RESEARCH

OXTR overexpression leads to abnormal mammary gland development in mice

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Abstract

Oxytocin receptor (OXTR) is a G-protein-coupled receptor and known for regulation of maternal and social behaviors. Null mutation (*Oxtr*^{-/-}) leads to defects in lactation due to impaired milk ejection and maternal nurturing. Overexpression of OXTR has never been studied. To define the functions of OXTR overexpression, a transgenic mouse model that overexpresses mouse *Oxtr* under β -actin promoter was developed (*++Oxtr*). *++Oxtr* mice displayed advanced development and maturation of mammary gland, including ductal distention, enhanced secretory differentiation and early milk production at non-pregnancy and early pregnancy. However, *++Oxtr* dams failed to produce adequate amount of milk and led to lethality of newborns due to early involution of mammary gland in lactation. Mammary gland transplantation results indicated the abnormal mammary gland development was mainly from hormonal changes in *++Oxtr* mice but not from OXTR overexpression in mammary gland. Elevated OXTR expression increased prolactin-induced phosphorylation and nuclear localization of STAT5 (p-STAT5), and decreased progesterone level, leading to early milk production in non-pregnant and early pregnant females, whereas low prolactin and STAT5 activation in lactation led to insufficient milk production. Progesterone treatment reversed the OXTR-induced accelerated mammary gland development by inhibition of prolactin/p-STAT5 pathway. Prolactin administration rescued lactation deficiency through STAT5 activation. Progesterone plays a negative role in OXTR-regulated prolactin/p-STAT5 pathways. The study provides evidence that OXTR overexpression induces abnormal mammary gland development through progesterone and prolactin-regulated p-STAT5 pathway.

Key Words

- ▶ OXTR
- ▶ mammary gland development
- ▶ prolactin
- ▶ p-STAT5
- ▶ progesterone
- ▶ RANKL

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Introduction

Abnormal mammary gland development is associated with many diseases including breast cancers (Cardiff *et al.* 2000). Mammary gland develops through several stages including embryonic development, puberty, pregnancy, lactation and involution (Macias & Hinck 2012). Mammary gland development requires hormones

including prolactin (PRL), progesterone (P4) and estrogen (E2). PRL controls alveologenesis and lactogenesis of mammary gland through phosphorylation of JAK2/STAT5 axis (Yamaji *et al.* 2009). PRL binds to prolactin receptor (PRLR) and activates STAT5 through JAK2-regulated phosphorylation. Activated STAT5 (p-STAT5) translocates

to nucleus and regulates the expression of alveolar epithelial differentiation-associated genes and milk protein genes including whey acidic protein (*Wap*) and β -casein (*Csn2*) (Schmitt-Ney *et al.* 1991, Gouilleux *et al.* 1994, Li & Rosen 1995, Liu *et al.* 1995, Ali & Ali 1998, Vonderhaar 1999). P4 is required for mammary epithelium proliferation, ductal side-branching and alveolar morphogenesis during pregnancy (Briskin *et al.* 1998, Mulac-Jericevic *et al.* 2003). Receptor activator of NF κ B1 ligand (RANKL) mediates mouse mammary epithelium proliferative response to P4 (Asselin-Labat *et al.* 2010, Beleut *et al.* 2010, Joshi *et al.* 2010). Moreover, RANKL is in the downstream of PRL signaling. Response of RANKL to both PRL and P4 may indicate RANKL is at the junction of two hormones (Srivastava *et al.* 2003, Fernandez-Valdivia *et al.* 2008). Many studies have demonstrated that local cues, including STAT3, leukemia inhibitory factor (LIF), insulin-like growth factor-binding protein-5 (IGFBP5), matrix metalloproteinase 2 (MMP2) and 3 (MMP3), regulate the phase of involution. Deletion of *Stat3* or *Lif* in mammary gland results in dramatic delay in involution (Chapman *et al.* 1999, Kritikou *et al.* 2003). Overexpression of *Igfbp5*, *Mmp2* and *Mmp3* in mammary gland causes premature involution (Simpson *et al.* 1994, Fata *et al.* 2001, Ning *et al.* 2007).

Oxytocin receptor (OXTR) is a G-protein-coupled receptor for neurotransmitter oxytocin (Kimura *et al.* 1992). In response to ligand binding, activated OXTR couples to G $_{\alpha q/11}$ and G $_{\beta \gamma}$ to stimulate phospholipase C- β isoforms, leading to various cellular events and increased intracellular [Ca $^{2+}$]. OXTR is known to be expressed in mammary gland (Kimura *et al.* 1998), uterine myometrium (Helmer *et al.* 1998), cardiac muscle of heart (Gutkowska *et al.* 1997), vascular endothelium layer (Thibonnier *et al.* 1999) and specific brain regions (Insel *et al.* 1993, Hidema *et al.* 2016). Many functions of OXTR have been studied using null mutation (*Oxtr* $^{-/-}$). *Oxtr* $^{-/-}$ mice elicit abnormal sexual, social behaviors (Takayanagi *et al.* 2005), obesity and thermoregulation disorder (Kasahara *et al.* 2015). *Oxtr* $^{-/-}$ mice exhibit normal reproduction but defective in lactation. All offspring from *Oxtr* $^{-/-}$ dams died within 24 h after birth due to impaired milk ejection and defect in maternal nurturing (Takayanagi *et al.* 2005, Hidema *et al.* 2016). However, either deficient or excessive gene expression can lead to pathologic development and disease. Whether overexpression of OXTR has any effect on mammary gland development and how OXTR regulates mammary gland development are still not clear.

