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Mice Null for Frizzled4 (*Fzd4*$^{-/-}$) Are Infertile and Exhibit Impaired Corpora Lutea Formation and Function[1]

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ABSTRACT

Previous studies showed that transcripts encoding specific Wnt ligands and Frizzled receptors including *Wnt4,* Frizzled1 (*Fzd1*), and Frizzled4 (*Fzd4*) were expressed in a cell-specific manner in the adult mouse ovary. Overlapping expression of *Wnt4* and *Fzd4* mRNA in small follicles and corpora lutea led us to hypothesize that the infertility of mice null for *Fzd4* (*Fzd4*$^{-/-}$) might involve impaired follicular growth or corpus luteum formation. Analyses at defined stages of reproductive function indicate that immature *Fzd4*$^{-/-}$ mouse ovaries contain follicles at many stages of development and respond to exogenous hormone treatments in a manner similar to their wild-type littermates, indicating that the processes controlling follicular development and follicular cell responses to gonadotropins are intact. Adult *Fzd4*$^{-/-}$ mice also exhibit normal mating behavior and ovulate, indicating that endocrine events controlling these processes occur. However, *Fzd4*$^{-/-}$ mice fail to become pregnant and do not produce offspring. Histological and functional analyses of ovaries from timed mating pairs at Days 1.5–7.5 postcoitus (p.c.) indicate that the corpora lutea of the *Fzd4*$^{-/-}$ mice do not develop normally. Expression of luteal cell-specific mRNAs (*Lhcgr*, *Prlr*, *Cyp11a1* and *Sfrp4*) is reduced, luteal cell morphology is altered, and markers of angiogenesis and vascular formation (*Efnb1, Efnb2, Ephb4, Vegfa, Vegfc*) are low in the *Fzd4*$^{-/-}$ mice. Although a recently identified, high-affinity FZD4 ligand Norrin (Norrie disease pseudoglioma homolog) is expressed in the ovary, adult *Ndph*$^{-/-}$ mice contain functional corpora lutea and do not phenocopy *Fzd4*$^{-/-}$ mice. Thus, *Fzd4* appears to impact the formation of the corpus luteum by mechanisms that more closely phenocopy *Prlr* null mice.

corpus luteum, corpus luteum function, Efnb, follicle, Fzd4, gene regulation, LH receptor, Ndph, Norrin, ovary, ovulation, Sfrp4, Vegf

INTRODUCTION

The development and maturation of ovarian follicles is dependent on the pituitary gonadotropins acting via their cognate receptors [1]. In addition to this classical pathway, many new regulators of ovarian cell function have been identified [2]. One of these is WNT4, a member of the Wnt family of secreted ligands, that has recently been shown to be essential for embryonic development of the ovary [3]. At birth, female mice null for *Wnt4* exhibit gonadal structures resembling testicular tubules, express genes associated with male gonad formation, and have reduced numbers of germ cells [3]. This switch in gonadal phenotype is related in part to the role of WNT4 in controlling steroidogenic and endothelial cell migration in the embryonic gonad [4]. Specifically, WNT4 prevents formation of the elaborate vascular network that develops in the testis on embryonic Day 14.5. Furthermore, *Wnt4*$^{-/-}$ mice die shortly after birth because of kidney defects, precluding a functional analysis of the signaling molecule in the adult mouse. Gene duplication of *WNT4* has been detected in phenotypic female individuals who have an XY genotype [5]. In these individuals, testis development is abnormal, thereby leading to the altered phenotype.

