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Progesterone induces expression of *Lrp2* in the murine uterus

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ABSTRACT

Progesterone (P4) and progesterone receptor (PR) have important functions in uterine environment. In previous studies, using high density DNA microarray analysis, we identified low density lipoprotein receptor-related protein 2 (*Lrp2*) is one of the genes upregulated by P4 and PR. In present studies, we examined the expression of *Lrp2* through real-time PCR, *in situ* hybridization and immunohistochemistry by P4-PR response. *Lrp2* mRNA transcript was significantly increased after P4 treatment in the luminal and glandular epithelium of the wild-type mice. However, *Lrp2* expression was not observed in the progesterone receptor knock out (PRKO) mice treated with P4. The expression of *Lrp2* expression is not regulated by estrogen. During early pregnancy, the expression of *Lrp2* was detected at 2.5 dpc and then significantly increased at 3.5 dpc in luminal and glandular epithelium. These results suggest that *Lrp2* is a novel target gene by P4 and PR.

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1. Introduction

The uterus is consisted of heterogeneous cell including myometrium and endometrium that consist of luminal, glandular epithelial cells and stromal cells [1,2]. It undergoes dynamic changes during menstrus or estrus cycle and to support successful pregnancy. During these phenomena, the regulation of progesterone (P4) and estrogen (E2) is required to maintain uterus health and support embryo development [3,4]. In early pregnancy, temporal E2 stimuli and P4 priming are essential for embryo implantation [5]. The P4 is a critical regulator of reproductive events associated with the embryo development and maintenance of pregnancy [6]. This action is mediated through its receptor, the progesterone receptor (PR), which exists as two predominant isoforms, PR-A and PR-B, generated from the identical gene [7,8]. The expression pattern of PR has changed in mice during the peri-implantation period. PR is upregulated in the luminal epithelium (LE) from 0.5 days post coitum (dpc) to 1.5 dpc and in both LE and stroma cells during 2.5 dpc and 3.5 dpc, and quickly disappeared from the LE in the postimplantation 4.5 dpc [4,9]. These patterns unequivocally demonstrated the vital importance of P4 and its receptor in establishment and maintenance of pregnancy [6,10]. However, despite the importance of P4-PR signaling, the precise mechanism is not fully understood on uterine function. The progesterone receptor knock-out (PRKO) mice have the several reproductive defects such as infertility and malfunction of ovulation, implantation and decidualization [6,10]. We identified target genes of PR in the mouse uterus through oligonucleotide microarray [11]. Among them, we found that the expression of low density lipoprotein receptor-related protein 2 (*Lrp2*) was upregulated in the response of P4.

Low density lipoprotein receptor-related protein 2 (*Lrp2*) is 600-kDa transmembrane protein belonging to the low-density lipoprotein (LDL)-receptor family. It is heavily expressed in epithelial cell types. *Lrp2* is an endocytic receptor and expressed in the number of steroid-responsive tissues, particularly in the male and female reproductive organs such as epididymis, prostate, ovaries, and uterus [12]. LRP2 has several ligands associated to various development process such as Sonic Hedgehog (Shh) and BMP4 mechanism, including the absorption of retinoic acid (vitamin A) and vitamin D, immune, and stress response [4,13]. Recently, to investigate the function of *Lrp2*, *Lrp2*-deficient mice have produced by gene targeting, exhibit severe forebrain abnormalities, lung defects as well as tubular reabsorption in the urine [14,15]. Furthermore, in the female reproductive systems, the function of *Lrp2* is related to alteration of the uterine architecture. In this study, we explored the spatiotemporal expression and regulation of *Lrp2* in the response to P4-PR and during early pregnancy.