To investigate the role of excessive OXTR in mammary gland development, we generated an OXTR

overexpression mouse model. OXTR overexpression produced distinct phenotypes including early mammary gland development and involution. The study describes the impact of OXTR on mammary gland development through hormonal changes.

Materials and methods

Materials and reagents

All general chemicals were purchased from Sigma and enzymes for molecular cloning were from New England Biolabs, USA and Takara.

Animals

The entire study was approved by the Animal Experimental Ethics Committee of Northeast Normal University (NENU/IACUC). All mouse work was conducted in accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of National Institutes of Health and UK legal requirements.

C57BL/6J (B6), DBA/2, CD1 mice were purchased from Vital River (A Charles River Company, Beijing, China). All animals were maintained under pathogen-free conditions at 21 $\pm$ 1°C, 50 $\pm$ 20% relative humidity and 12:12-h light/darkness cycle. Mice were housed in IVC cages (5 per cage) with free access to regular chow and water. Females at various stages of mammary gland development were used in the study and WT littermates were used as control. Mice were anesthetized with 1% pentobarbital sodium at a dose of 10 mg/kg intraperitoneally before experiments.

Generation of β -actin-*Oxtr* ($^{++}$ *Oxtr*) mice

Plasmid construction

Briefly, 1.2 kb mouse *Oxtr* coding region was PCR-amplified from mouse mammary gland cDNA and inserted after β -actin promoter of pCAGGS (LMBP2453, Laboratory of Molecular Genetics, Institute of Medical Science, University of Tokyo). A 3.5 kb fragment including β -actin promoter, mouse *Oxtr* cDNA and β -Globin intron and polyA was excised and purified using Qiagen Gel Extraction Kit (Qiagen). The purified DNA fragment was diluted with injection buffer to a 2 ng/ μ L concentration.

Zygotes collection, microinjection, and transferring

Transgenic mice overexpressing mouse oxytocin receptor gene ($^{++}$ *Oxtr*) were generated by pronuclear microinjection.

Six-week-old and sexually emergent F1 (B6×DBA/2) females were superovulated by intraperitoneal injection of 5 IU PMSG followed by 5 IU hCG at an interval of 46 h. Mating was set with C57BL/6J stud male for overnight. Zygotes were collected from the infundibulum region of oviduct into M2 medium in the morning, digested with hyaluronidase and transferred into M16 medium. DNA solution (2 ng/μL) was pronucleus-injected into C57/BL6J/DBA zygotes using an Olympus IX71 inverted microscope equipped with Narishige microinjector. More than 120 zygotes of two-cell stage were obtained after injection and overnight culture, and then oviduct-transferred to pseudo-pregnant CD1 females. The pseudo-pregnant females were prepared by mating with C57BL/6J/DBA males.

Genotyping

Transgenic founders were identified by PCR. Genomic DNA was extracted from tail tips. In brief, samples were digested with GNTK buffer (50 mM KCl, 1 M MgCl₂, 10 mM Tris-HCl, pH 8.5, 0.01% gelatin, 0.45% Nonidet P-40, 0.45% Tween20 and 100 mg/mL proteinase K) at 55°C overnight (Malumbres *et al.* 1997). The lysates were diluted and boiled for 15 min. PCR was performed at 94°C for 2 min, followed by 30 cycles of denaturing at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 1 min. Final extension was at 72°C for 10 min. The PCR primers are located in rb_glob_PA_terminator of pCAGGS vector, pCAGGS Forward (1785–1806): AATGCCCTGGCTCACAAATAC; pCAGGS Reverse (2240–3363): GGGACAGCTATGACTGGGAGTAG. Eight pups were found carrying the transgene (founders). Transgenic mice were switched to C57/BL6J genetic background (≥98%) by consecutive mating with C57/BL6J mice for more than five generations.

Real-time PCR

Total RNA was extracted from mouse tissues using TRIzol reagent (Takara). One microgram of total RNA was reversibly transcribed with Prime Script cDNA Synthesis Kit from Takara according to manufacturer's instruction. Real-time PCR was carried out using SYBR Green Mix (Takara) following standard procedures. Quantification was calculated using the 2^{−ΔΔCT} value method. All PCR results were normalized to expression level of 18S ribosomal RNA. PCR primers were listed in Supplementary Table 1 (see section on [supplementary data](#) given at the end of this article).

Histology analysis and immunohistochemistry

For histological analysis, 5 μm sections of 4% paraformaldehyde (PFA)-fixed, paraffin-embedded mammary glands were stained with hematoxylin and eosin (H&E) (Marshall & Horobin 1973). For immunostaining (Ramos-Vara 2005), a 15-min antigen heat retrieval in EDTA (pH 8.0) was performed. Primary antibodies: rabbit anti-OXTR (ab181077, Abcam, 1:500) and rabbit anti-Phospho-STAT5 (9359s, Cell Signaling Technology (CST), 1:600). The slides were incubated with primary antibodies overnight at 4°C. Secondary antibody was HRP-conjugated anti-rabbit IgG (8114P, CST, USA). The slides were incubated with secondary antibodies for 30 min at room temperature. Peroxidase signal was revealed with DAB (CST, USA). The slides were counterstained with hematoxylin, and positively stained cells appeared brown.

Mammary gland whole mount

One of the 4rd inguinal mammary glands was dissected and spread on a glass slide. After fixation with Carnoy solution (ethanol, chloroform, glacial acetic acid; 6:3:1), gland was rehydrated gradually through a series of diluted ethanol and immersed in carmine aluminum solution (1 g carmine and 2.5 g aluminum potassium sulfate were dissolved into 500 mL distilled water by boiling for 20 min) (Plante *et al.* 2011) for staining overnight at room temperature. Dehydrated gland through serial ethanol baths (50, 70, 95 and 100%) was cleared in xylene and examined under an Olympus IX71 inverted microscopy.

