BIOLOGY OF REPRODUCTION **85,** 386–396 (2011)
Published online before print 20 April 2011.
DOI 10.1095/biolreprod.111.091769

Postnatal Deletion of *Wnt7a* Inhibits Uterine Gland Morphogenesis and Compromises Adult Fertility in Mice[1]

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ABSTRACT

The success of postnatal uterine morphogenesis dictates, in part, the embryotrophic potential and functional capacity of the adult uterus. The definitive role of *Wnt7a* in postnatal uterine development and adult function requires a conditional knockout, because global deletion disrupts müllerian duct patterning, specification, and cell fate in the fetus. The *Wnt7a*-null uterus appears to be posteriorized because of developmental defects in the embryo, as evidenced by the stratified luminal epithelium that is normally found in the vagina and the presence of short and uncoiled oviducts. To understand the biological role of WNT7A after birth and allow tissue-selective deletion of *Wnt7a*, we generated loxP-flanked exon 2 mice and conditionally deleted *Wnt7a* after birth in the uterus by crossing them with *Pgr^{Cre}* mice. Morphological examination revealed no obvious differences in the vagina, cervix, oviduct, or ovary. The uteri of *Wnt7a* mutant mice contained no endometrial glands, whereas all other uterine cell types appeared to be normal. Postnatal differentiation of endometrial glands was observed in control mice, but not in mutant mice, between Postnatal Days 3 and 12. Expression of morphoregulatory genes, particularly *Foxa2*, *Hoxa10, Hoxa11, Msx1,* and *Wnt16*, was disrupted in the *Wnt7a* mutant uteri. Conditional *Wnt7a* mutant mice were not fertile. Although embryos were present in uteri of mutant mice on Day 3.5 of pregnancy, blastocyst implantation was not observed on Day 5.5. Furthermore, expression of several genes (*Foxa2, Lif, Msx1,* and *Wnt16*) was reduced or absent in adult *Wnt7a*-deleted uteri on Day 3.5 postmating. These results indicate that WNT7A plays a critical role in postnatal uterine gland morphogenesis and function, which are important for blastocyst implantation and fertility in the adult uterus.

developmental biology, female reproductive tract, pregnancy, transgenic/knockout model, uterus

INTRODUCTION

The histological organization of the adult uterine endometrium consists of a simple columnar luminal epithelium (LE) supported by stromal cells that contain coiled endometrial glands lined by simple columnar epithelial cells or glandular epithelium (GE) [1–3]. Circular and longitudinal layers of smooth muscle, termed the myometrium, surround the endometrium. Development of the uterus begins prenatally with formation, patterning, and then fusion of the müllerian ducts [4–6]. Although the organogenetic development and differentiation of most female reproductive tract organs from the müllerian ducts are complete at birth, the uterus is neither fully developed nor differentiated at birth. Establishment of tissue-specific uterine histoarchitecture is only completed postnatally in laboratory rodents, domestic animals, and humans [1, 7–9]. Postnatal radial patterning morphogenesis establishes the three classic histological elements of the uterine wall: the endometrium, the myometrium, and the perimetrium. Events common to postnatal morphogenesis of uteri include coordinated development of the endometrial glands from the LE, organization and stratification of endometrial stroma, and differentiation and growth of the myometrium [1, 6–8].

At birth, the neonatal mouse uterus lacks endometrial glands and consists of a simple LE supported by relatively undifferentiated mesenchyme [3]. Between birth (Postnatal Day [PND] 0) and PND 3, the three layers of mesenchyme are distinctly segregated into radially oriented endometrial stroma and inner circular and prospective outer longitudinal myometrial layers. By PND 6, epithelial invaginations appear that represent formation of GE buds [10]. By PND 12, endometrial glands extend from the LE into the surrounding endometrial stroma, and the outer longitudinal layer of the myometrium is fully organized into bundles [3]. The basic adult configuration of the uterus in mice is established by PND 15 [11]. Postnatal uterine morphogenesis is a critical period, because disruption of endometrial adenogenesis and mesenchymal specification and differentiation can cause permanent fertility problems in the adult [12–14]. Moreover, proper development of the endometrial stroma and myometrium is crucial for endometrial receptivity and decidualization as well as for expulsion of the fetus at term [15].

Postnatal uterine morphogenesis is complex and governed by intrinsic stromal-epithelial interactions that are precisely orchestrated by multifactorial gene networks as well as endocrine hormones after puberty [16]. *Wnt* genes encode secreted glycoproteins that are homologous to the *Drosophila*

[1]Supported by National Institutes of Health grants 1 R21 HD054679 (to T.E.S.) and 1 R01 HD30284 (to R.R.B.). Portions of this work will be presented at the 44th Annual Meeting of the Society for the Study of Reproduction, July 31–August 4, 2011, Portland, Oregon.
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Received: 15 February 2011.
First decision: 8 March 2011.
Accepted: 22 March 2011.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

segment polarity gene *wingless* (*wg*) and regulate stem cell fate, cell differentiation, and tissue growth [17] via canonical and noncanonical signaling pathways [18]. The canonical WNT signaling pathway involves binding of frizzled (FZD) receptors and inhibition of catenin (cadherin-associated protein), beta 1 (CTNNB1) degradation, resulting in nuclear translocation and activation of target genes. Noncanonical WNT signaling encompasses a variety of signaling pathways that involve different WNT receptors (i.e., ROR2 and RYK) and mediators (i.e., calcium, MAPK8/9/10, and CAMK2) that regulate cell migration and movement. A subset of *Wnts* (*Wnt4*, *Wnt5a*, and *Wnt7a*) is involved in müllerian duct patterning and differentiation during development of the female reproductive tract in the embryo [14, 19, 20]. Postnatal uterine morphogenesis also involves a number of different *Wnts* and their signaling pathways. Total ablation of *Wnt7a* and *Wnt5a* or conditional ablation of *Wnt4* and *Ctnnb1* in the uterus after birth alters postnatal uterine development [21–23].

Adult *Wnt7a*-null mice are viable but infertile and exhibit malformations in the female reproductive tract, including shortened and uncoiled oviducts, hypoplastic uterine horns, and a vaginal septum tract [23, 24]. *Wnt7a*-null female mice display abnormal morphogenesis along the anteroposterior and radial axes of the uterine horn during postnatal development [20]. The most prominent feature is an absence of uterine glands, which are normally derived from the LE, and a reduction in the mesenchymally derived uterine stroma so that the inner circular and outer longitudinal layers of myometrium, which are present but reduced and disorganized, are much closer to the endometrial LE. Thus, in the absence of *Wnt7a* from conception, both epithelial and mesenchymal differentiation is disrupted in the uterus. Available evidence supports the idea that the abnormalities in epithelial growth and tubulogenesis in the uteri of *Wnt7a*-null mice is caused by müllerian duct dysgenesis in the embryo [25, 26]. The female reproductive tract of adult *Wnt7a*-null mice appears to be posteriorized, in which the posterior oviduct is more similar to the uterus and the uterus has characteristics of the vagina [23]. The *Wnt7a*-null uterus has a stratified LE (in contrast to simple columnar LE in the wild-type uterus) surrounded by a shallow stromal layer that does not contain glands. A stratified type of LE is characteristic of the vagina but not of normal uterus or oviduct. The myometrium appears to be hyperplastic and disorganized by 3 mo of postnatal development, and by 6 mo, the endometrial stroma is displaced by the myometrium [23, 27]. Although to our knowledge it has not been investigated in the uterus, WNT7A signals through the canonical WNT/catenin, beta 1 signaling pathway in other cells and organs [28–31]. Recent studies indicate that the canonical WNT-catenin signaling is required for mesenchymal differentiation into stroma and myometrium [32], endometrial gland development [21], estrogen-induced uterine growth [33], and implantation in adult mice [34].