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2. Materials and methods

2.1. Animals and tissue collection

All procedures for animal study were approved by the institutional animal care guidelines at Michigan State University. To evaluate *Lrp2* expression by steroid hormone regulation, wild-type C57BL/6 mice and PRKO mice at 6 weeks age were ovariectomized and 2 weeks later, the mice were injected with one of the following: vehicle (sesame oil), P4 (1 mg/mouse), or E2 (0.1 μg/mouse) ($n = 3$ per genotype per treatment per time point). The injections subsequently repeated every 24 h. The mice were anesthetized with Avertin (2,2,-tribromoethanol, Sigma–Aldrich, St. Louis, MO) and euthanized by cervical dislocation under anesthetic and uteri were collected at 6 h, or 3 days. For early pregnancy study, wild-type C57BL/6 mice at 8 weeks age were mated with wild-type male mice and different days of pregnant uterine samples were obtained and the morning of vaginal plug was designated as day 0.5 days post coitum (dpc) ($n = 3$). Uterine tissues were immediately frozen at the time of dissection and stored at −80 °C for RNA or fixed with 10% (v/v) formalin for *in situ* hybridization and 4% (v/v) paraformaldehyde for immunohistochemistry.

2.2. Quantitative real-time PCR

RNA was extracted from the uterine tissues using the RNeasy total RNA isolation kit (Qiagen, Valencia, CA). Expression levels of *Lrp2* mRNA were measured by real-time PCR TaqMan analysis with the Applied Biosystems StepOnePlus™ system according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Real-time PCR primers for *Lrp2* (01328172), and *18S* rRNA (4319413E) were purchased from Applied Biosystems. The cDNA was produced from 1 μg of total RNA using random hexamers and M-MLV (Invitrogen Corp., Carlsbad, CA). The real-time PCR was performed using RT-PCR Universal Master Mix reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. All real-time PCR was performed using three independent RNA sets. The mRNA quantities were normalized against *18S* RNA with ABI rRNA control reagents. Statistical analyses used one-way ANOVA followed by Turkey post hoc multiple range test or Student *t*-test using the Instat package from GraphPad (San Diego, CA).

2.3. In situ hybridization

The protocol for *in situ* hybridization was essentially as described previously by Simmons et al. (1989). Uterine tissues were fixed in 10% (v/v) formalin. After overnight fixation at room temperature, tissues were dehybrated through a series of ethanol and then processed for paraffin embedding. Paraffin sections were mounted onto poly-L-lysine-coated slides (VWR Scientific Products, West Chester, PA), and used for *in situ* hybridization. The riboprobes were generated by *in vitro* transcription of amplified DNA products containing the T7 polymerase promoter sequence flanking the desired nucleotide primer sequence, using ^{35}S-UTP (Promega, Fitchburg, WI). Slides were incubated for 7 min at room temperature in Proteinase K (20 μg/ml) in a buffer containing 50 mM Tris and 5 mM EDTA (pH 8). Slides were then acetylated with acetic anhydride, dehydrated and exposed to either denatured antisense or sense probes in hybridization buffer (50% (v/v) formamide, 10% (w/v) dextran sulfate, 5 Denhardt's solution, 300 mM NaCl, 5 mM EDTA (pH 8), 20 mM Tris (pH 8) and 0.05 mg/ml yeast tRNA). Hybridization was performed at 55 °C