WNTs act by binding Frizzled (FZD) receptors, members of a specific class of seven pass transmembrane receptors [6]. To determine whether *Wnt4* mRNA was present in the postnatal mouse ovary and coexpressed with transcripts encoding specific *Fzd* receptors, we analyzed the expression of several members of the WNT signaling network [7]. Our results documented that *Wnt4* mRNA is expressed in granulosa cells of small follicles in the postnatal ovary and that this expression is increased in preovulatory follicles as well as in association with ovulation and luteinization. Among the FZD receptor family, *Fzd4* and *Fzd1* mRNAs were expressed. Whereas *Fzd1* transcripts were selectively increased in periovulatory follicles, *Fzd4* mRNA was expressed throughout development and appeared to be preferentially increased during ovulation and luteinization [7]. Mice null for *Fzd1* have not been described. However, mice null for *Fzd4* (*Fzd4*$^{-/-}$) have been generated and, unlike the *Wnt4*$^{-/-}$ mice, they are viable and function normally with the exception of developmental defects observed in cerebellar, auditory, ocular and esophageal tissues [8–10]. Moreover, recent studies have shown that FZD4 not only binds specific WNT ligands but also binds Norrin (Norrie disease pseudoglioma [NDP]), a small secreted protein with a cysteine knot motif encoded by the NDP gene [8]. Careful analysis of NDP homolog (NDPH) in the mouse linked *Ndph* to the abnormal retinal phenotype of *Fzd4*$^{-/-}$ mice, and documented that the absence of *Fzd4* altered endothelial migration along the retinal surface and prevented secondary and tertiary arborization of the retinal blood vessels in a manner similar to that seen in *Ndph*-deficient mice [8, 11]. These observations indicated that in specific tissues, FZD4 and NPDH regulated vascular development.

In addition to these published results, preliminary observations suggested that *Fzd4* was essential for normal reproductive function because both male and female *Fzd4*$^{-/-}$ mice appeared to be infertile (Y. Wang and J. Nathans, personal communi-

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cation). Because our results indicated that *Fzd4* mRNA was expressed in the adult ovary and more specifically in growing follicles and corpora lutea [7], we hypothesized that defects in ovarian function might be one cause of infertility in the $Fzd4^{-/-}$ mice and potentially related to the expression and function of angiogenic factors. Because the formation of the corpus luteum is associated with the angiogenesis and the vascularization of the granulosa cell layer [12], we hypothesized that the $Fzd4^{-/-}$ mice might exhibit impaired luteal cell function.

The formation and function of the murine corpus luteum is dependent on the luteotropic actions of the pituitary hormone prolactin and the induction of specific genes, including P450scc (*Cyp11a1*) and 3β-hydroxysteroid dehydrogenase (*Hsd3b6*), that are essential for progesterone production [13]. In addition, prolactin is known to induce expression of transcripts encoding the LH receptor (*Lhcr*) [14], and the Wnt signaling molecule secreted Frizzled-Related Protein 4 (*Sfrp4*) [15]. Mice null for prolactin (*Prl*) and the prolactin receptor (*Prlr*) fail to form functioning corpora lutea [16]. The latter have been shown to exhibit impaired expression of prolactin-regulated genes and exhibit impaired vascular formation [16]. Thus, we further hypothesized that $Fzd4^{-/-}$ mice might exhibit impaired prolactin signaling and reduced progesterone production, and thus impaired implantation.

To examine these hypotheses, follicular development and ovulation were analyzed in ovaries of prepubertal $Fzd4^{-/-}$ mice before and following treatment with eCG to stimulate follicular growth and hCG to stimulate ovulation. Fertility was further assessed using timed mating experiments with young adult mice. The data presented provide the first report that a FZD receptor impacts reproductive success in the adult mouse. Although follicular development appears normal and ovulation occurs in both wild-type and $Fzd4^{-/-}$ mice, implantation sites were never observed in the mutant mice. Rather, our results show for the first time that mice null for *Fzd4* fail to form functional corpora lutea; thereby delineating when FZD4 appears to be important. This is a novel observation because it provides the first documentation that FZD4 signaling affects ovarian function and may be associated with defective prolactin signaling and impaired vascular development.