Lactation analysis and mammary gland development in lactation

Neonatal growth and survival, milk yield, mammary gland morphology, major milk protein gene expression and involution-associated gene expression were analyzed to assess changes in lactation (Palmer *et al.* 2006).

To analyze milk yield and neonatal growth, embryos from B6 were transferred in WT and *++Oxtr* dams to eliminate the effect of pups' genotypes using procedures described 'Zygotes collection, microinjection and transferring' section. Milk yield was accessed using daily litter weight gain of pups. The number of pups nursed by WT dam was the same as that nursed by the *++Oxtr* dam. Pups from each litter were weighed every 24 h, and litter weights and average body weights were recorded. Cross-fostering experiments were used to rule out possible pup-dependent effects where pups from WT dams were placed

on *++Oxtr* dams, and pups from *++Oxtr* dams were placed on WT dams.

Whole-mount and histological staining was used to define the progressive changes in mammary gland morphology during lactation. The major milk protein gene expression (*Csn2* and *Wap*) was used to examine mammary gland differentiation.

Mammary gland transplantation

This procedure is used to assess phenotypes of mammary gland development determined by the effects of genotype in mammary epithelial or a supportive micro-environment. Alveolar and ducts repopulating into mammary fat pad were evaluated. The endogenous mammary epithelium was removed from the 4rd inguinal glands of 3-week-old WT or *++Oxtr* virgin females to provide a cleared mammary fat pad (supportive micro-environment). The donor epitheliums were prepared as follows: the 4rd mammary gland of 3-week-old WT or *++Oxtr* virgin females were harvested and minced into 1 mm³ fragments in DMEM/F12. The 1 mm³ fragments of donor epitheliums were inserted into the cleared mammary fat pads. Donor epithelium from WT was inserted to the left side and *++Oxtr* on the right side. Eight weeks after transplantation, fat pads were excised for whole-mount staining (Dunphy *et al.* 2010).

Hormone treatment

Progesterone (Sigma, P-7556; Sigma Chemical) and prolactin (BBi Life Sciences, D824BC0272, China) were diluted in sterile saline (0.9% NaCl) to the final concentration of 3 mg/mL and 10 ng/ μ L respectively. WT and *++Oxtr* virgin females were treated with 300 μ g progesterone subcutaneously daily for 7 days. *++Oxtr* females at late pregnancy were treated with 300 μ g progesterone subcutaneously or 50 ng/g PRL intraperitoneally for three consecutive days (P18.5, P19.5, L1). Control littermates at the same stage were treated in parallel with saline.

ELISA for hormone measurements

Serum samples were collected at various stages and prepared by clotting for 30 min and centrifuging at 400g room temperature. ELISA assays were performed according to manufacturer's instruction. The optical density was measured using microplate reader (SpectraMax, Molecular Device, USA). ELISA kit for mouse progesterone (Cat DEV9988, DEMEDITEC Diagnostics GmbH, Germany)

has the sensitivity of 0.04 ng/mL. The intra-assay and inter-assay variation are <9.9% and <7.6%, respectively. ELISA kit for mouse prolactin (Cat AB100736, Abcam) has the sensitivity of 30 pg/mL. The intra-assay and inter-assay variation are <10% and <12%, respectively. ELISA kit for mouse Estradiol (Cat ADI-900-174, Enzo, USA) has the sensitivity of 14 pg/mL. The intra-assay and inter-assay variation are <7.1% and 14.2%, respectively.

Immunoblotting

Ovary, uterus and mammary gland protein was extracted in RIPA buffer. 40 μ g of total protein was separated on 10% SDS-PAGE and transferred to PVDF membranes (Towbin *et al.* 1979). The membranes were incubated with primary antibodies overnight (12 h) at 4°C. The primary antibodies used were rabbit anti-OXTR (Abcam, ab181077; 1:5000), rabbit anti-Phospho-STAT5 (CST, USA, 9359s; 1:1000), goat anti-RANKL (R&D Systems, AF462; 1:2000) and rabbit anti-GAPDH (Bioworld, USA, AP0063, 1:10,000). Following three washes with TBST, the membranes were incubated with secondary antibodies for 1 h at room temperature. The secondary antibodies used were horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG (GE Healthcare, USA, 1:3000) and HRP-conjugated rabbit anti-goat IgG (Bioworld, USA, BS30503, 1:10,000). Amersham™ ECL™ (GE Healthcare, USA) was used as signal detection (Renart *et al.* 1979). GAPDH is served as a loading control.

Statistical analysis

All values reported are presented as means $\pm$ standard error of means (S.E.M.). *P* value was calculated with one-way ANOVA to compare three or more groups, unpaired two-tailed Student's *t*-tests to compare two groups and log-rank (Mantel–Cox) test for survival analysis. Statistically significant differences are indicated with asterisks (**P*<0.05; ***P*<0.01; ****P*<0.001).

Results

OXTR overexpression and localization in developing mammary gland

To explore the role of OXTR in mammary gland development, *Oxtr* cDNA was transgenically expressed under β -actin promoter (*++Oxtr*). OXTR expression was examined in female reproductive system, brain, heart, kidney and white adipose tissue (WAT) (Fig. 1A, B and C).

Real-time PCR (RT-PCR) (Fig. 1A) and immunoblotting (Fig. 1B) results confirmed *Oxtr* expression was upregulated by 14-folds in *++Oxtr* mammary gland ($P=0.0008$), 6.4-folds in ovary ($P=0.009$), 6.6-folds in uterus ($P=0.008$), 206-folds in brain ($P=0.0004$), 21500-folds in heart ($P=0.0003$), 235-folds in kidney ($P=0.0006$) but not in WAT (Fig. 1C).