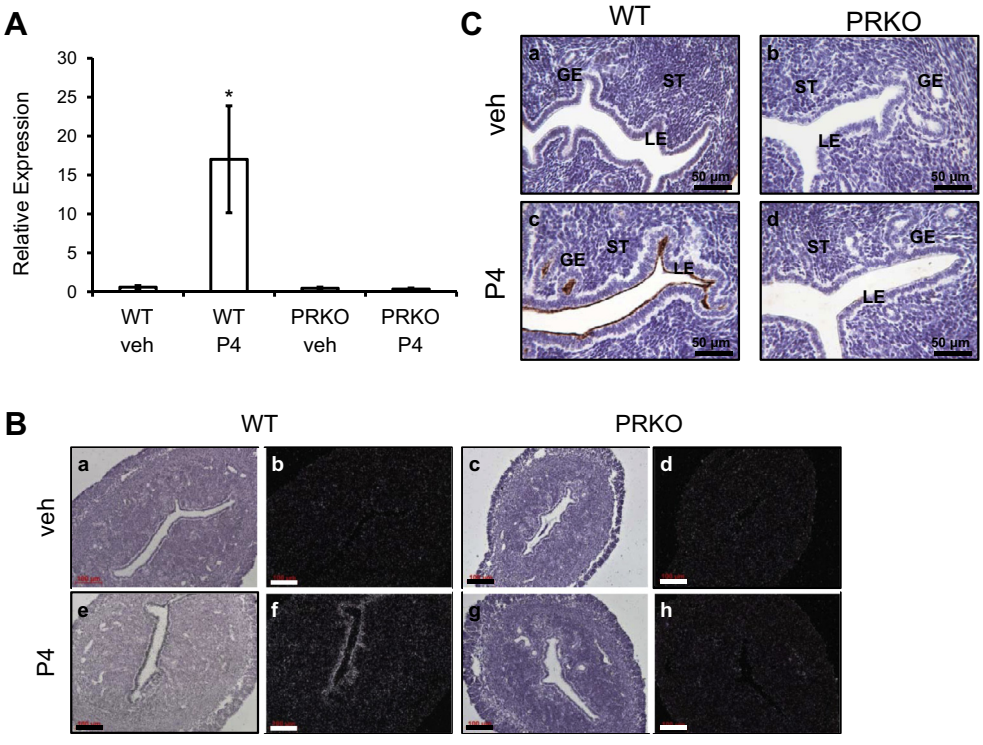

Fig. 1. The expression of *Lrp2* in wild-type (WT) or progesterone receptor knock-out mouse (PRKO) mice. (A) The expression pattern of *Lrp2* from progesterone (P4) treated WT or PRKO uteri by real-time PCR. Total RNA used for the real-time PCR assay was prepared from wild-type or PRKO uteri that were treated with P4 or vehicle (sesame oil) for 6 h (hrs). The results represent the mean ± SEM of three independent RNA sets. $^{*}p < 0.05$. (B) The localization pattern of *Lrp2* transcription by *in situ* hybridization in the WT and PRKO mice. Uterine sections from WT or PRKO uteri were treated with P4 or vehicle for 6 h. Nuclei were lightly counterstained with hematoxylin. Scale bar = 100 μm. (C) The localization pattern of LRP2 by immunohistochemistry in the vehicle or P4-treated uteri. Uterine sections were collected from P4 or vehicle treated WT and PRKO mice for 6 h. Nuclei were counterstained with hematoxylin. LE, luminal epithelium; GE, glandular epithelium; ST, stroma cells.

overnight in a humidity chamber containing 5 SSC and 50% (v/v) formamide. Hybridized slides were exposed to 20 μg/ml RNase A for 30 min at 37 °C. Slides were washed in 50% (v/v) formamide, 2 SSC at 55 °C for 30 min, dehydrated in a graded series of ethanol in 0.3 M ammonium acetate, and following morning, slides were dipped in autoradiography emulsion (Amersham, Pittsburgh, PA) and placed at 4 °C in a light-proof box for several days. After development, slides were counterstained with hematoxylin.

2.4. Immunohistochemistry

Uterine sections from paraffin-embedded tissue were cut at 6 μm and mounted on silane-coated slides, deparaffinized, and dehydrated in a graded alcohol series. Sections were preincubated with 10% normal rabbit serum in PBS (pH 7.5) and then incubated with primary antibody diluted in 10% normal rabbit serum in PBS (pH 7.5) overnight at 4 °C at the following dilution: 1:1000 for LRP2 (Santa Cruze, Santa Cruz, CA). On the following day, sections were washed in PBS and incubated with 1:500 diluted secondary antibody, and washed three times with PBS. Horseradish peroxidase-conjugated streptavidin (Vector Laboratories, Burlingame, CA) at a dilution of 1:1000 was added to the slides and incubated for 30 min. Immunoreactivity was detected using DAB (Vector Laboratories). After this, the sections were counterstained with hematoxylin for 30 s. The slides were subsequently washed in water and rehydrated, then mounted. The immunostained sections were observed under a microscope.