MATERIALS AND METHODS

Experimental Animals

Frizzled4 (*Fzd4*) wild-type (+/+; $Fzd4^{+/+}$), heterozygous (+/–; $Fzd4^{+/-}$), and null (–/–; $Fzd4^{-/-}$) female mice (129/SVJ X C57BL/6J background) [10] were generated by intercrosses of heterozygous Fz4 mice (generously provided by Jeremy Nathans, Johns Hopkins University, Baltimore, MD). Offspring were genotyped by polymerase chain reaction (PCR) analysis using specific primer pairs to detect the *Fzd4* wild-type and null alleles. Sense and antisense primers for the wild-type *Fzd4* allele were 5′-TGGAAAGGCTAATGGTCAA-GATCGG-3′ and 5′-TTCTGATGCTGAGTTGGGTGAGTGG-3′, respectively. Sense and antisense primers for the gene-targeted allele were 5′-GCTTCCTCGTGCTTTACGGTATCG-3′ and 5′-CTCAGAAGCCATA-GAGCCCACCGC-3′, respectively. PCR was carried out for 30 cycles using conditions of 94°C (1 min) for denaturing, 60°C (2 min) for annealing, and 72°C (3 min) for extension. Predicted PCR product sizes are 449 and 349 bp for the wild-type and null alleles, respectively. In addition, mice of the *Ndph* knockout line, which have been described previously, [17] were obtained by crossing of either wild-type (+/*y*) or hemizygous (–/*y*) male mice with heterozygous (+/–) female mice. All animals were treated in accordance with the NIH Guide for Care and Use of Laboratory Animals as approved by the Animal Care and Use Committee, Baylor College of Medicine, Houston, TX.

Hormone Treatment

Immature (Day 23) female mice were injected i.p. with 4 IU eCG (Gestyl; Professional Compounding Center of America) to stimulate follicular growth. After 48 h, the mice were injected i.p. with 5 IU hCG (Pregnyl; Organon Special Chemicals), an LH-like molecule used to promote ovulation and luteinization. Some females were mated overnight with wild-type males and checked the following morning for vaginal plugs. Oocytes were collected from the oviducts 50–52 h post-hCG and counted.

Timed Mating Experiments

For timed mating experiments, $Fzd4^{+/+}$ and $Fzd4^{-/-}$ females were housed overnight with wild-type males and checked the following morning designated Day 0.5 postcoitus (p.c.), for a vaginal plug, Ovaries were collected on Days 1.5, 5.5, and 7.5 after observation of a vaginal plug. For each female, RNA was extracted from one ovary and used in RT-PCR analyses, and the other ovary was fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned at 7 μm for in situ hybridization and hematoxylin-eosin staining. Fertilization was confirmed by finding embryos at the 2- and 4-cell stage from the oviducts of females on Day 1.5 p.c., and pregnancy was determined by evidence of implantation sites in the uteri on Days 5.5 and 7.5 p.c. Blood was collected from these animals by cardiac puncture, and serum isolated using Microtainer Brand serum separators (Becton Dickinson). The serum samples were sent to the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core Laboratory (Charlottesville, VA) for progesterone RIA assays. Mouse prolactin was measured by RIA in the laboratory of the National Hormone and Peptide Program.