The definitive role of *Wnt7a* in postnatal uterine development and function requires a conditional knockout, because global deletion of this gene in the embryo disrupts fetal müllerian duct patterning, specification, and cell fate. To understand the biological role of WNT7A in the neonate and adult, we generated loxP-flanked exon 2 mice and conditionally deleted *Wnt7a* after birth by crossing them with Pgr^{Cre} mice. The conditional ablation of *Wnt7a* after birth disrupted endometrial gland development and expression of morphoregulatory genes in the neonate, which resulted in a failure of blastocyst implantation in the adult. Thus, *Wnt7a* has an important biological role in postnatal uterine development.

MATERIALS AND METHODS

Animals and Tissue Collection

Mice were maintained in the designated animal care facility at Texas A&M University-College Station according to the institutional guidelines for the care and use of laboratory animals, and animal procedures were approved by the Institutional Animal Care and Use Committee. Postnatal samples were obtained after parturition, and the day that pups were observed was considered to be PND 1. Pregnancy samples were obtained by the mating of mice, and the day that a vaginal plug was observed was considered to be Gestational Day (GD) 0.5. At the time of necropsy, reproductive tract tissues were flash-frozen and stored at −80°C or fixed with 4% (vol/vol) paraformaldehyde and paraffin embedded.

Construction of the Targeting Vectors and Generation of Chimeric Mice

The *Wnt7a* gene contains four exons and three introns, and targeted deletion of exon 2 was used to create the original *Wnt7a*-null allele [24]. A targeting construct was made using a bacterial artificial chromosome (BAC) library clone (RPCI-23: Female (C57BL/6J) Mouse BAC Library) containing mouse *Wnt7a* from BACPAC Resources (Children's Hospital Oakland Research Institute). The targeting construct, containing *Wnt7a* fragment with exons 1 and 2, with 2 kb of the 5′ arm and 2 kb of the 3′ arm homology regions (Supplemental Fig. S1A, all Supplemental Data are available online at www.biolreprod.org), was transfected into $5–10 \times 10^7$ E14TG2a embryonic stem cells from 129Sv strain using a Bio-Rad gene pulser. Embryonic stem cell clones were selected positively with G418 (200 μg/ml; Mediatech, Inc.) for presence of the PGK-neo cassette and negatively with ganciclovir (2 μM; InvivoGen) for absence of the MC1TK cassette. Southern blot analysis was used to screen genomic DNA with *Xba*I digestion using 3′ external probes (Supplement Fig. S1B). Correctly targeted clones were microinjected into blastocysts derived from C57BL/6 mice. The F_1 agouti male offspring mice were analyzed by PCR genotyping and Southern blot analysis to validate germ line transmission of the *Wnt7a* flox-neo ($Wnt7a^{flneo}$) allele.

Generation of a Wnt7a Conditional Null Allele

B6.129-$Pgr^{tm2(cre)Lyd}$ (Pgr^{Cre}) mice were provided by Drs. Franco DeMayo and John Lydon of Baylor College of Medicine [35]. B6.Cg-Tg(ACTFLPe)9205Dym (FLPe, Jax 005703) were obtained from the Jackson Laboratory. A PGK-neo cassette flanked by two Frt sites was removed from the $Wnt7a^{floxneo/+}$ allele by crossing the mice to FLPe mice [36], which resulted in generating the $Wnt7a^{fl/+}$ allele. $Wnt7a^{fl/fl}$ mice were then obtained by intercrossing heterozygous $Wnt7a^{fl/+}$ mice. Both the $Wnt7a^{floxneo/+}$ and the $Wnt7a^{fl/+}$ mice were viable and did not display any abnormalities.

The PCR analysis was used to genotype DNA extracts from tail biopsy specimens. Genomic DNA was extracted from mouse tail biopsy specimens via digestion with 300 μl of tail lysis buffer (5 mM ethylenediaminetetra-acetic acid, 200 mM NaCl, 100 mM Tris, and 0.2% SDS) with 0.25 mg of Proteinase K (Sigma Chemical Co., St. Louis, MO) overnight at 55°C. Following incubation, 1 ml of 100% ethanol was added to each tube, and samples were then vortexed and centrifuged at $16\,000 \times g$ for 30 min. Pelleted DNA was washed with 1 ml of 70% ethanol and resuspended in 150 μl of Tris-EDTA buffer (10 mM Tris, 50 mM EDTA).

Genomic DNA from each sample was reverse transcribed in a total reaction volume of 50 μl. Briefly, DNA (50 ng) was combined with appropriate primer mix (W1 and W2 or P1, P2, and P3; Integrated DNA Technologies), dNTP MIX (2.5 mM each; TaKaRa Bio, Inc.), 10× ExTaq Buffer (TaKaRa Bio, Inc.), and TaKaRa Ex Taq (TaKaRa Bio, Inc.). Primers P1, P2, and P3 (P1, 5′-ATG TTT AGC TGG CCC AAA TG-3′; P2, 5′-TAT ACC GAT CTC CCT GGA CG-3′; P3, 5′-CCC AAA GAG ACA CCA GGA AG-3′) were used to amplify the *Pgr* wild-type (285-bp) and *Cre* (590-bp) alleles (Supplementary Fig. S2A). To detect the *Wnt7a* flox allele, primers W1 and W2 (W1, 5′-CAC AGC CAC CCC TAG AGA GCT CAA TT-3′; W2, 5′-ATG CTT TGC CAG GGA ACA CCC-3′) were designed to amplify the fragments from the wild-type (135-bp) and floxed (180-bp) alleles (Supplemental Fig. S2B). All PCR reactions were carried out for 35 cycles of 95°C for 45 sec, 61°C for 1 min, and 72°C for 1 min.

RNA Isolation and Quantitative Real-Time RT-PCR Analysis

To investigate the effect of *Wnt7a* deletion on changes of gene expression in the uterus, quantitative real-time RT-PCR analysis was conducted on RNA extracted from uterine tissues using the Qiagen RNeasy Mini Kit. Total RNA

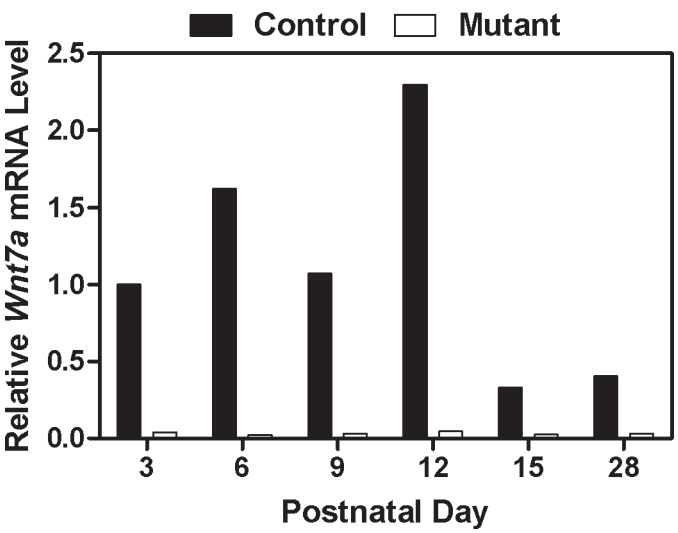

FIG. 1. Analysis of *Wnt7a* conditionally ablated in the uterus. The expression level of *Wnt7a* was measured in the uterus of $Pgr^{+/+}Wnt7a^{f/f}$ control and $Pgr^{Cre/+}Wnt7a^{f/f}$ mutant mice by real-time RT-PCR. The primers were specific for exon 2, which is floxed and deleted by Cre. Data are presented as the fold-change relative to the *Wnt7a* mRNA level on PND 3 in uteri from control mice.