2.5. Statistical analysis

Statistical analyses were performed using one-way ANOVA analysis followed by Tukey's post hoc multiple range test using the Instat package from GraphPad (GraphPad Software, Inc., San Diego, CA). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Spatial expression pattern of Lrp2 as progesterone target gene in murine uterus

We identified *Lrp2* as a potential P4- and PR-regulated gene in the murine uterus by using DNA microarray analysis [11]. In this study, we analyzed the expression of *Lrp2* mRNA and protein *by* the P4- and PR-regulation in the murine uterus. We performed real-time PCR in the vehicle (sesame oil) or P4-treated uterine samples of ovariectomized wild-type and PRKO female mice treated with P4 for 6 h. As shown in Fig. 1A, the mRNA transcript of *Lrp2* was significantly upregulated in the uteri of P4 treated wild-type mice compared to vehicle treated in wild-type mice. To investigate whether the P4 regulation of *Lrp2* is relevant to PR, we examined the expression of the *Lrp2* in the uteri of wild-type and PRKO mice. After P4 treatment, induction of the *Lrp2* was not detected in the PRKO mice (Fig. 1A). These results show that *Lrp2* mRNA expression is regulated by P4 and PR in uterus.

To analyze the spatial expression of *Lrp2* by P4 in the uterus, we performed *in situ* hybridization in the vehicle or P4-treated wild-type and PRKO mice. Consistent with the real-time PCR outcomes, we observed *Lrp2* signal in the glandular and luminal epithelium of the uterine section obtained from P4-treated wild-type uterus in the results of *in situ* hybridization (Fig. 1B). The *Lrp2* signals were not detected in the vehicle treated wild-type uterine sections and vehicle or P4 treated PRKO uterine sections. To further demonstrate the expression of LRP2 protein, we performed the immunohistochemistry for LRP2 in vehicle or P4 treated wild-type and

