxtR (21). *OxtR*^{-/-} osteoblasts stably transfected with *OxtR-EGFP*, when stimulated with Oxt, displayed a colocalization with arrb1/2 in proximity to the plasma membrane within 2–3 min (Fig. 3B). No colocalization was observed at later time points, confirming rapid receptor endocytosis. Similar results were obtained in WT osteoblasts, where the endogenous OxtR was labeled with an anti-OxtR antibody.

It is known that, after receptor endocytosis, Arrb rapidly detaches from the vesicle, which then associates with Rab proteins; the latter proteins are small GTPases that regulate intracellular trafficking of several GPCRs (22). We investigated OxtR interaction with Rab5, a GTPase specifically involved in endosome sorting of clathrin-coated vesicles. We noted that the OxtR-EGFP fusion protein colocalized with Rab5 in ~5 min (Fig. 3C). Importantly, no such interaction was observed between OxtR-EGFP and Rab7, indicating that the receptor complex was not sorted toward degradation. This observation is not inconsistent with OxtR-Rab7 interactions noted in cells that do not normally express the OxtR (23).

Nuclear transport of proteins and RNA occurs through the nuclear pore complex and is mediated by a superfamily of transport receptors known collectively as karyopherins or importins (24). Karyopherins bind to their cargoes by recognizing specific nuclear localization signals or nuclear export signals. Transport through the nuclear pore complex is facilitated by transient interactions between the karyopherins and the nuclear pore complex. Of these molecules, Tnp1 escorts proteins into the nucleus through interactions with a nuclear localization signal, which is composed of short stretches of basic amino acids (25). We studied the subcellular distribution of Tnp1 and the OxtR-EGFP in *Oxt*^{-/-} osteoblasts; use of the latter cells avoids receptor activation by cell-secreted Oxt. Without Oxt stimulation, OxtR and Tnp1 were found to not colocalize. However, significant colocalization was noted beginning 5 min after exogenous Oxt (10⁻⁸ M). This cytosolic colocalization was in vesicle-like structures that eventually were found to be clustered in the perinuclear region. Dramatically, at 20 min post-Oxt, whereas Tnp1 remained cytosolic, the OxtR-EGFP fusion protein was almost exclusively nuclear. These experiments clearly indicate Tnp1 as a primary carrier for OxtR nuclear transport.

We examined whether various steps of OxtR internalization were required for the anabolic action of Oxt. For this experiment, primary mouse calvarial osteoblasts were transfected with *arrb1*, *arrb2*, or *Tnp1* siRNA (50 nM). The silenced cells were then stimulated with Oxt (10⁻⁸ M) and lysed for RNA and proteins extraction. Of note was that the stimulation of Erk remained unchanged on *Arrb1* or *Arrb2* knockdown, which is different from that noted by others in murine uterine myocytes (26). However, the Oxt-induced induction of osteoblast differentiation genes, namely *osterix* (*Sp7*), *Atf4*, *bone sialoprotein* (*Ibsp*), and *osteocalcin* (*Bglap*), was dramatically suppressed in *Arrb1/2* siRNA-transfected cells at 6 h (Fig. 4A). This result suggested that the effects of Oxt on osteoblast differentiation were Arrb1/2-dependent but independent of Erk activation.