Semiquantitative RT-PCR

RNA was isolated from ovaries using Trizol reagent (Invitrogen) and from other tissues using the RNeasy mini kit (Qiagen Sciences), as directed by the respective manufacturers. Total RNA was reverse-transcribed using 500 ng poly-dT (Amersham Pharmacia Biotech) and 0.25 U avian myeloblastosis virus-reverse transcriptase (Promega Corp.) at 42°C for 75 min and 95°C for 5 min. For specific primers used, the cycles of amplification were as follows:Aromatase (*Cyp19*), sense, 5′TGCACAGGCTCGAGTATTTCC-3′, and antisense, 5′-ATTTCCACAATGGGGCTGTCC-3′, 25 cycles; LH receptor (Lhcgr), sense, 5′-CTTATACATAACCACCATACCAG-3′, and antisense, 5′-ATCCCA-GCCACTGAGTTCATTC-3′ [18], 25 cycles; P450scc (*Cyp11a1*), sense, 5′-AGAAGCTGGGCAACATGGAGTCAG-3′, and antisense, 5′-TCACATCCCAGGCAGCTGCATGGT-3′ [19], 21 cycles; PRLR (*Prlr*), sense, 5′-ATACTGGAGTAGATGGGGCCAGGAGAAATC and antisense, 5′-CTTCCATGACCAGAGTCACTGTCAGGATCT [20], 25 cycles; Wnt4 (Wnt4) sense, 5′-TTCTCACAGTCCTTTTGGACG-3′, and antisense, 5′- TCTGTATGTGGCTTGAACTGTG-3′) [7], 25 cycles; sFRP4 (*Sfrp4*), sense, 5′-CATCAAGCCCTGCAAGTCTG-3′, and antisense, 5′-TAAGGGTGGCTCCATCACAG-3′) [15], 18 cycles; Ephrin B1 (*Efnb1*), sense, 5′-ATCGCAAGCATACACAGCAG-3′, and antisense, 5′-CTGGGCCTTCAAACCTTGTA-3′, 25 cycles; Ephrin B2 (*Efnb2*), sense, 5′-CTAACCTCTCCTGCGCATTC-3′, and antisense, 5′- GACGCACAGGA-CACTTCTCAATG-3′, 26 cycles; EphB4 (*Ephb4*), sense, 5′- CTTCCCATTG-GATTGCACTT-3′, and antisense, 5′- TGGTCACCCTTTCTCTTTGG-3′, 26 cycles; VEGFA (Vegfa) sense, 5′-CCTCCGAAACCATGAACTTTCTGCTC-3′, and antisense, 5′-CAGCCTGGCTCACCGCCTTGGCTT-3′, 28 cycles; VEGFC (*Vegfc*) sense, 5′-CTACAGATGTGGGGGTTGCT-3′, and antisense, 5′-GCTGCCTGACACTGTGGTAA-3′, 26 cycles; L19 (*Rpl19*) sense, 5′-CTGAAGGTCAAAGGGAATGTG-3′; antisense, 5′-GGACAGAGTCTT-GATGATCTC-3′ [21, 22] 22 cycles. Each RT reaction mixture was separated into two aliquots: 500 ng of the primer pairs for the gene of interest were added to one aliquot, and primers for L19 were added to the other aliquot as the control. [α^{32}P]dCTP (2 μCi; ICN), 2.5 UTaq polymerase, and thermocycle buffer (Promega Corp.) were included in the PCR reactions as described previously [7, 15, 19]. Preliminary experiments were performed for each gene to ensure that the cycle numbers selected fell within the linear range of PCR amplification (not shown). PCR amplification conditions were 94°C for denaturing (1 min), 60°C for annealing (2 min), and 72°C for extension (3 min) for aromatase, LHR, P450scc, PRLR and sFRP4 and L19. All others were amplified according to 94°C for denaturing (30 sec), 60°C for annealing (45 sec), and 72°C for extension (1 min), and then a final 5 min at 72°C for extension. The amplified cDNA products were resolved on a 5% polyacrylamide gel and subsequently quantified using a Storm 860 PhosphorImager (Molecular Dynamics, Inc.).

For analyses of NDP (*Ndph*) and Frizzled4 (*Fzd4*) message in various tissues and ovarian samples, the One-Step RT-PCR system with Platinum Taq kit (Invitrogen) and 100-ng samples of total RNA was used. Briefly, reactions were formulated as directed by the manufacturer, except that 0.625μCi of [α-^{32}P]dCTP (specific activity 3000 Ci/mmol; MP Biomedicals) were added to each reaction to generate quantifiable radioactive signals and to increase assay sensitivity. Oligonucleotides used were sense 5′-ATGAGAAATCATGTAC-TAGCTGCAT C-3′ and antisense 5′-TCAGGAGCTGCATTCCTCACAGT-3′