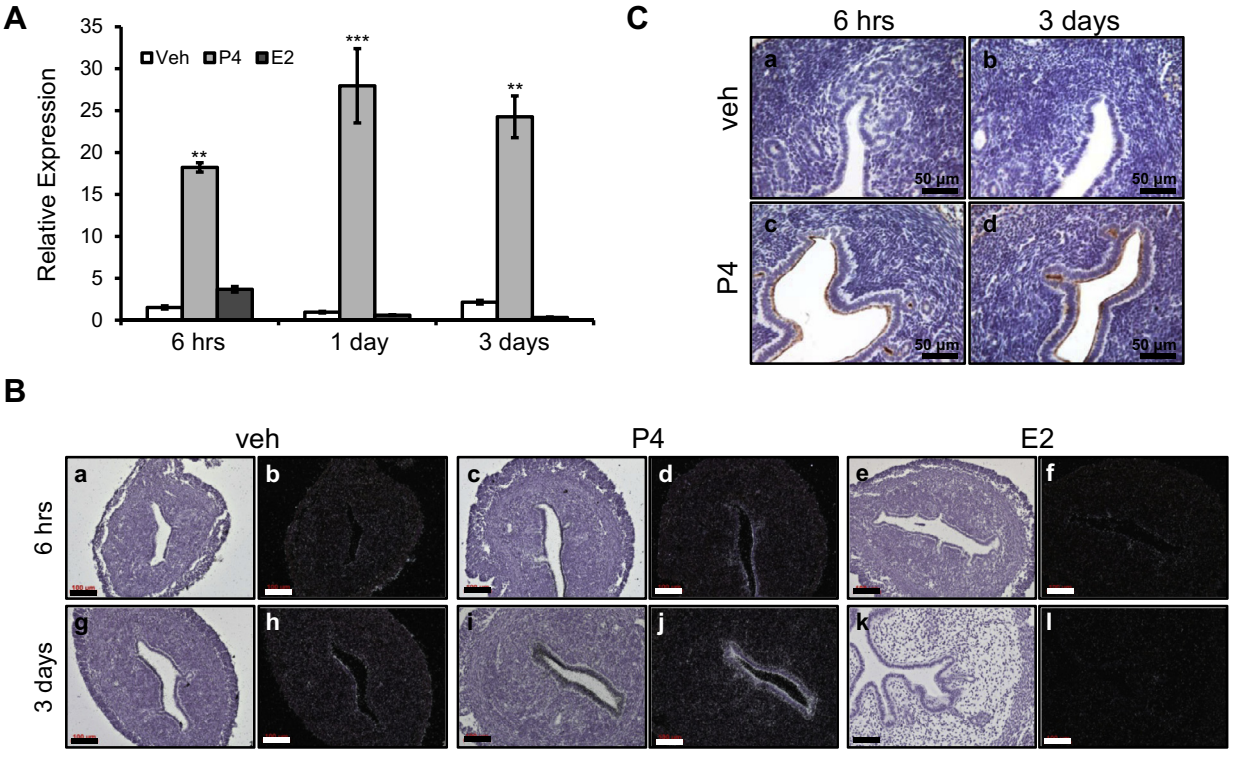

Fig. 2. The expression of *Lrp2* by steroid hormones. (A) The expression level of *Lrp2* from vehicle, P4, or esterogen (E2) treated uteri by real-time PCR. Total RNA used for the real-time PCR assays was prepared from WT mice treated with vehicle, P4, or E2 for 6 h 1 day or 3 days. $^{**}p < 0.01$; $^{***}p < 0.001$. (B) The localization pattern of *Lrp2* transcription by *in situ* hybridization in with vehicle, P4, or E2 treated uteri for 6 h, or 3 days. Nuclei were lightly counterstained with hematoxylin. Scale bar = 100 μm. (C) The localization pattern of LRP2 by immunohistochemistry in vehicle, or P4 treated wild-type mice uteri for 6 h, or 3 days. Nuclei were counterstained with hematoxylin.

PRKO uterine sections. The pattern of LRP2 immunostaining was also similar to that of *Lrp2* mRNA signal. The LRP2 was highly expressed in luminal and glandular epithelial surface of P4 treated wild-type uterus, whereas there is no signal in the vehicle treated wild-type uterine sections and vehicle or P4 treated PRKO uterine sections (Fig. 1C). These results indicate that the regulation of P4 and PR is essential for *Lrp2* gene regulation in luminal and glandular epithelium cells.

3.2. Regulation of Lrp2 by estradiol and progesterone in the uterus

To investigate whether steroid hormone differences contribute differenced in *Lrp2* expression, we treated ovariectomized female mice with vehicle (sesame oil), P4 (1 mg/mouse), or E2 (0.1 μg/mouse). Uteri were collected from the mice 6 h, or 3 days after hormone treatment, and the expression of *Lrp2* was investigated by real-time PCR. As shown in Fig. 2A, the *Lrp2* mRNA level was significantly increased after P4 treatment in the wild-type mice compared with other groups. However, the expression of *Lrp2* was not induced in the wild-type mice after E2 treatment. Next, we performed *in situ* hybridization to identify the spatial expression of the *Lrp2* expression (Fig. 2B). *Lrp2* signal was seen in luminal and glandular epithelium after P4 treatment. However, we could not detect any signal in vehicle or E2 treated mice. We also identified LRP2 protein expression in the uteri of vehicle or P4 treated mice by immunohistochemistry. LRP2 protein was highly expressed in the epithelial apical surface in P4 treated the wild-type mice uteri. However, no signal was seen in vehicle treated the wild-type mice uteri (Fig. 2C). These results suggest that E2 could not induce the expression of *Lrp2* but P4 regulates *Lrp2* expression in uteri.