from each sample was reverse transcribed in a total reaction volume of 20 μl. Briefly, total RNA (800 ng) was combined with primer mix containing oligo(dT) primer (0.2 μg/ml), random hexamer primer (300 μg/ml; Invitrogen), and dNTP MIX (10 mM each) and incubated at 65°C for 5 min. An RT mix containing 5× First-Strand Buffer, 0.1 M dithiothreitol, and SuperScript II Reverse Transcriptase (Invitrogen) was added to the reaction, and reverse transcription was performed under the following conditions: 25°C for 10 min, 42°C for 60 min, and 70°C for 5 min. Control reactions in the absence of reverse transcriptase were prepared for each sample to test for genomic DNA contamination. Real-time PCR analysis was performed using an ABI prism 7900HT system (Applied Biosystems). Expression levels of *Fzd6, Fzd10, Hoxa10, Hoxa11, Ihh, Lif, Msx1, Msx2, Wnt4, Wnt5a, Wnt7a, Wnt11, Wnt16,* and *Vangl2* were measured with Power SYBR Green PCR Master Mix (Applied Biosystems) using specific oligonucleotide primers designed by the Oligo 5 program (Molecular Biology Insights, Inc.) (Supplemental Table S1). Mouse *Rpl13a* was used as a reference gene. Expression of *Foxa2* was determined by real-time RT-PCR TaqMan analysis with TaqMan Gene Expression Master Mix (Applied Biosystems) and normalized against *Rn18s*. Real-time probes and primers for *Foxa2* and *Rn18s* were purchased from Applied Biosystems. Each individual sample was run in triplicate using the following conditions: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 sec and 60°C for 1 min. A dissociation curve was generated at the end of amplification to ensure that a single product was amplified. The threshold line was set in the linear region of the plots above the baseline noise, and threshold cycle (Ct) values were determined as the cycle number at which the threshold line crossed the amplification curve.

Immunohistochemistry

Uteri were fixed overnight in 4% (vol/vol) paraformaldehyde, followed by thorough washing in 70% ethanol, and tissues were processed, embedded in paraffin, and sectioned. Uterine sections from paraffin-embedded tissue were cut at a thickness of 5 μm and mounted on slides, deparaffinized, and rehydrated in a graded alcohol series. Sections were subjected to antigen retrieval using boiling citrate buffer, preincubated with 10% normal goat serum in PBS (pH 7.5), and then incubated with appropriate primary antibody. Sections were incubated with either rabbit anti-FOXA2 antibody (catalog no. WRAB-FOXA2; Seven Hills Bioreagents) diluted 1:2000 in 10% normal serum in PBS (pH 7.5) or rabbit anti-MKI67 antibody (catalog no. ab66155; Abcam) diluted 1:750 in 1% bovine serum albumin in PBS (pH 7.5). On the following day, sections were washed in PBS and incubated with biotinylated secondary antibody (5 μg/ml; Vector Laboratories) for 1 h at room temperature. Immunoreactive protein was detected using the Vectastain Elite ABC Kit (Vector Laboratories) and diaminobenzidine tetrahydrocholoride as the chromagen.

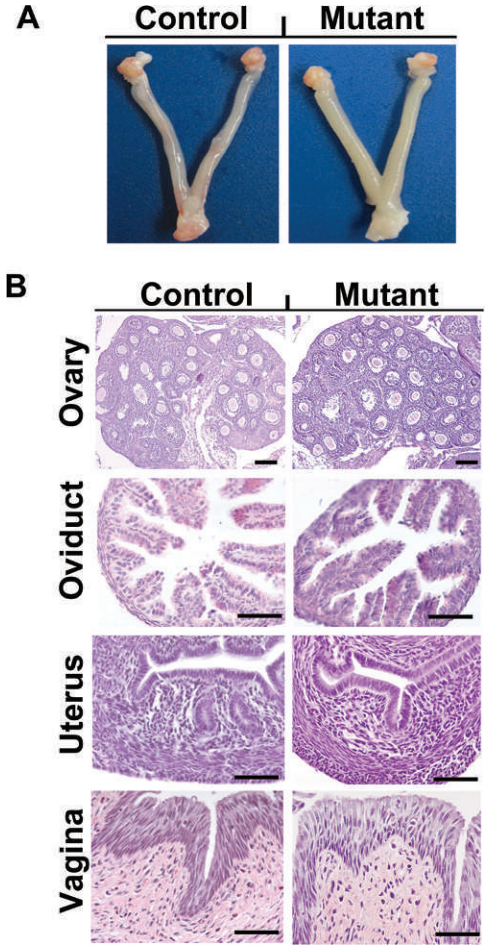

FIG. 2. Postnatal ablation of *Wnt7a* disrupts uterine development. **A**) Gross anatomy of the female reproductive tract of $Pgr^{+/+}Wnt7a^{+/+}$ control and $Pgr^{Cre/+}Wnt7a^{f/f}$ mutant mice on PND 28. **B**) Histological analysis of the ovary, oviduct, uterus, and vagina of $Pgr^{+/+}Wnt7a^{+/+}$ control and $Pgr^{Cre/+}Wnt7a^{f/f}$ mutant mice on PND 28. Reproductive tract tissue sections were stained with hematoxylin and eosin. No differences in histoarchitecture of the ovary, oviduct, or vagina were observed between control and mutant mice. Note the absence of endometrial glands in the uteri of mutant as compared to control mice. Bar = 50 μm.

Statistical Analyses

All quantitative data were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System (SAS Institute, Inc.). In all analyses, error terms used in tests of significance were identified according to the expectation of the mean squares for error. For analysis of real-time PCR data, the Ct values of the target mRNA were analyzed for effects of day, genotype (control or mutant), and their interaction with the *Rpl13a* values used as a covariate. Significance ($P < 0.05$) was determined by probability differences of least-square means (LSMs). The data are presented as the LSM and overall SEM.

RESULTS

Generation of Mice with Conditional Ablation of Wnt7a *in the Uterus*

Ablation of *Wnt7a* leads to disruption of paramesonephric duct differentiation in the fetus [23]. To further investigate the biological role of *Wnt7a* in the uterus after birth, the *Wnt7a* gene was floxed and used to create *Wnt7a* floxed ($Wnt7a^{f/+}$) mice (see Supplemental Fig. S1A for the targeting strategy). Conditional ablation of *Wnt7a* in the uterus was conducted by crossing $Wnt7a^{f/f}$ mice with $Pgr^{Cre/+}$ mice [35]; Pgr^{Cre} mice