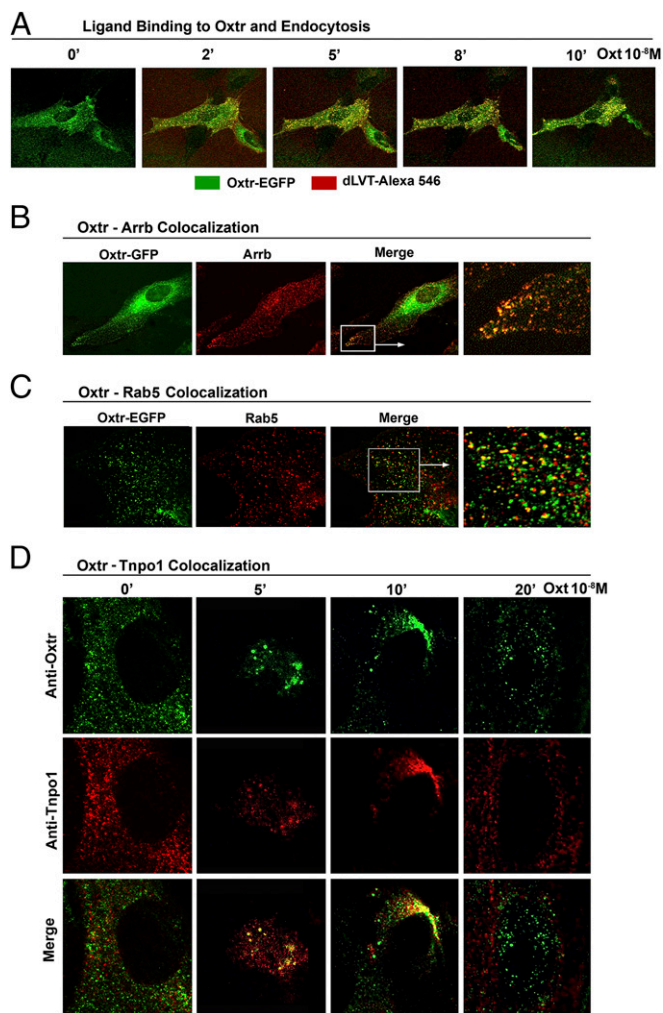


Fig. 3. Oxt intracellular trafficking mediated by Arrb, Rab5, and Tnpo1. (A) Shown are time-lapse confocal micrographs of primary *Oxtr*^{-/-} osteoblasts transfected with *Oxtr*-EGFP, which appeared at the cell surface and in the cytosol (Movie S1). When a red fluorescent agonist of Oxt (dLVT-Alexa-546) was added, diffuse fluorescence was detectable in the medium. On agonist binding to Oxt on the membrane surface, yellow vesicles were visible in the cells within 2 min, indicating the internalization of dLVT-Alexa-546-bound *Oxtr*-EGFP. At 10 min, all of *Oxtr*-EGFP (green) was (A) sequestered into cytoplasmic vesicles and (B) colocalized with Arrb1/2 (red) within 2–3 min in proximity of the plasma membrane. (C) Oxt also colocalized with Rab5 (red) 5 min after Oxt. Stimulation by Oxt for 5, 10, or 20 min resulted in colocalization of Oxt (green) and Tnpo1 (red). In the absence of stimulation, Oxt and Tnpo1 appeared apart, but within 5 min, they localized in vesicle-like structures and at 10 min, became crowded in proximity of the nucleus. (D) At 20 min, Oxt and Tnpo1 again appeared separated, with Oxt accumulating in the nuclear compartment and Tnpo1 accumulating in the cytoplasm. ', minute.

In complementary experiments, rather than inhibiting Arrb expression, we prevented its recruitment by the Oxt. The Oxt has three Ser clusters in the C-terminal tail that are targets for phosphorylation by GPCR kinases, an event that recruits Arrb. Mutations that prevent Ser phosphorylation, thus, inhibit Oxt–Arrb interactions and receptor endocytosis (21). We generated an Oxt mutant, in which two of three Ser clusters were mutated to Ala residues (*Oxtr*^{mut}). *Oxtr*^{-/-} osteoblasts were then transfected with WT or mutated *Oxtr*. Fig. 4B shows that the *Oxtr*^{mut} is partially retained at the plasma membrane after Oxt stimulation, indicating the dependence of receptor internalization on Ser phosphorylation and Arrb recruitment. This effect was also independent on Erk phosphorylation, which remained unchanged

in *Oxtr*^{mut}-transfected osteoblasts compared with cells transfected with WT *Oxtr*. Importantly, however, transfection with *Oxtr*^{mut} inhibited the induction of osteoblast gene expression by Oxt. This experiment together with the *Arrb1/2* siRNA data (Fig. 4A) establish that Oxt internalization followed by Arrb recruitment is required for Oxt-induced osteoblast differentiation.

Having established Tnpo1 as a primary carrier for the nuclear transport of Oxts, we evaluated the significance of this interaction in Oxt-induced osteoblast differentiation. Attenuated osteoblastic gene expression was noted in cells transfected with *Tnpo1* siRNA at 6- and/or 12-h time points (Fig. 4C). Using the nuclear subfraction of MC3T3.E1 cells, we found no Oxt localization in the *Tnpo1* siRNA transfectants, compared with those transfected with scrambled siRNA (48 h) (Fig. 4C). *Oxtr*^{-/-} osteoblasts expectedly showed no nuclear Oxt localization. The data indicate that association with Tnpo1 is necessary for Oxt nuclear localization and that this process is required for Oxt-induced osteoblast differentiation.