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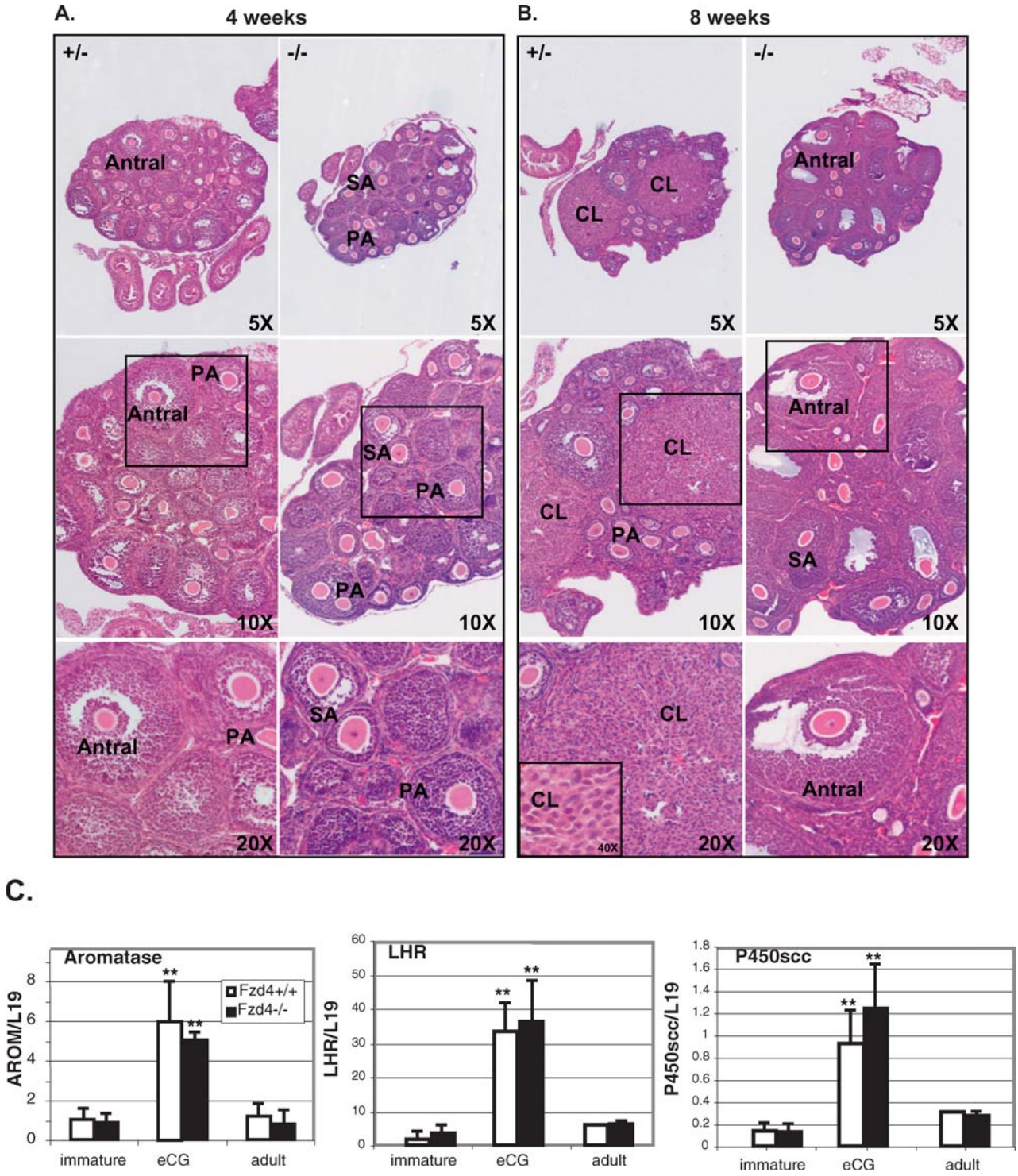