We further investigated the expression and localization of OXTR in mammary glands at different developmental stages: non-pregnancy 8 weeks and 12 weeks (8 W, 12 W), pregnancy day 3.5 and 9.5 (P3.5, P9.5) and lactation day 1, 4 and 18 (L1, L4, L18). RT-PCR results indicated that *Oxtr* mRNA expression in *++Oxtr* mammary gland was constantly higher than WT ($P<0.001$ for each time point) (Fig. 1D). Immunoblotting and immunohistochemistry (IHC) results displayed that OXTR in WT mammary gland was hardly detectable at non-pregnancy, increased during pregnancy and reached a peak in lactation. Compared to WT, *++Oxtr* OXTR expression was higher at non-pregnancy ($P=0.0005$ for 8 W, $P=0.0004$ for 12 W) and early pregnancy ($P=0.006$ for P3.5, $P=0.002$ for P9.5), but lower in lactation ($P=0.016$ for L1, $P=0.0001$ for L18) (Fig. 1E and F). IHC results indicated that OXTR was localized in developing alveolar and ductal epithelial cells of both WT and *++Oxtr* mammary gland (Fig. 1F). Premature mammary gland development and early involution were clearly demonstrated in *++Oxtr* mice (Fig. 1F). Collectively, expression of OXTR in developing mammary gland is altered in *++Oxtr* mice, and β -actin promoter-driven OXTR overexpression induces abnormal mammary gland development.

OXTR overexpression leads to accelerated mammary gland development and milk production at non-pregnancy and early pregnancy

To examine the impact of OXTR overexpression on mammary gland development, whole-mount staining of mammary glands at different stages was performed. Slight increase in branching of duct and terminal end buds (TEB) numbers was detected in *++Oxtr* mice at 3 and 8 weeks (Fig. 2Aa, b, c, d). *++Oxtr* mice exhibited markedly precocious secretory alveolar differentiation and distension of mammary ducts at 12 weeks (12 W) and pregnancy (P3.5, P9.5) (Fig. 2Ae, f and B). The calibers (diameters) of *++Oxtr* mammary ducts were significantly increased at 12 W and pregnancy compared to WT ($P<0.0001$ for 12 W, P3.5 and P9.5) (Fig. 2C). Hematoxylin and eosin (H&E) staining results showed accelerated lobuloalveolar development and distended ducts full of proteinaceous material in *++Oxtr* mammary

gland (Fig. 2D and Supplementary Fig. 1A). Imaging results of macroscopic mammary morphology revealed presence of liquid milk filled in mammary ducts of non-pregnant and early pregnant *++Oxtr* mice (Fig. 2E). Higher mRNA expression of *Csn2* ($P<0.0001$ for 12 W, P3.5 and P9.5) and *Wap* ($P<0.0001$ for 12 W, P3.5 and P9.5) (two major milk protein genes) in *++Oxtr* mice than WT was in accordance with the morphological changes and precocious secretory differentiation (Fig. 2F). These results demonstrate that OXTR overexpression induces early secretory alveolar maturation at non-pregnancy and early pregnancy.

Early secretory lobuloalveolar involution results in postnatal lactation failure

All offspring from *++Oxtr* dams died during lactation, regardless of the genotype of offspring ($P<0.001$) (Fig. 3A). In order to eliminate the effect of pups' genotypes, WT embryos were oviduct transferred to pseudo-pregnant WT and *++Oxtr* females. Milk yield was accessed using daily litter weight gains of pups after embryos transfer (ET) (Fig. 3B). The number of pups nursed by WT dam was the same as that nursed by *++Oxtr* dam. *++Oxtr* dams had a significantly lower milk yield than WT dams during lactation ($P<0.001$ for each time point) (Fig. 3B). Pups from each litter were weighed every 24 h and average body weights were recorded. Pups nursed by *++Oxtr* dams showed a significantly lower average body weight than pups nursed by WT dams ($P=0.0013$ for L4, $P=0.0011$ for L5, $P<0.001$ for L6–L14) (Fig. 3C). For cross-fostering experiments, newborn pups of *++Oxtr* dams were successfully fostered by WT mothers regardless of pup's genotype, but *++Oxtr* mothers failed to foster (Fig. 3D). When pups of *++Oxtr* dams were replaced with newborns from CD1 mothers, all of the pups showed signs of dehydration and stunted growth, compared to their littermates nurtured by CD1 mothers (Supplementary Fig. 1B). There was no evident sign of abnormal behavior from *++Oxtr* mothers with normal crouching over the pups. Survival failure of newborns is probably caused by insufficient milk secretory of *++Oxtr* mothers. This is supported by the reduced mRNA expression of *Csn2* and *Wap* in *++Oxtr* mammary gland during lactation at L1, L4 and L18 (Fig. 3E).