Discussion

We have shown previously that osteoblasts express abundant plasma membrane Oxts that drive cell differentiation to a mature, mineralizing phenotype in response to pituitary- and bone marrow-derived Oxt (4, 6). However, the mechanism of this effect has remained uncertain. Here, we show that the skeletal action of Oxt is mediated by and dependent on the internalization of Oxts and their subsequent translocation to the nucleus. Nuclear localization of the Oxt on ligand binding has previously been reported in breast cancer cells and primary fibroblasts (20). In osteosarcoma cells, however, the process is constitutively active (20). Our data support these original observations but in addition, establish the precise mechanism of nuclear import. Notably, we find that, after Arrb-mediated internalization, Oxts interact with Rab5 and then binds to the karyopherin Tnpo1, which facilitates nuclear transport. Knockdown of either *Arrb* or *Tnpo1* or the inhibition of Arrb recruitment abrogates the action of Oxt on gene expression.

The effects of Oxt on osteoblast differentiation, exerted through its nuclear translocation, seem independent of its effects on activating the MAPK pathway. Notably, despite knocking down Arrb isoforms, which abrogate the effects of Oxt on gene expression, the pErk signal persists. Likewise, blocking the recruitment of Arrb by mutating Oxts, which prevents their internalization, fails to inhibit Oxt-induced Erk phosphorylation. In osteoblasts, therefore, the rapid Erk phosphorylation seems separate from relatively protracted effects of internalized Oxts on gene expression. The Erk response could arise from an interaction of Oxt with the vasopressin receptor Avpr1, the activation of which, as we have shown, also causes Erk phosphorylation (27). However, this effect is in contrast to uterine myocytes, wherein pErk is reduced in mice lacking either Arrb1 or Arrb2 (26). The intactness of the pErk response in osteoblasts could be explained by the known activation of MAPKs by G_{αi} and G_{αq} (28); this actin could account for the positive effect of Oxt on osteoblast viability that we have shown earlier (4).

Our data are fully consistent with growing evidence of the presence of GPCRs within the nucleus (14, 15). In fact, molecules that have traditionally been considered part of the plasma membrane and cytosol have also been widely described in the nucleus. Nuclear localization has been documented for the CCR2, VPAC1, S1P1, and CXCR4 receptors, which in certain cases, are mediated by Tnpo1 (17–19). In contrast, GnRHR, mGluR5, and βARs have been found in the nuclear membrane. Importantly, these GPCRs have been shown to be signaling-efficient (29–31). The activation of GnRHR, for example, induces Hist1h3 acetylation and phosphorylation, whereas mGluR5 activation triggers nuclear Ca²⁺ signals and CREB phosphorylation (29, 30). Likewise, G proteins have been found in the nucleus.