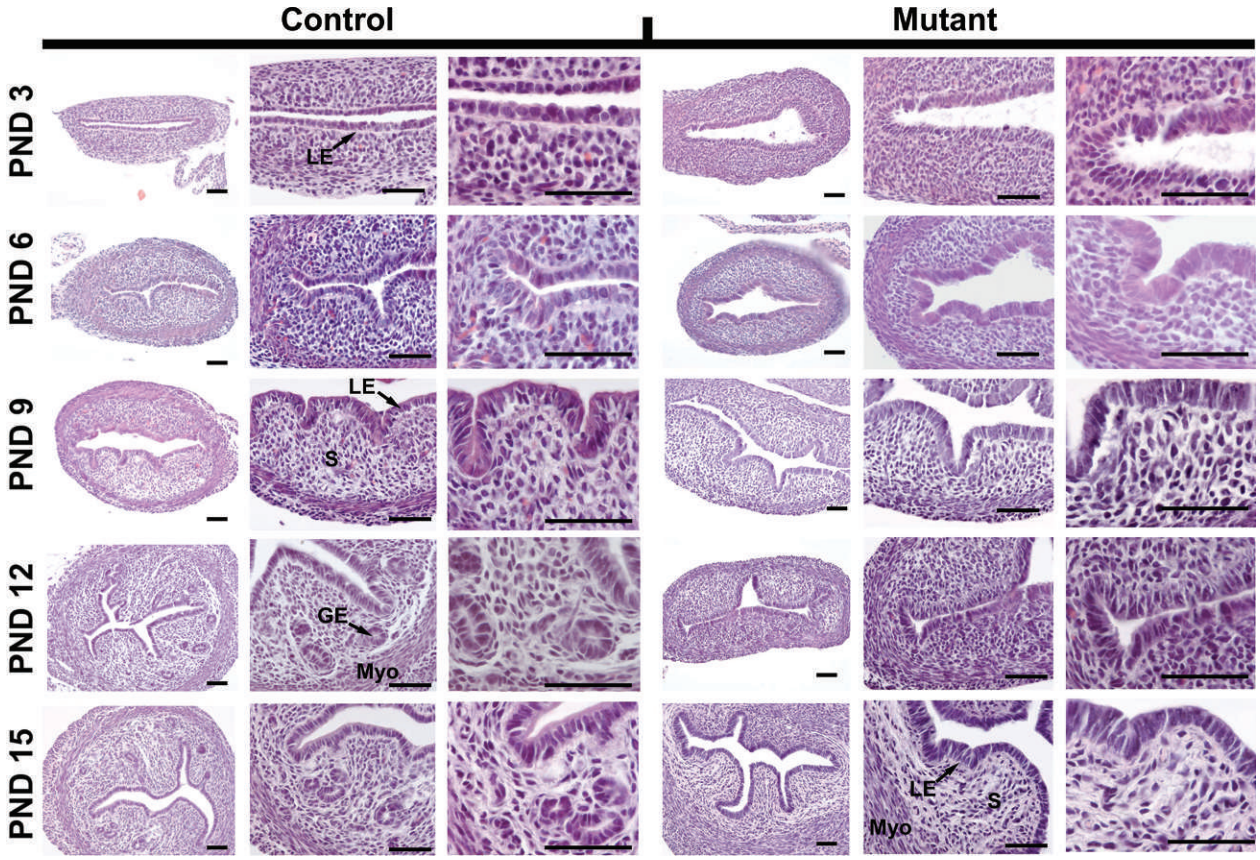

FIG. 3. Endometrial gland development is disrupted by postnatal ablation of *Wnt7a*. Histological analysis of uteri collected on PNDs 3, 6, 9, 12, and 15 from $Pgr^{+/+}Wnt7a^{f/f}$ control mice (**left**) and $Pgr^{Cre/+}$ $Wnt7a^{f/f}$ mutant mice (**right**). Myo, myometrium; S, stroma. Bar = 50 μm.

are an excellent model to conditionally ablate genes in the uterus after birth [37]. Cre excision activity in the Pgr^{Cre} mouse model is restricted to cells that express the PGR after birth, including the uterus, ovary, oviduct, pituitary gland, and mammary gland [35]. In the neonate, PGR expression is initiated after birth in the uterine epithelia by PND 3 and in the stroma by PND 6 [37, 38]. As expected, real-time PCR analysis confirmed the deletion of *Wnt7a* expression in the developing postnatal uterus of $Pgr^{cre/+}Wnt7a^{f/f}$ mutant mice as compared to $Pgr^{+/+}Wnt7a^{f/f}$ control mice (Fig. 1).

Impact of Conditional Ablation of Wnt7a *on Female Fertility*

To determine the effect of conditional ablation of WNT7A on female fertility, female control mice ($Pgr^{+/+}Wnt7a^{+/+}$, $Pgr^{cre/+}Wnt7a^{+/+}$, and $Pgr^{+/+}Wnt7a^{f/f}$) and female $Pgr^{cre/+}Wnt7a^{f/f}$ mutant mice were bred to $Pgr^{+/+}Wnt7a^{+/+}$ male mice. During the time of observation, control mice exhibited normal fecundity, whereas the $Pgr^{cre/+}Wnt7a^{f/f}$ mutant mice were completely infertile (Table 1). Thus, postnatal ablation of WNT7A after birth in the uterus is detrimental to female fertility.

Postnatal Ablation of Wnt7a *Inhibits Endometrial Gland Development in the Uterus*

Because the Pgr^{Cre} mouse model recombines alleles in multiple reproductive tissues [35], we first assessed the impact of *Wnt7a* ablation on postnatal development of the female reproductive tract. On PND 28, no gross anatomical differences were observed in the female reproductive tract of control and mutant mice (Fig. 2), and weight of the uterus was not different ($P > 0.10$) (data not shown). As illustrated in Figure 2, no histoarchitectural differences were observed in the ovary and oviduct from control and mutant mice on PND 28. Although the uteri of control mice contained many glands in the endometrium, the uteri of mutant mice contained no endometrial glands. No other consistent histoarchitectural differences were noted in the endometrium or myometrium of the uterus from control and mutant mice. Furthermore, no obvious differences were observed in the ovary or oviducts between control and mutant mice (Supplemental Fig. S3).

In the developing mouse uterus, endometrial glands begin to differentiate on PND 5 or 6 and are well developed by PND 15 [11, 16]. In control mice of the present study, endometrial glands were absent on PND 3 but were budding from the LE by PND 6 and present in the stroma by PND 12 (Fig. 3). In contrast, endometrial gland development was not observed in the uteri of mutant mice. As noted previously, no other histoarchitectural differences were observed in either the

TABLE 1. Effect of conditional ablation of *Wnt7a* on female fertility.

Female genotype	No. of females	No. of pups	No. of litters	Average pups per litter[a]	Average litters per female[a]
$Pgr^{+/+}Wnt7a^{+/+}$	12	156	23	7.4 ± 0.5	1.9 ± 0.2
$Pgr^{+/+}Wnt7a^{f/f}$	9	101	16	7.2 ± 0.5	1.8 ± 0.3
$Pgr^{cre/+}Wnt7a^{+/+}$	8	84	13	7.6 ± 0.6	1.6 ± 0.3
$Pgr^{cre/+}Wnt7a^{f/f}$	13	0	0	NA	NA

[a] NA, not available.