To investigate what caused the insufficient milk yield, whole-mount and histological analysis of lactating mammary glands was performed. Results exhibited sparse alveolar units, small alveolar size and early involution of the epithelial cells into adipose tissue in *++Oxtr* mammary gland (Fig. 3F, G and Supplementary

Figure 1

Overexpression of OXTR and localization during mammary gland development. Total RNA and protein were isolated from mammary gland (4rd pair), ovary, uterus, brain, heart, kidney and white adipose tissue (WAT) of WT and *Oxtr*^{+/+} littermates at 12 weeks. (A) *Oxtr* mRNA expression in female reproductive system by RT-PCR, $n=4$ for each tissue. (B) OXTR expression in female reproductive system by Immunoblotting. GAPDH is served as a loading control. Protein quantifications using Image J, $n=4$ for each tissue. (C) *Oxtr* mRNA expression in brain, heart, kidney and WAT by RT-PCR, $n=3$ for each tissue. Mammary glands (4rd pair) were harvested from *Oxtr*^{+/+} and WT littermates at 8 and 12 weeks (8 W and 12 W), pregnancy (P3.5 and P9.5) and lactation (L1, L4 and L18). (D) RT-PCR analysis of *Oxtr* mRNA expression in mammary glands during development, $n=3$ for each time point. (E) Immunoblotting analysis of OXTR in mammary gland. Protein quantifications using Image J, $n=4$ for each time point. (F) Immunohistochemistry staining of OXTR in mammary sections (5 μ m) and quantifications of immunostaining using Image Pro Plus. Nuclei were stained blue with hematoxylin. Scale bar: 100 μ m. Original magnifications: $\times 20$, $n=3$ for each time point. Data are represented as mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, calculated using two-tailed unpaired t test.

Figure 2

OXTR overexpression accelerates mammary gland development and milk production at non-pregnancy and pregnancy. Mammary glands (4rd pair) of ++Oxtr and WT mice at 3, 8 and 12 weeks (3, 8, 12 W), pregnancy (P3.5, P9.5) were harvested. (A) Whole-mount staining of mammary gland at non-pregnancy (3, 8 and 12 W). Scale bar: 1 mm. Original magnifications: $\times 1.25$. (B) Whole-mount staining of mammary gland at P3.5 and P9.5. Scale bar: 500 μ m. Original magnifications: $\times 4$. (C) Mammary duct caliber (mm) of 12 W, P3.5 and P9.5 WT and ++Oxtr mice. Quantifications from whole-mount staining image using CAD software, $n=5$ for each time point. (D) H&E staining of mammary gland from 12 W, P3.5 and P9.5 WT and ++Oxtr mice, duct full of proteinaceous material (arrows). Scale bar: 200 μ m. Original magnifications: $\times 10$. (E) Macroscopic images of mammary gland (3rd pair), duct full of milk (arrows). Scale bar: 1 cm. (F) Gene expression of major milk protein *Csn2* and *Wap* during development (12 W, P3.5 and P9.5), $n=3$ for each time point. Data are represented as mean $\pm$ S.E.M. *** $P<0.001$, calculated using two-tailed unpaired t test.

Fig. 1A). Involution-related genes (*Stat3*, *Lif*, *Igfbp5*, *Mmp2* and *Mmp3*) were upregulated in the mammary glands of ++Oxtr mice relative to WT in lactation (L1, L18) (Fig. 3H and Supplementary Fig. 1C), suggesting the early involution may have led to lactation failure.

Prolactin and progesterone pathways are major players in OXTR-induced maldevelopment

To determine whether the increase of OXTR in mammary gland or other organs caused abnormal mammary

gland development, mammary gland transplantation experiments were performed. Whole-mount staining results showed that WT or ++Oxtr donor epithelium did not make marked difference in mammary gland development (Fig. 4A). Instead, ++Oxtr fat pad showed better support for both WT and ++Oxtr donor mammary epithelium with increased outgrowth coverage (area of the fat pad repopulated by mammary epithelium) (Fig. 4A and B) and duct distension (Fig. 4A and C) than WT. These results indicate that the abnormal mammary gland development is mainly derived from the microenvironmental changes

Figure 3

Impaired lobuloalveologenesis and early involution results in lactation failure of ++Oxtr mice. (A) Survival analysis of newborn pups (n=51) from WT dams (n=7) and newborn pups (n=50) from ++Oxtr dams (n=9). (B) Milk yield by daily litter weight gain of newborn pups after embryos transferring (ET). 5 pups per litter were nursed by WT or ++Oxtr dams (n=4). (C) Average body weight of pups. 5 pups per litter were nursed by WT or ++Oxtr dams (n=4). (D) Survival analysis of pups (n=37) nursed by WT dams (n=5) and pups (n=34) nursed by ++Oxtr dams (n=5) by cross-fostering experiments. (E) RT-PCR analysis of *Csn2* and *Wap* mRNA in ++Oxtr and WT mammary glands at P9.5, L1, L4, L18, n=6 for each time point. (F) Whole-mount staining of mammary gland and quantification of lobuloalveolar size in L4 and L14. Scale bar: 500µm. Original magnifications: x4, n=4 for each time point. (G) H&E staining of mammary gland in L1 and L18. Scale bar: 200µm. Original magnifications: x10. (H) RT-PCR analysis of mammary gland involution-related gene expressions in L1 and L18, n=6 for each time point. Mammary glands (4rd pair) were harvested from ++Oxtr and WT mice in lactation (L1, L4, L14 and L18). Data are represented as mean±s.e.m. *P<0.05; **P<0.01; ***P<0.001, calculated using two-tailed unpaired t test and Log-rank (Mantel-Cox) test.

Figure 4

Mammary gland cross transplantation. The transplanted mammary (4rd pair) fat pads were harvested 8 weeks post transplantation from WT or *++Oxtr* mice. (A) Whole-mount staining of transplanted mammary gland. Scale bar: 500µm. Original magnifications: $\times 4$. (B) Outgrowth coverage of the repopulated fat pad (percentage of duct and alveolar staining area) using Image Pro Plus, $n=3$. (C) Average duct calibers, $n=3$. Data are represented as mean $\pm$ s.e.m. * $P<0.05$; ** $P<0.01$, calculated with one-way ANOVA.

in *++Oxtr* mice but not from the increased expression of OXTR in mammary gland.