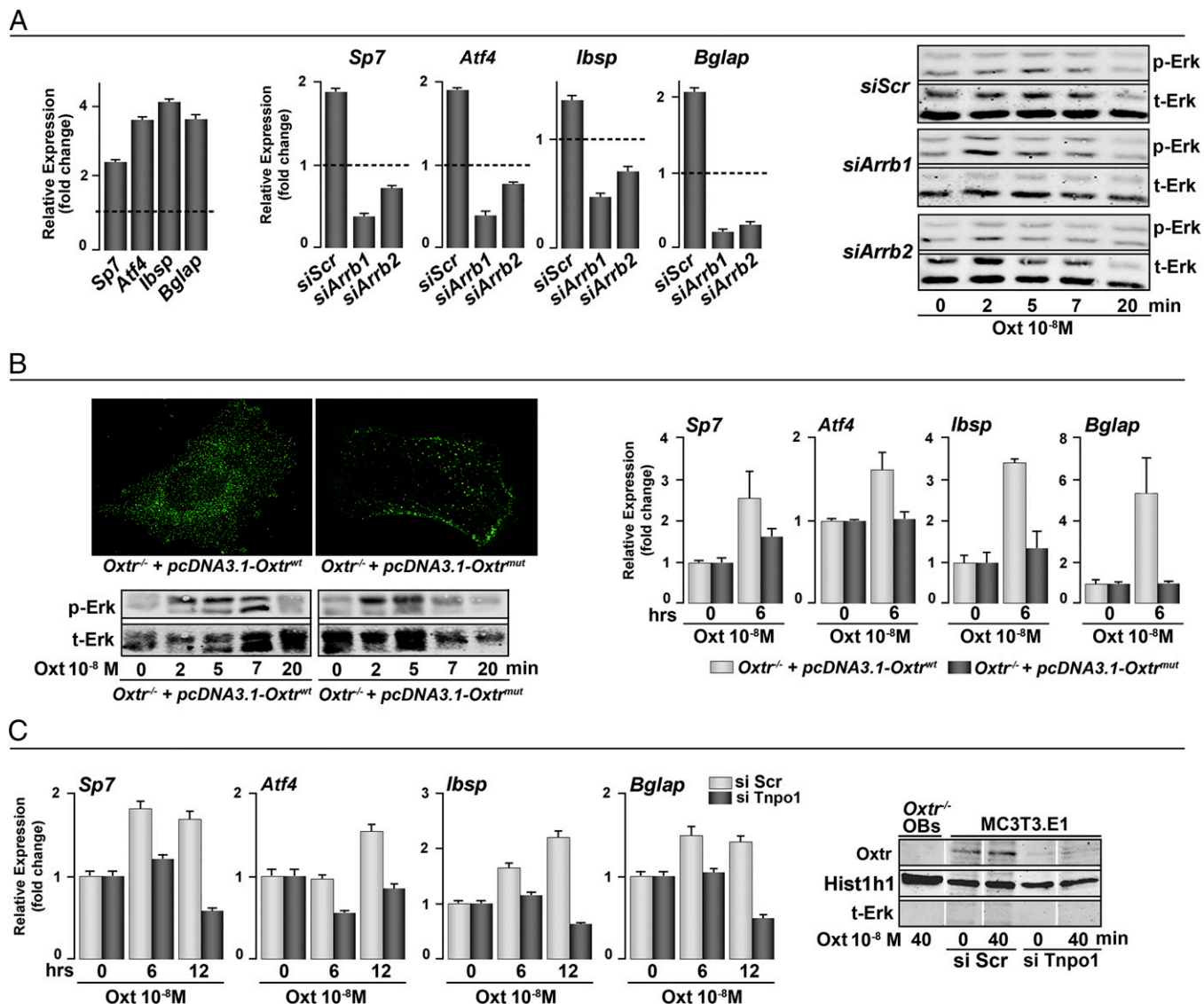


Fig. 4. Nuclear Oxt_r modulates osteoblastic gene expression through nuclear trafficking. In control primary osteoblasts, Oxt up-regulates the expression of *osterix* (*Sp7*), *Atf4*, *bone sialoprotein* (*Ibsp*), and *osteocalcin* (*Bglap*). (A) siRNA knockdown of *Arrb1/2* abolished this up-regulation without affecting Erk phosphorylation. We created an Oxt_r construct (*pcDNA3.1-Oxtr^{mut}*) that was unable to bind stably to *Arrb* for subsequent endocytosis. *Oxtr^{-/-}* osteoblasts were stably transfected with *pcDNA3.1-Oxtr^{wt}* or *pcDNA3.1-Oxtr^{mut}* and stimulated with Oxt (10^{-8} M) for 15 min. (B) Immunostaining showed that mutant receptor appears mostly at the plasma membrane compared with WT receptor. Erk phosphorylation was not affected; however, gene up-regulation was dampened in the absence of endocytosis. (C) Nuclear membrane translocation of Oxt_r was *Tnpo1*-dependent, because Oxt_r was not detected in the nucleus after siRNA knockdown of *Tnpo1*. This knockdown also abolished the up-regulation of osteoblastic gene expression. Note that only relevant lanes in the Western blots are shown. Intervening lanes are replaced with white gaps. siScr, scrambled siRNA (control).