3.3. The expression of the Lrp2 during early pregnancy

The above analysis demonstrated the regulation of *Lrp2* by P4. During early pregnancy, P4 and temporal E2 induction are important for preparing blastocyst attachment. To investigate the expression profile of *Lrp2* mRNA in mouse uteri during early pregnancy, we performed real-time PCR. The initiation of pregnancy was marked by the presence of the postcoital vaginal plug (0.5 dpc). As shown in Fig. 3A, the expression *Lrp2* was detected on 2.5 days post coitum (dpc) and then its expression was significantly increased at 3.5 dpc ($p < 0.001$) compared to 0.5 dpc. *Lrp2* mRNA levels were downregulated from 4.5 dpc to 7.5 dpc. Next, to observe the spatial expression profile of LRP2, we assayed the expression profile of LRP2 by immunohistochemistry from 0.5 dpc to 7.5 dpc uteri of natural pregnancy (Fig. 3B). On 3.5 dpc, LRP2 was strongly expressed in the apical surface of luminal and glandular epithelial cells. These spatial patterns of LRP2 expression are similar to the ones observed in the uteri of P4 treated wild-type mice. These results suggest that the expression of *Lrp2* may have an important role during early pregnancy.

4. Discussion

Steroid hormones participate in the development and reproduction, and bind to carrier proteins that deliver hormones to target cells and subsequently, induce the transcription of correlated target genes. P4 is a crucial steroid hormone in the female reproductive system associated with the establishment and maintenance of pregnancy [8,16–18]. P4 regulation could be accomplished through progesterone receptors (PRs). The PRs are