Mammary gland development is mostly dependent on the hormonal environment including 17β -estradiol (E2), progesterone (P4) and prolactin (PRL). To determine whether these hormones play roles in the OXTR-induced changes, serum levels at various stages were analyzed by ELISA. No difference was found in E2 between virgin *++Oxtr* and WT (Supplementary Fig. 2A). However, progesterone was low through all stages in *++Oxtr* mice. In WT mice, serum progesterone reached a peak at pregnancy and dropped in lactation (Fig. 5A). WT prolactin increased during pregnancy and maintained a high level in lactation. In contrast, *++Oxtr* prolactin exhibited constantly higher level at non-pregnant stage ($P<0.0001$ for 12 W, 28 W) but lower levels during pregnancy ($P=0.038$ for P3.5, $P=0.006$ for p9.5) and lactation ($P=0.034$ for L1, $P=0.006$ for L14) compared to WT (Fig. 5B). We examined RANKL expression at various stages by immunoblotting. In WT mice, RANKL expression was undetectable at non-pregnancy, gradually increased to a peak during pregnancy and dropped to a barely detectable level in lactation. In *++Oxtr* mice, RANKL expression was higher than WT at non-pregnancy and early pregnancy, but barely detectable in lactation (L1) and increased again with mammary involution in L18 (Fig. 5C). Results suggest that progesterone/RANKL, prolactin/RANKL pathways are both involved in the OXTR-induced mammary gland development.

To investigate whether OXTR overexpression has any effect on p-STAT5, we examined the expression and

distribution of activated p-STAT5 (Tyr 694) in developing mammary glands. No difference was found in *Stat5* (*Stat5a* and *Stat5b*) mRNA levels between WT and *++Oxtr* mammary gland (Supplementary Fig. 2B and C). However, IHC results showed that OXTR overexpression induced higher levels of nuclear p-STAT5 in non-pregnant ($P=0.006$ for 12 W) and early pregnant *++Oxtr* mammary epithelial cells ($P=0.023$ for P3.5, $P=0.004$ for p9.5) than WT (Fig. 5Da, b, c, d, e, f and E). This result is consistent with the morphological changes and early milk production at non-pregnancy and pregnancy. Although nuclear p-STAT5 was detected in both WT and *++Oxtr* mammary epithelia during lactation, an earlier downregulation of p-STAT5 ($P=0.048$ for L1, $P=0.002$ for L18) was evident in *++Oxtr* mice than WT (Fig. 5Dg, h, i, j and E). This result is in agreement with the early morphological involution and low milk protein secretion in lactation. Immunoblotting analysis of p-STAT5 was in accord with IHC result (Fig. 5C). These patterns are correlated well with the functional changes, the early milk production at non-pregnancy and early termination in lactation. Results strongly suggest that prolactin/p-STAT5 signaling mediates the OXTR-induced abnormal mammary gland development.

Progesterone can reverse the OXTR-induced changes through inhibition of PRL/p-STAT5 pathway

To determine whether OXTR-induced changes were regulated by P4, 11-week-old virgin WT and *++Oxtr* females were treated with 300µg (3 mg/mL) P4 subcutaneously

Figure 5

Progesterone/RANKL and prolactin/p-STAT5 are altered during mammary gland development in +Oxtr mice. (A) Serum progesterone levels in WT and +Oxtr mice at various stages, $n=5$ for each time point. (B) Serum prolactin levels in WT and +Oxtr mice at various stages, $n=5$ for each time point. (C) Immunoblotting analysis of p-STAT5 and RANKL in developing mammary gland from +Oxtr and WT mice. GAPDH is served as a loading control, $n=4$ for each time point. (D) Immunohistochemistry staining of p-STAT5 in developing mammary gland. The enlarged images at the lower left corner show the state of p-STAT5. Nuclei were stained blue with hematoxylin. Scale bar: 100 μm. Original magnifications: ×20. (E) Quantifications of immunostaining using Image Pro Plus, $n=4$ for each time point. The mammary glands (4rd pair) were harvested from +Oxtr and WT littermates at 12W, P3.5, P9.5, L1 and L18. Data are represented as mean ± s.e.m. * $P<0.05$; ** $P<0.01$, *** $P<0.001$, calculated using two-tailed unpaired t test.

for seven consecutive days. P4 treatment significantly inhibited serum prolactin level in both WT ($P=0.0072$) and +Oxtr mice ($P<0.0001$) and reversed OXTR-induced hyperprolactinemia to a WT-like phenotype (Fig. 6A). P4 administration also rescued the decreased expression of its receptor (*Pgr*) mRNA seen in +Oxtr mice (Fig. 6B). RT-PCR and immunoblotting results both confirmed OXTR repression in mammary gland by P4 ($P=0.0003$) (Fig. 6C and D). P4 repressed nuclear p-STAT5 but enhanced RANKL expression (Fig. 6D). Whole-mount

staining of mammary gland was performed to access the corresponding morphological changes. +Oxtr mice with P4 treatment exhibited attenuation of mammary gland development and morphologically similar to WT (Fig. 6E). P4-treated +Oxtr mice did not show distension of mature ducts filled with milk (Fig. 6F and G). Consistent with p-STAT5 expression and morphology, major milk protein gene expression (*Wap* and *Csn2*) was inhibited by P4 treatment ($P<0.0001$) (Fig. 6H). Overall, results demonstrate that progesterone can reverse the excessive