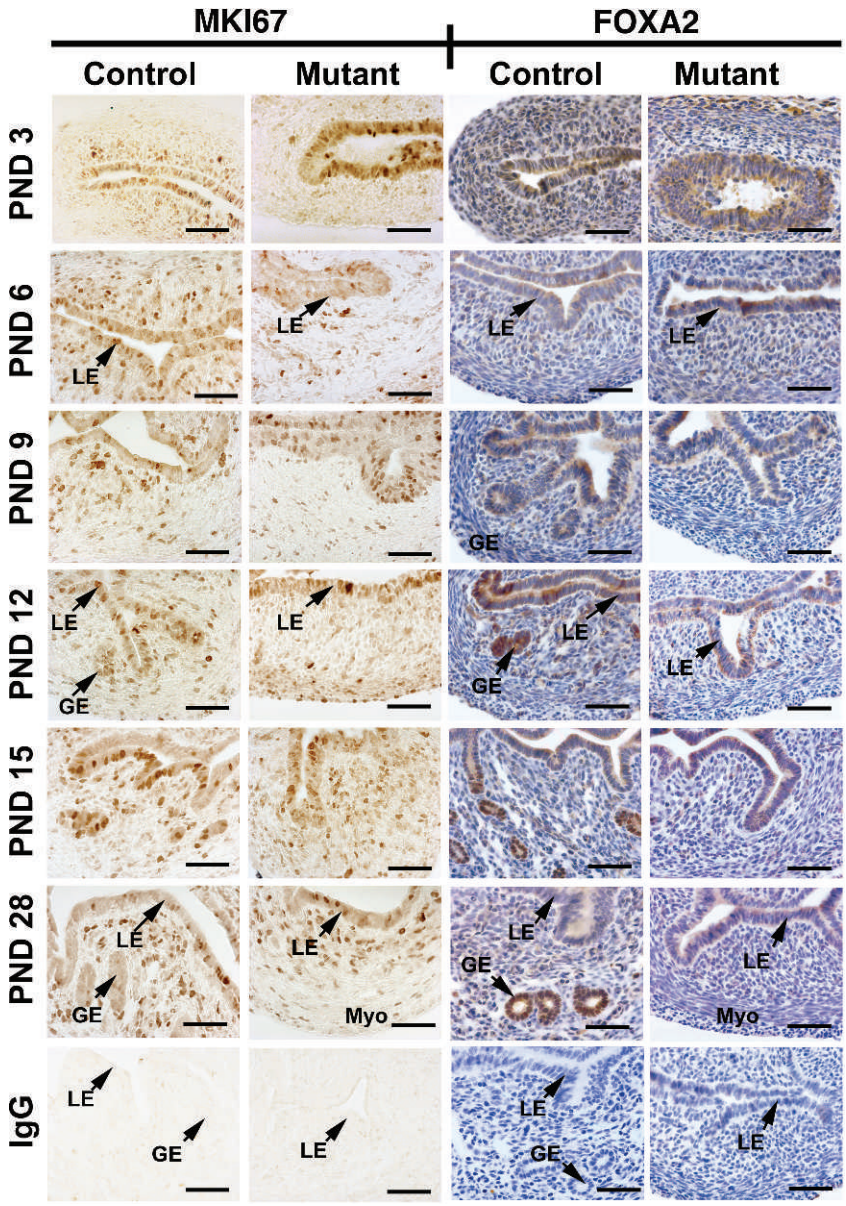

FIG. 4. Immunohistochemical localization of MKI67 and FOXA2 protein in the uteri of $Pgr^{+/+}Wnt7a^{f/f}$ control mice (**left**) and $Pgr^{Cre/+}$ $Wnt7a^{f/f}$ mutant mice (**right**). Cell proliferation was assessed by immunostaining uteri for MKI67. Sections were not counterstained with hematoxylin and eosin after MKI67 localization but were counterstained after FOXA2 localization. Myo myometrium; S, stroma. Bar = 50 μm.

endometrium or myometrium. Therefore, conditional deletion of *Wnt7a* in the developing mouse uterus after birth disrupts endometrial gland morphogenesis, resulting in a glandless uterus in the adult.

Postnatal Deletion of Wnt7a *in the Uterus Does Not Affect Cell Proliferation But Alters Gene Expression*

Null mutation of *Wnt7a* in the embryo alters cell proliferation and apoptosis in the postnatal reproductive tract [39]; therefore, we assessed effects of conditional deletion of *Wnt7a* on cell proliferation using immunohistochemistry for MKI67 (Ki67), a cellular marker of proliferation. In control mice, immunoreactive MKI67 protein was observed in the nuclei of both LE and stromal cells in the developing uterus (Fig. 4). Note the abundance of proliferating LE and GE cells in the PND 12 and PND 15 uteri from control mice as shown in Figure 4. Postnatal deletion of *Wnt7a* had no discernible effects on cell proliferation in any compartment of the uterus.

A carefully orchestrated interplay of *Hox* and *Wnt* genes regulate the prenatal morphogenesis of the female paramesonephric duct [4, 5, 40]. Several of those genes (*Hoxa10, Hoxa11, Wnt4, Wnt5a,* and *Wnt7a*), their signaling pathways, and target genes such as *Msx1* are hypothesized or have been found to regulate endometrial gland development as well as myometrial differentiation in the postnatal uterus [41]. As illustrated in Figure 5, real-time RT-PCR analysis revealed that postnatal deletion of *Wnt7a* disrupted normal patterns of *Fzd10, Hoxa10*, *Hoxa11, Msx1, Msx2, Wnt4, Wnt5a, Wnt11,* and *Vangl2* expression in the uterus (day × genotype, $P < 0.01$). Note that *Msx1* expression is essentially lost after PND 3 in the uteri of mutant mice. In contrast, relative levels of *Msx2* and *Wnt4* mRNA were elevated in mutant uteri during postnatal development (day × genotype, $P < 0.01$). Furthermore, expression patterns of *Fzd6* and *Wnt16* were not different ($P > 0.10$) in uteri of control and mutant mice. CTNNB1 protein was present predominantly in the uterine epithelia and was not different between control and mutant uteri during postnatal development (data not shown).

Recently, Jeong et al. [12] found that conditional deletion of *Foxa2* in the postnatal uterus using Pgr^{Cre} resulted in a complete loss of endometrial glands and that FOXA2 protein was present solely in the endometrial glands of the adult uterus.