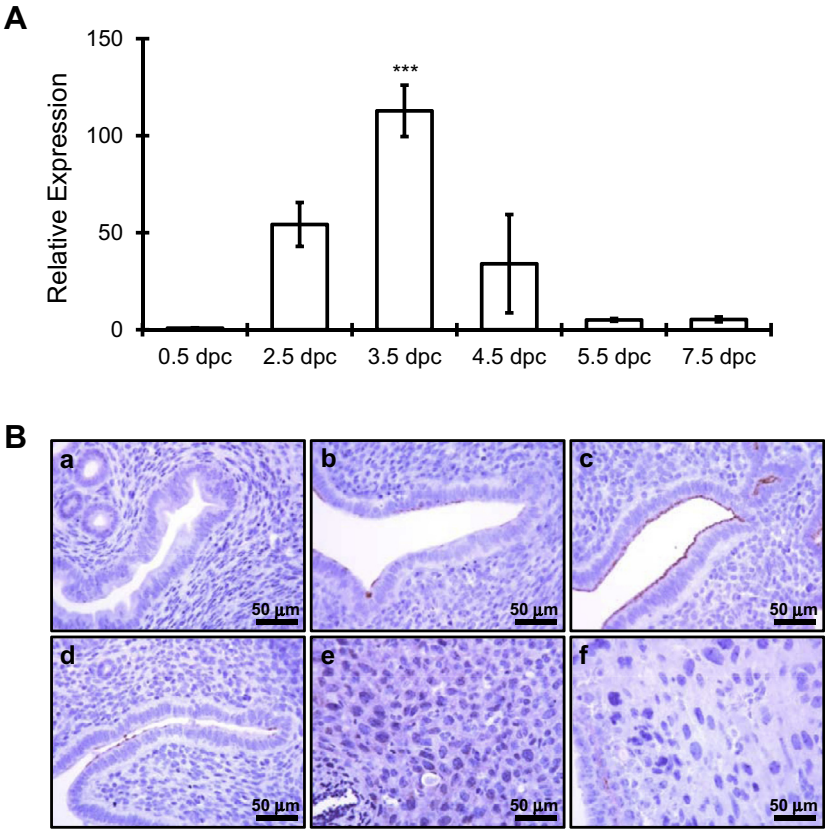

Fig. 3. The expression level of *Lrp2* in pseudopregnancy and the localization pattern of LRP2 during natural pregnancy. (A) The expression level of *Lrp2* in uteri of pseudopregnancy by real time PCR. Total RNA used for real-time PCR assays was prepared from pseudopregnant uteri. The results represent the mean ± SEM of three independent RNA sets. ***$p < 0.001$. (B) Protein expression of LRP2 during natural pregnancy by immunohistochemistry staining. These figures show the LRP2 expression at 0.5 dpc (a), 2.5 dpc (b), 3.5 dpc (c), 4.5 dpc (d), 5.5 dpc (e), and 7.5 dpc (f). LRP2 expression peaks on day 3.5 of pregnancy. Nuclei were counterstained with hematoxylin.

constituted with two isoforms, PRA and PRB, which are produced from the single gene containing alternative translation initiation site. In 1995 and 1996, Lydon et al. investigated the P4-regulated pathways by using the mouse model carrying a null mutation of the progesteron receptor (PRKO) [6,10]. The binding of P4 to PR leads translocation of PR to nucleus and stimulates P4 target genes. Although the genes play important roles in embryo implantation and maintain uterine health, these have underscored and only a few P4-PR regulated genes have identified. We have identified P4-regulated genes by microarray analysis. Recently, Rubel et al. successfully identified the PR regulated mechanism and downstream targets thorough chromatin immunoprecipitation followed by deep sequencing (ChIP-seq) [19]. This study demonstrates important dataset that will be useful in the identification of novel regulated transcription factors in normal uterine regulation through P4-PR mechanism and identifies an apparent majority of PR transcription regulation was occurred after acute treatment with P4. Among them, *Lrp2* was the strongest increased gene when exposed to P4. In the present study, we also provide clear evidence that the expression of *Lrp2* is changed by P4-PR response in the murine uterus.

LRP2 (also termed megalin) has huge molecular mass (600 kDa), belongs to LDLR family, and has been known as an endocytic receptor for various ligands, including lipoprotein, steroid-binding protein, and retinoid. LRP2 has been detected in multiple tissues including the male reproductive tract, uterus, oviduct, and intestinal brush border [20–22]. LRP2 is associated to proteolytic activity between the transmembrane and cytoplasmic domain. The combination of ligand and LRP2 activates shedding of its ectodomain by protein kinase C (PKC)-involved matrix metalloproteinase. Cytoplasmic C-terminal fragment is cleaved by β-secretase, and released into the cytoplasm, whereby presumably the function of LRP2 as a transcriptional regulator in the nucleus [23,24]. In detail, the role of *Lrp2* has been identified in embryonic renal development, vitamin D homeostasis, sex hormone uptake, and holoprosencephaly (HPE). Also, there is a report that *Lrp2* participates in the development of female reproductive organ. In wild-type mice, the vaginal cavity open 4–5 weeks after birth [25], but megalin-deficient mice possess a closed vaginal cavity, resulting in gross inflation of the uterus owing to fluid accumulation [26]. The histology appearance of megalin-deficient mice suggests that the defective signaling of steroid hormones is responsible for this malformation. However, the definitive functions such as steroid hormone regulation and embryo implantation have not been studied in female reproductive system.

Previously, Pan et al. identified that *Lrp2* mRNA levels are unregulated by P4 and E2 responses compared to E2 [27]. In our study, (The *Lrp2* mRNA and LRP2 protein levels were significantly upregulated by P4 in wild-type mice, but not in PRKO (Fig. 1). The increase of *Lrp2* also investigated in P4 treated uterine section. Both P4 and E2 are important for maintenance of female reproductive system and implantation. Especially, P4 is required throughout pregnancy. Embryo implantation depends on hormone regulation and endometrium status that are the proliferation of stroma cells and the differentiation of endometrial cells to prepare the blastocyst attachment. In mice uterine section of early pregnancy, the LRP2 expression is the highest at 3.5 dpc (Fig. 3) in the membrane of epithelial cells. These results suggest that *Lrp2* may provide direct regulation mediator by P4-PR response and be important in the uterus of mouse during early pregnancy.

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