Figure 6

Progesterone can reverse the PRL/p-STAT5 effect at non-pregnancy. Eleven-weeks WT and +*Oxtr* mice were treated with a vehicle or 300 μ g (3 mg/mL) P4 for 7 days. The mammary glands (4rd pair) were harvested from +*Oxtr*, hormone-treated +*Oxtr* and WT littermates at 12 W. (A) Serum prolactin levels of virgin WT and +*Oxtr* mice after P4 treatment, $n=3$. (B) RT-PCR analysis of *Pgr* expression in response to hormonal treatment, $n=3$. (C) RT-PCR analysis of *Oxtr* expression after P4 treatment, $n=3$. (D) Immunoblotting analysis of OXTR, p-STAT5 and RANKL in virgin mammary gland after P4 treatment. (E) Whole-mount staining of mammary glands in response to hormonal treatment. Scale bar: 500 μ m. Original magnifications: $\times 4$. (F) Image of mammary gland (3rd pair) morphology in response to P4 treatment. Scale bar: 1 cm. (G) Quantification of mammary duct caliber (mm) in response to P4 treatment, $n=3$. (H) RT-PCR analysis of major milk protein gene expression in mammary glands in response to P4 treatment, $n=3$. Data are represented as mean $\pm$ S.E.M. ** $P<0.01$; *** $P<0.001$, calculated with one-way ANOVA.

effect of OXTR on mammary gland morphology, gene expression and function by inhibiting prolactin-induced p-STAT5 pathway.

Progesterone exacerbates but prolactin rescues the reduced PRL/p-STAT5 pathway during late pregnancy and lactation

To investigate whether the early involution was caused by reduced prolactin, +*Oxtr* mice were treated with

300 μ g (3 mg/mL) P4 subcutaneously or 50 ng/g PRL intraperitoneally for three consecutive days at late pregnancy (P18.5, P19.5) and early lactation (L1). RT-PCR and immunoblotting results revealed that OXTR expression in mammary gland was inhibited by P4 ($P<0.0001$), but not affected by PRL (Fig. 7A and C). *Prlr* mRNA was also inhibited by P4 ($P<0.0001$), but not affected by PRL (Fig. 7B). In addition, immunoblotting results also confirmed that p-STAT5 was repressed by P4 but enhanced by PRL (Fig. 7C). IHC results indicated

Figure 7

Progesterone can exacerbate but prolactin can rescue STAT5 pathway at late pregnancy and lactation. *++Oxtr* mice were treated with 300 μ g (3 mg/mL) P4 or 50 ng/g PRL for 3 days at late pregnancy (P18.5, P19.5) and L1. The mammary glands (4rd pair) were harvested from *++Oxtr*, hormone-treated *++Oxtr* and WT littermates in lactation (L1). (A) RT-PCR analysis of *Oxtr* mRNA expression at L1 in response to hormonal treatment, $n=3$. (B) RT-PCR analysis of *Prlr* mRNA expression at L1 in response to hormonal treatment, $n=3$. (C) Immunoblotting analysis of OXTR, p-STAT5 and RANKL in L1 mammary gland after hormonal treatment. (D) Whole-mount staining of mammary glands in response to hormonal treatment. Scale bar: 200 μ m. Original magnifications: $\times 10$. (E) Quantification of alveolar size (mm^2), $n=3$. (F) RT-PCR analysis of major milk protein gene expression in mammary glands in response to hormonal treatment, $n=3$. Data are represented as mean $\pm$ s.e.m., calculated with one-way ANOVA.

that PRL rescued impaired morphological changes and restore the nuclear p-STAT5 expression (Supplementary Fig. 3A). RANKL was scarcely detected in lactation (L1) in either WT or *++Oxtr* mice. P4 treatment slightly enhanced RANKL expression, but not PRL (Fig. 7C). Whole-mount staining of mammary gland was performed to assess alveolar morphological changes following hormonal treatment. P4 treatment promoted alveolar proliferation but exacerbated the impaired alveolar differentiation and led to smaller alveolar size in *++Oxtr* mice (Fig. 7D, b, c and E). This enhanced impairment resulted in death of all pups within 24 h after birth (Supplementary Fig. 3B). After prolactin treatment of *++Oxtr* mice, alveolar development was restored to the phenotype similar to WT (Fig. 7D

and E). Similar to p-STAT5 expression and morphological changes, *Wap* and *Csn2* mRNA was intensely inhibited by P4 but restored by PRL (Fig. 7F). These results demonstrate that P4 exacerbates alveolar development in *++Oxtr* mice, while PRL can rescue it through PRL/p-STAT5 pathway during lactation.

Discussion

In this study, we have confirmed that OXTR regulates mammary gland development. OXTR overexpression caused early mammary gland development and involution through disrupting hormone-regulated pathways.

Overexpression of OXTR increased prolactin-induced STAT5 activation, leading to premature secretory differentiation and early milk production at non-pregnancy and early pregnancy. In lactation, *++Oxtr* mice exhibited decreased prolactin-induced STAT5 phosphorylation, resulting in a severe impairment in mammary gland differentiation, early involution of alveolar and failure of nurturing.

PRL/p-STAT5 pathway functions as a link between OXTR and mammary gland development in the study. PRL/p-STAT5 pathway is involved in alveolar development with lactogenic differentiation during pregnancy and lactation (Han *et al.* 1997, Cui *et al.* 2004). OXTR overexpression induced continuous higher PRL in non-pregnant *++Oxtr* mice. This altered micro-environment-augmented PRL-induced phosphorylation as well as nuclear translocation of p-STAT5. Increased nuclear p-STAT5 contributed to differentiation of alveolar, demonstrated by transcription of milk protein genes (*Csn2* and *Wap*) and early milk production at non-pregnancy and early pregnancy. Prolactin overproduction with unexpected milk secretion (galactorrhea) is the symptoms of hyperprolactinemia (Mancini *et al.* 2008). From pregnancy to lactation, WT PRL rose and maintained at very high levels. *++Oxtr* mice, instead, exhibited insufficient PRL at pregnancy and lactation, leading to low nuclear p-STAT5, impaired alveolar development, early involution and lactogenic failure. Indeed, PRL treatment rescued the impaired alveolar development by activation of PRL/p-STAT5 axis. Lactation failure in *++Oxtr* mice is similar to the phenotypes lacking prolactin (*Prl*^{-/-}),

its receptor (*Prlr*^{-/-}), Jak2 (*Jak2*^{-/-}) and lack of activated STAT5 (Horseman *et al.* 1997, Liu *et al.* 1997, Ormandy *et al.* 1997, Parganas *et al.* 1998, Teglund *et al.* 1998, Wagner *et al.* 2004). In summary, OXTR overexpression under β -actin promoter induces abnormal mammary gland development through PRL-induced STAT5 phosphorylation.