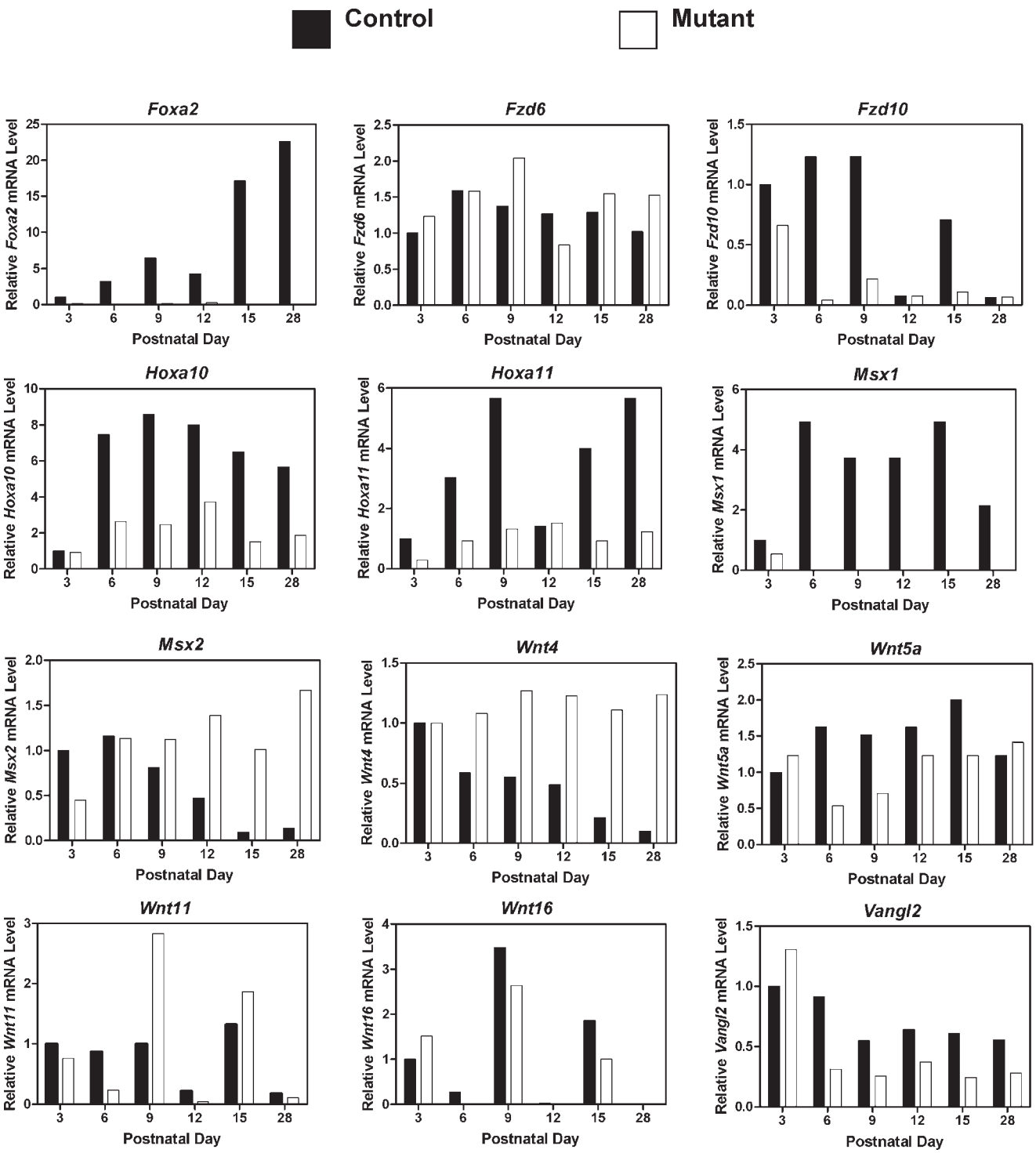

FIG. 5. Postnatal ablation of *Wnt7a* alters gene expression in the developing uterus. The relative mRNA levels of the indicated genes was measured in the uterus of $Pgr^{+/+}Wnt7a^{f/f}$ control and $Pgr^{Cre/+}Wnt7a^{f/f}$ mutant mice by real-time RT-PCR. Data are presented as the fold-change relative to the mRNA level on PND 3 in uteri from control mice.

As illustrated in Figure 5, *Foxa2* mRNA levels increase substantially in the uteri of control mice after PND 3 in association with the development of endometrial glands. Note the presence of FOXA2 protein in the nuclei of endometrial glands in control mice after PND 9 as shown in Figure 4. Interestingly, FOXA2 protein is observed in the cytoplasm of LE cells on PNDs 3–15 and in some uterine mesenchymal and endometrial stromal cells. However, FOXA2 protein is restricted to the nuclei of endometrial glands in PND 28 control uteri. In contrast, no increase in *Foxa2* expression was found (day × genotype, $P < 0.001$) in the uteri of mutant mice that lack endometrial glands (Fig. 5). Although cytoplasmic FOXA2 protein was observed in the LE of both control and mutant mice from PND 3 to PND 28, FOXA2 protein was only observed in the nuclei of endometrial glands after PND 9 in uteri of control mice (Fig. 4).

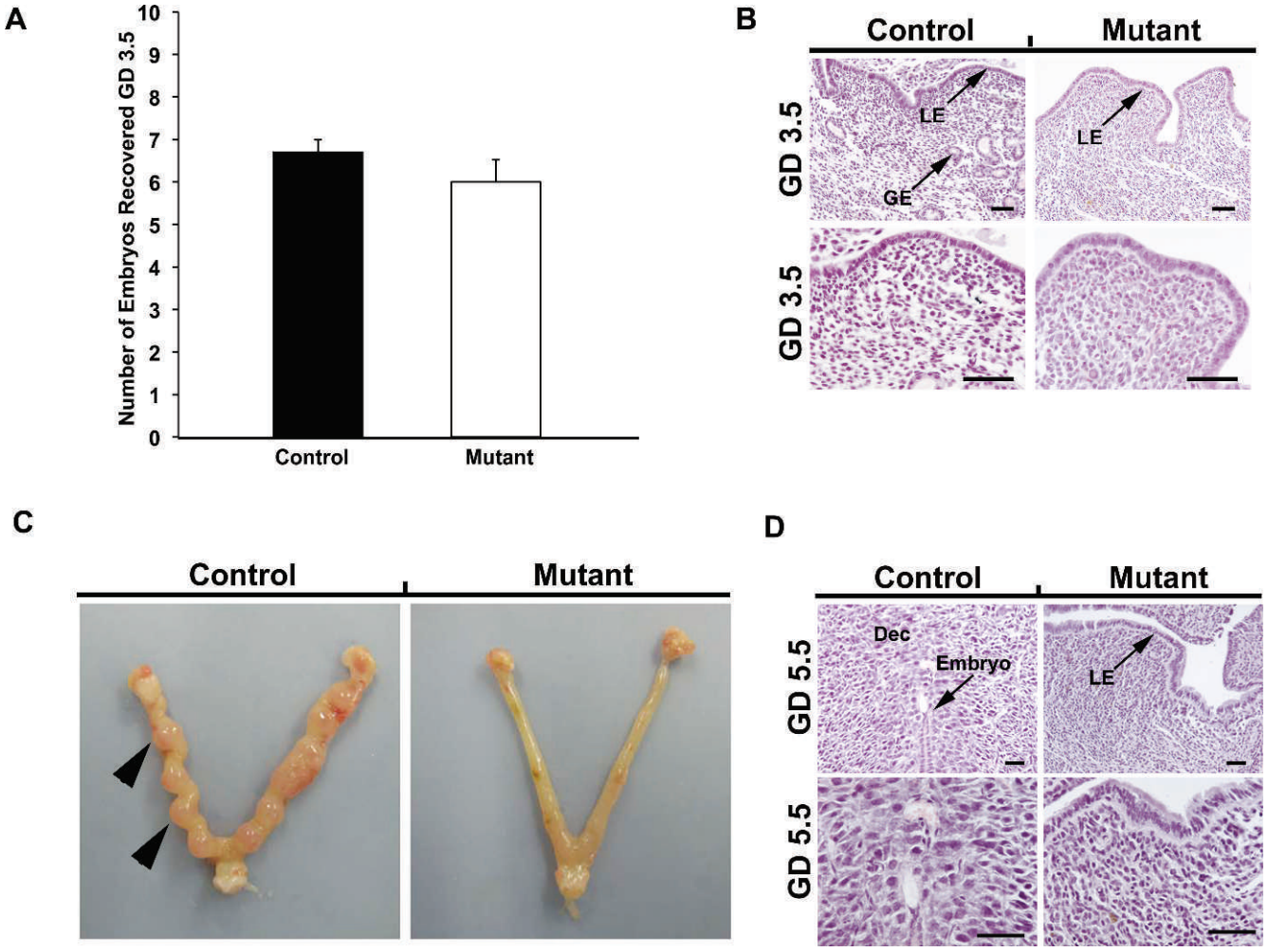

FIG. 6. Implantation defect in the $Pgr^{Cre/+}Wnt7a^{f/f}$ mutant mice. **A**) Embryos recovered from and histology of the control and mutant uterus on GD 3.5. The error bar denotes standard error of the mean number of embryos. **B**) Note the absence of endometrial glands (GE) in the uteri of mutant mice. **C**) Implantation sites in the control and mutant uterus on GD 5.5. The results represent results of five independent mice per genotype. Gross anatomy of the mutant uteri shows an absence of implantation sites (arrows) compared with controls. **D**) Implanted blastocysts are observed in the uteri of control mice on GD 5.5 but not in $Pgr^{Cre/+}Wnt7a^{f/f}$ mutant uteri. Dec, decidua; S, stroma. Bar = 50 μm.