Immunoprecipitation studies show that the CXCR4 binds to a nuclear $G_{\alpha i}$ protein (19). Its activation by the ligand SDF-1 α elicits intranuclear Ca^{2+} signals that are sensitive to inhibition by the $G_{\alpha i}$ inhibitor pertussis toxin—this effect testifies to the presence of a fully functional CXCR4/ $G_{\alpha i}$ apparatus within the nucleus (19). Similarly, we found that the enzyme CD38, hitherto considered an extracellular cyclase that generates the second messenger cADPr, was localized to the inner nuclear membrane (32). At this location, it triggered Ca^{2+} signals through ryanodine receptor-gated Ca^{2+} channels, which we and others found were also resident within the inner nuclear membrane (33, 34). It has been shown recently that insulin triggers the nuclear localization of an otherwise cytosolic protein, caveolin-2; this effect, in turn, prevents histone methylation (35). We remain unsure whether a fully functional Oxt_r/ G protein exists in the nucleus.

Our data raise the possibility that membrane GPCRs, although signaling relatively rapidly through traditional phosphorylation cascades, may interact directly with the transcription machinery of a cell after nuclear localization. Furthermore, it might be worthwhile examining whether nuclear Oxt_rs, in addition to mediating the skeletal actions of Oxt, also contribute to other more delayed actions of Oxt in social bonding. Likewise and more generally, it would be meaningful to further study whether certain unexplained actions of GPCRs, particularly long-term responses to agonist stimulation, are mediated by a nuclear mechanism.

At the physiologic level, albeit speculatively, these differences might translate into distinct regulatory actions of Oxt on calcium homeostasis during pregnancy and lactation. Oxt_rs are present on osteoclast precursors and osteoclasts (36), and their stimulation results in increased osteoclastogenesis but inhibited bone

resorption (4). Rapid rises in Oxt during periods of calcium stress (1) might enable calcium mobilization followed by the rapid cessation of bone resorption through a brake on active osteoclasts (4). Thereafter, the maternal skeleton recovers, and we speculate that the proosteoblastic action of the hormone mediates bone rebuilding (5, 37, 38). We find that bone formation during pregnancy is inhibited in *Oxtr*^{-/-} mice and that *Oxtr*^{-/-} pups display reduced trabecular mineralization (5). The extent to which nuclear Oxt actions mediate these putative physiological effects requires additional studies. It is also important to determine if osteoclasts and osteoblasts respond differently to the range of concentrations of circulating Oxt during pregnancy and lactation. For example, whereas higher plasma Oxt levels may permit calcium mobilization by the osteoclast, falling concentrations postlactation may trigger reparative anabolic response.

Materials and Methods

Osteoblast-enriched cultures were performed using calvaria from newborn mice after sequential collagenase digestion (details in *SI Materials and Methods*). Intact nuclei were isolated as described in the work by Adebajo et al. (32). The outer nuclear membrane and nucleoplasts (nuclei without outer membranes) were separated (32). For immunofluorescence microscopy,

cells or nucleoplasts were fixed and incubated with antibodies to Oxtr (gift from Fabio Malavasi, University of Turin, Turin, Italy), Arrb1/2 (Santa Cruz), Tpn01, Kpnb1 (Abnova), and Rab5 (Cell Signaling). Fluorescent-labeled goat anti-mouse or anti-rabbit secondary antibodies (Alexa-488 and Alexa-568, respectively; Invitrogen) were used for detection. Nuclei were counterstained with Quinolinium 4-[3-(3-methyl-2(3H)-benzothiazolylidene)-1-propenyl]-1-[3-(trimethylammonio)propyl]-diiodide (TO-PRO). For immunogold staining, we followed the protocol detailed in *SI Materials and Methods* and examined the sections using a Zeiss EM 109 electron microscope and a Gatan CMS camera. MS analysis of nuclear extracts is detailed in *SI Materials and Methods*. For mutagenesis, we created pcDNA3.1-*Oxtr*^{mut}, a mouse *Oxtr* mutant construct with two clusters of Ser residues mutated to Ala residues (S365A, S366A, and S367A and S376A, S377A, and S378A). *Oxtr*^{-/-} mouse osteoblasts were stably transfected with pcDNA3.1-*Oxtr*^{mut} or pcDNA3.1-*Oxtr*^{wt} after geneticin selection. Western immunoblotting and quantitative PCR were performed using standard methods.

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