A link between OXTR and progesterone/RANKL is also emerged from our study. The progesterone-RANKL axis is involved in mammary epithelium proliferation (Beleut *et al.* 2010). OXTR overexpression caused low serum progesterone at various development stages, indicating OXTR plays a negative role in the regulation of progesterone. Surprisingly, RANKL as a downstream mediator of progesterone to promote cell proliferation was upregulated in non-pregnant and early pregnant *++Oxtr* mammary gland. Since RANKL expression is regulated by both progesterone and prolactin (Fata *et al.* 2000, Mulac-Jericevic *et al.* 2003), it is likely that prolactin axis causes the increase of RANKL despite low progesterone at non-pregnancy and early pregnancy. Undetectable RANKL in lactation may be due to low progesterone and blockage of prolactin axis to induce RANKL. The hypothesis is supported by the fact that progesterone treatment increased RANKL expression and promoted proliferation, but prolactin treatment had no effect on RANKL expression in lactation. RANKL likely serves as a balance between progesterone and prolactin action in mammary gland. Progesterone treatment of *++Oxtr* females at different development stages

Figure 8

Role model of OXTR in mammary gland development. OXTR regulates mammary gland development through progesterone/RANKL axis and PRL/pSTAT5 axis. OXTR regulates PRL/p-STAT5 pathway. Activated STAT5 (p-STAT5) translocates to nucleus to regulate the transcription of *Csn2* and *Wap*. The expression of milk protein *Wap* and *Csn2* marks the mammary epithelium differentiation and maturation. OXTR overexpression inhibits progesterone level. Progesterone/RANKL pathway mediates mammary epithelium proliferation but inhibits PRL/p-STAT5-induced differentiation. RANKL is regulated by both prolactin and progesterone, but prolactin-induced RANKL is prevented in lactation. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-18-0356>.

suppressed OXTR expression in mammary gland. This is consistent with the view that progesterone acts as a negative regulator of OXTR in mammary gland (Grazzini *et al.* 1998).

A crosstalk is present between progesterone/RANKL and PRL/p-STAT5 in OXTR-induced abnormal mammary gland development. In WT, progesterone and prolactin both rose during pregnancy and orchestrated proliferation and differentiation of mammary epithelium in preparation for lactation. Prolactin was maintained at a high level, while a precipitous decrease of progesterone happened at parturition (Fig. 5A and B). The drop is necessary for prolactin-controlled events associated with lactogenic differentiation (Kuhn 1969, Virgo & Bellward 1974). Progesterone treatment of *++Oxtr* mice at late pregnancy and lactation aggravated the inhibition on PRL/p-STAT5 axis, worsen the impaired alveolar differentiation and led to offspring death within 24h of birth (Supplementary Fig. 3B). Activated PRL/p-STAT5 axis and increased secretory differentiation in non-pregnant *++Oxtr* mice may be due to the low progesterone. Indeed, progesterone treatment at non-pregnancy reversed the OXTR-induced secretory activation by inhibiting PRL/p-STAT5 axis. Progesterone plays a negative role in OXTR-regulated prolactin/STAT5 pathways. Since RANKL can inhibit prolactin-induced lactogenic activity through STAT5 (Cordero *et al.* 2016), blockage of prolactin-induced RANKL at late pregnancy and lactation is important. Collectively, mammary gland development depends on the balance of proliferation and differentiation. OXTR overexpression disrupts this balance.

The results of mammary gland transplantation demonstrated that abnormal mammary gland development was mainly dependent on OXTR-induced hormonal changes but not OXTR in mammary gland. OXTR in brain responds to exogenous oxytocin and stimulates prolactin release from pituitary lactotroph (Tabak *et al.* 2010, Kennett & McKee 2012). Neuron-specific overexpression of OXTR may shed light on this.

Investigation of OXTR on mammary gland development provides a working model that OXTR-induced accelerated mammary gland development at non-pregnancy and early pregnancy but lactation failure through progesterone/RANKL and PRL/p-STAT5 pathway (Fig. 8). OXTR regulates PRL/p-STAT5 pathway to act on mammary epithelium differentiation, mammary gland maturation and milk production. OXTR overexpression inhibits progesterone secretion and disrupts the balance between progesterone and PRL/p-STAT5. Progesterone/RANKL pathway mediates mammary epithelium

proliferation but inhibits PRL/p-STAT5 induced differentiation. RANKL is regulated by both prolactin and progesterone, but prolactin-induced RANKL is prevented in lactation. OXTR induces abnormal mammary gland development through hormonal changes.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-18-0356>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Y W Z, D L, X C F, L Q Z and Y J conceived and designed the experiments. D L, Y J, C L Z, Y P Y, M J S, J Z and M J Z carried out experiments. D L, H H J and A L Y analyzed the data. D L and Y C contributed reagents/materials/analysis tools. Y W Z and D L wrote the paper. All authors were involved in revising the manuscript and had final approval of the submitted and published versions.

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