Postnatal Deletion of Wnt7a *Impacts Adult Uterine Function and Compromises Blastocyst Implantation*

Conditional ablation of *Wnt7a* after birth resulted in complete infertility (Table 1). To determine the underlying cause of the infertility, control and mutant mice were bred and the uterus flushed on GD 3.5. As illustrated in Figure 6A, no difference ($P > 0.10$) was found in the number of embryos recovered from the uterus of control and mutant mice. The embryos recovered from both control and mutant mice were blastocysts of normal size and morphology. The uteri of both control and mutant mice were histoarchitecturally similar except that mutant uteri lacked endometrial glands (Fig. 6B). Implantation was observed in the uteri of control mice on GD 5.5 but not in the uteri of mutant mice (Fig. 6C). Note the presence of an implanted embryo and decidualized stroma in the uteri of control mice on GD 5.5 as shown in Figure 6D.

The expression of a number of genes that regulate endometrial receptivity and/or blastocyst implantation (*Foxa2, Hoxa10, Hoxa11, Ihh, Lif, Msx1,* and *Wnt4*) as well as *Wnts* and their receptors were measured in the uteri of control and mutant mice on GD 3.5 (Fig. 7). A striking reduction ($P < 0.05$) was found in expression of *Foxa2, Lif, Msx1, Wnt7a*, and *Wnt16* in the uteri of mutant mice. In contrast, expression of *Wnt11*, *Msx2*, *Wnt4*, *Wnt5a,* and *Hoxa10* was increased ($P < 0.05$) in the uteri of mutant as compared to control mice. No difference in expression of *Ihh* or *Hoxa11* was detected ($P > 0.10$) in the uteri of control as compared to mutant mice.

DISCUSSION

To understand the role of WNT7A in postnatal development of the uterus, we generated mice in which *Wnt7a* was ablated in the uterus using mice with floxed *Wnt7a* alleles and the Pgr^{Cre} mouse model [35]. The resulting female mutant mice were infertile, with an inability of the uterus to support embryo implantation. We observed that loss of *Wnt7a* in the uterus after birth in the $Pgr^{Cre/+}Wnt7a^{f/f}$ neonatal mutant mice disrupted differentiation and growth of the endometrial glands without altering differentiation of the stroma or myometrium or inducing stratification of the LE. The uterine dysgenesis observed in the *Wnt7a* conditional mutant mice is less severe than that in global *Wnt7a*-null mice, likely because the uterine mesenchyme has already differentiated into endometrial stroma and myometrium by PND 3 and *Wnt5a* and *Wnt7a* expression becomes restricted to the uterine portion of the female

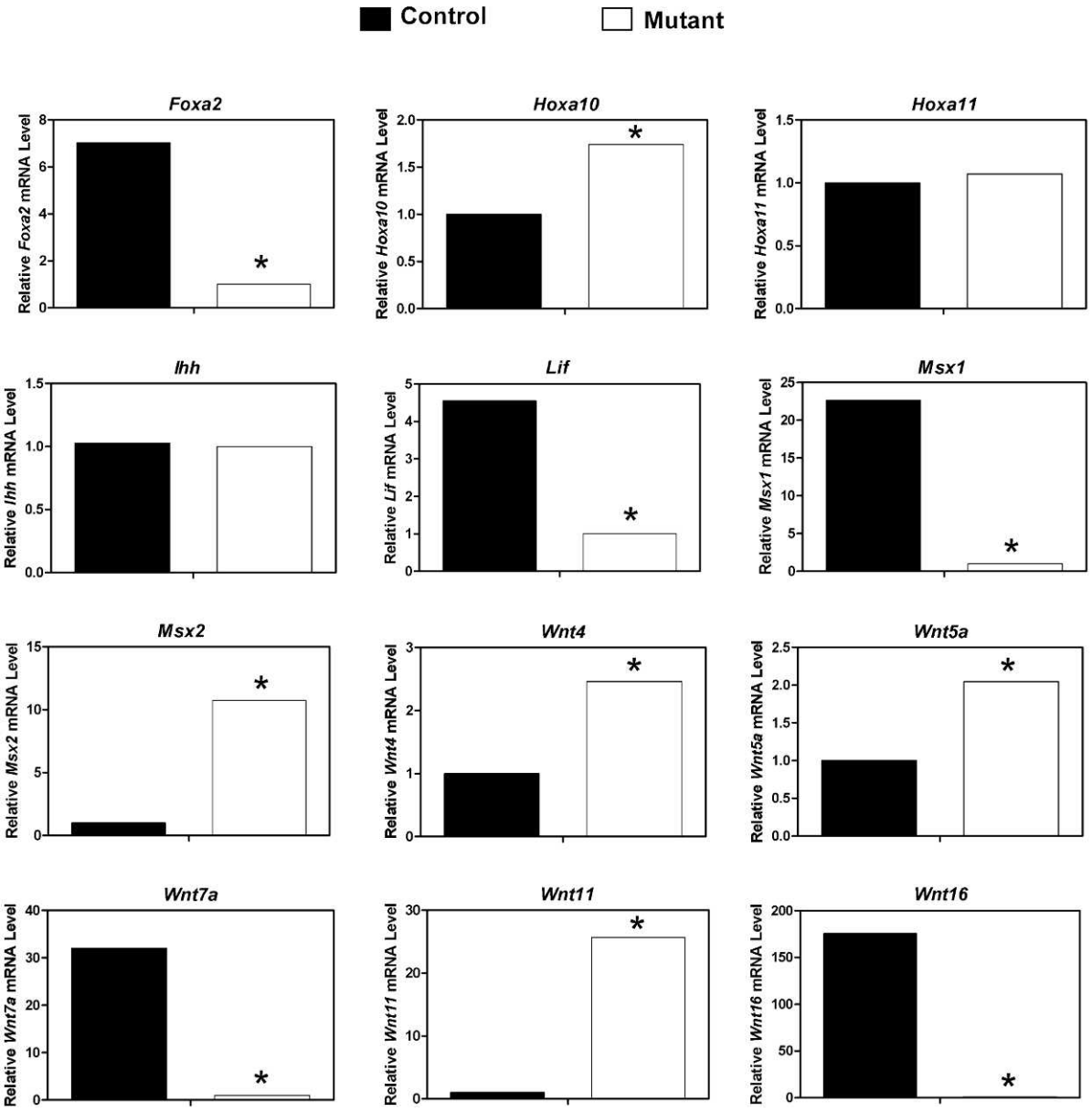

FIG. 7. Postnatal ablation of *Wnt7a* alters expression of genes in the uterus that are important for blastocyst implantation. The relative mRNA level for the indicated genes was measured in the uterus of $Pgr^{+/+}Wnt7a^{f/f}$ control and $Pgr^{Cre/+}Wnt7a^{f/f}$ mutant mice on GD 3.5 by real-time RT-PCR. Data are presented as the fold-change relative to the mRNA level on GD 3.5 in uteri from control mice. The asterisk denotes a significant difference ($P < 0.05$) in mRNA levels.

reproductive tract after birth [20]. Indeed, the uteri of adult *Wnt7a*-null mice appear to be posteriorized, as evidenced by the stratified LE that is normally found in the vagina and the presence of short and uncoiled oviducts [23, 27]. Collectively, these results support the ideas that WNT7A is a secreted protein from the LE that acts on the mesenchyme in a paracrine manner during fetal life to regulate cell fate, differentiation, and survival in the müllerian duct and that global deletion of *Wnt7a* in the embryo disrupts epitheliomesenchymal interactions that elicit defects in all uterine cell types as well as other müllerian duct-derived structures, including the oviduct, cervix, and vagina, given that *Wnt7a* is expressed throughout müllerian duct epithelia [39].

Until recently, little was known about the cellular and molecular mechanisms governing uterine gland differentiation and development. However, a recent study found that conditional ablation of *Foxa2* using the Pgr^{Cre} mouse model results in an absence in uterine glands [12]. *Foxa2* has an important role in epithelial budding and morphogenesis in many different epitheliomesenchymal organs, including the liver, lung, and prostate [42, 43]. FOXA2 likely has a biological role in gland specification via transcriptional regulation of differentiation genes [12]. One possible signaling pathway that either regulates FOXA2 or is regulated by FOXA2 is the WNT signaling pathway, because mouse models in which *Wnt7a*, *Wnt5a,* and *Ctnnb1* are ablated all lack uterine glands [21–23, 39]. Indeed, FOXA2 has been shown to regulate the expression of multiple *Wnts*, including *Wnt3a*, *Wnt8a,* and *Wnt7b* [44–46], and a reciprocal interaction between FOXA2 and WNT signaling has been noted in that CTNNB1, a downstream effector of canonical WNT signaling, can promote *Foxa2* [47]. WNT7A signals through the

canonical WNT/catenin, beta 1 signaling pathway in other cells and organs [28–31]. In mice with conditional ablation of *Foxa2*, no differences in *Wnt7a* or *Wnt5a* expression were observed in the adult uterus [12]. The low levels of *Foxa2* mRNA in the *Wnt7a* conditional mutant uterus is primarily due to the lack of endometrial glands that abundantly express *Foxa2* in uteri of control mice. Whether or not WNT7A is upstream of FOXA2 in the regulatory cascade governing uterine adenogenesis remains to be determined.

In the developing neonatal mouse uterus, *Hoxa10, Hoxa11, Wnt4*, *Wnt5a*, and *Wnt16* are expressed in the endometrial stroma, whereas *Msx1, Wnt7a, Wnt11, Fzd6*, *Fzd10,* and *Vangl2* are expressed in the uterine epithelia of neonatal mice [40, 41, 48]. In the present study, analyses of gene expression revealed that several genes were reduced (*Fzd10, Hoxa10, Hoxa11, Msx1, Wnt5a, Wnt11, Wnt16,* and *Vangl2*) or increased (*Msx2, Wnt4,* and *Wnt11*) by *Wnt7a* ablation and, thus, may be implicated in endometrial gland dysgenesis observed in the conditional mutant mice. The complete absence of *Msx1* and *Wnt16* in the neonatal and adult uteri of conditional mutant mice indicates that they, along with *Foxa2* governed by WNT7A in the postnatal uterus, are primary genes. The phenotype of *Wnt16* ablated mice has not been reported, but *Wnt16* is expressed in the stroma of the developing and adult mouse uterus [41, 49]. In other cells, *Msx1* and several *Fzd*s are regulated by canonical WNT signaling [50], and conditional deletion of *Ctnnb1* using the Pgr^{Cre} mouse model ablates uterine gland differentiation and induces stratification and squamous cell metaplasia of the LE [21]. Null mutation of the *Msx1* gene results in perinatal lethality [51]. To our knowledge, conditional mutation of *Msx1* has not been reported, although it has been implicated as a regulator of implantation in the mouse [26]. Alterations in other genes that are implicated in uterine development in the fetus and neonate, such as *Hoxa10*, *Hoxa11,* and *Vangl2* [48, 52–54], were also reduced only in the uteri of neonatal *Wnt7a* mutant mice in the present study. In contrast, *Hoxa10* and *Hoxa11* were reduced in the uterine stroma of *Wnt7a*-null adult mice [23]. Interestingly, the conditional loss of *Wnt7a* in the present study increased expression of *Msx2*, *Wnt4,* and *Wnt11* in both the neonate and adult, suggesting that *Wnt7a* negatively regulates their expression. Conditional ablation of *Wnt4* using the Pgr^{Cre} mouse model results in reduced numbers of uterine glands as well as stratification of the LE and defects in decidualization and embryo implantation. In contrast, conditional ablation of *Wnt11* using the Pgr^{Cre} mouse model had no effects on uterine gland development or fertility [41]. However, the effect of overexpression of *Msx2*, *Wnt4,* and *Wnt11* on uterine development and function has not been reported. Available studies support the idea that WNT7A from the LE in the developing postnatal uterus acts on the stroma and, perhaps, the LE itself and signals via the canonical catenin, beta 1 pathway to orchestrate changes in gene expression governing endometrial gland differentiation and development in the neonate. The conditional *Wnt7a* mouse should be useful to determine genes and regulatory cascades important for tissue development and growth.

The major histological defect in the uterus was the absence of endometrial glands. Because the Pgr^{Cre} mouse model recombines alleles in all compartments of the uterus as well as the ovary, oviduct, vagina, and pituitary [35], we determined if infertility was caused by the uterine defect or, perhaps, pituitary or ovarian defects. No histological or functional defects were observed in the ovary, oviduct, vagina, or pituitary, because unimplanted and morphologically normal embryos could be recovered on Day 3.5 postmating. Therefore, the lack of uterine glands is likely the key defect that underlies the infertility and lack of embryo implantation in the mutant mice. Indeed, the lack of endometrial glands in *Foxa2* conditional mutant mice [12] as well as uterine gland knockout ewes [13] results in severe subfertility or infertility, respectively, that manifests in a lack of embryo implantation. Whether or not delayed embryo attachment is observed in mice lacking *Wnt7a* or *Foxa2* remains to be determined. Endometrial glands and their secretions are critical regulators of peri-implantation survival of the conceptus and implantation as well as establishment of uterine receptivity and decidualization [13, 55–57]. Several signaling pathways necessary for implantation have been identified [37, 58]. *Foxa2* and *Lif* mutant mice exhibit defects in both implantation and decidualization [12, 59]. Leukemia inhibitory factor (LIF) is secreted by the uterine glands in response to nidatory estrogen on GD 3.5 [60, 61] and is also expressed in the subluminal stroma at the implantation site [62]. In the present study, *Foxa2* and *Lif* were much reduced in the GD 3.5 uterus of *Wnt7a* mutant mice, which is likely caused by the absence of endometrial glands. Indeed, uterine gland production of LIF is critical for embryo implantation [61]. Thus, the absence of uterine glands and LIF is probably the cause of embryo implantation failure in the *Wnt7a* conditional mutant. Future studies will determine the impact of *Wnt7a* conditional ablation on decidualization, which is likely given that decidualization defects occur in the *Foxa2* conditional mutant [12]. Thus, products of the endometrial glands are definitively required for embryo implantation as well as subsequent decidualization that regulates placental growth and development.

In summary, the present results concerning conditional ablation of *Wnt7a* in the uterus support the hypothesis that WNT7A is a critical regulator of postnatal uterine morphogenesis and, in particular, plays a pivotal role in the specification, differentiation, and development of the GE within the endometrium. The lack of endometrial glands resulted in an inability of the uterus to support embryo implantation. Future studies are needed to determine the regulatory cascades promulgated by WNT7A that regulate the critical process of endometrial morphogenesis after birth and potential roles of WNT7A in adult uterine function. The *Wnt7a* conditional mouse will be a particularly valuable model to undertake those studies as well as determine the role of WNT7A in other organs.

ACKNOWLEDGMENTS

The authors appreciate the assistance and advice of Dr. Allison Stewart of the University of Texas M.D. Cancer Center and Dr. Heather Franco of Baylor College of Medicine.

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