FIG. 1. Histological analysis of ovaries from *Fzd4*$^{+/-}$ and *Fzd4*$^{-/-}$ mice. Tissue sections from ovaries of 4-wk-old **(A)** and 8-wk-old (**B**) mice were stained with hemotoxylin and eosin. Stained sections are shown at increasing magnification in each column and within the selected squares. **A)** Preantral (PA), small antral (SA) and antral follicles are observed in ovaries from 4-week-old Fzd4+/– and Fzd4–/– mice. **B)** Corpora lutea (CL) are present in the ovaries of the 8-week-old heterozygote mice. Corpora lutea were variably present or absent (as shown) in ovaries of some Fzd4–/– mice at the same age. Images in the boxed areas of the middle panels are enlarged in the lower panels and luteal cells of the Fzd4+/– mice are shown in the bottom panel. **C)** Semiquantitative RT-PCR analyses of aromatase, LHR, and P450scc demonstrate that induction of these genes is similar in Fzd4+/– and Fzd4–/– ovaries obtained from immature mice and immature mice stimulated with eCG. Data are presented as mean ± SEM. Each point represents the mean ± SEM of three separate RNA samples (derived from three ovaries of three separate mice) run in triplicate. **Statistically significant difference ($P < 0.05$) compared to immature samples.

for *Ndph*, and sense 5′-GCATCGTAGCCACAC TTGAGAACA-3′ and antisense 5′-CGGCTGCTCCAGCCAGCTGCA-3′ for *Fzd4* (Sigma-Genosys). Oligonucleotides used for ribosomal protein L19 were as previously described [22]. Cycling conditions were 50°C for 30 min and 94°C for 2 min, followed by 18 (L19), 30 (*Ndph*) or 24 (*Fzd4*) cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 1 min. A final extension step of 72°C for 7 min was also performed. Samples were separated by electrophoresis on 2% TAE-agarose gels, dried and exposed to Biomax XAR film (Eastman Kodak) for 30–240 min at −70°C to

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TABLE 1. Ovulation and fertilization can be induced by hormones in *Fzd4*$^{-/-}$ mice (n = 2 mice per group).[a]

Genotype	Ovulated oocytes (%)	No. of fertilized oocytes
Fzd4$^{+/+}$	15	100
Fzd4$^{+/+}$	22	59
Fzd4$^{+/-}$	7	86
Fzd4$^{+/-}$	10	100
Fzd4$^{-/-}$	22	100
Fzd4$^{-/-}$	23	79

[a]No differences were observed in ovulation rate or fertilization among the groups.

generate the presented images. The relative radioactive signal strengths from the RT-PCR products were subsequently quantified as described above.

In Situ Hybridization

The clone for *Cyp11a1* was the generous gift of Keith Parker (University of Texas Southwestern Medical School, Dallas, TX) and that for *Sfrp4* was previously described [15]. In situ hybridization was performed as described by Wilkensen [23] and as previously shown in our laboratory [7, 15, 24]. Briefly, ovaries were fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned at 7 μm onto Fisherbrand Superfrost Plus microscope slides (Fisher Scientific). Tissue sections were deparaffinized, rehydrated, treated with 20 μg/ml proteinase K and 0.1 M triethanolamine/acetic anhydride, and dehydrated before overnight incubation with radiolabeled probe at 55°C. On the next day, slides were washed under highly stringent conditions and dried. The specificity and intensity of the radioactive signal was determined by exposing slides overnight to X-OMAT film (Eastman Kodak). Afterwards, each slide was dipped in photographic NTB-2 emulsion (Eastman Kodak) and exposed at 4°C for 24 h. Slides were developed with D-19 developer and fixer (Eastman Kodak) and stained with hematoxylin. Tissue histology and the mRNA probe were visualized under light- and dark-field illumination, respectively.

Immunohistochemical analyses. Ovaries of *Ndph*$^{+/+}$ and *Ndph*$^{-/-}$ mice were fixed in 4% paraformaldehyde and embedded in paraffin as described above. Tissue sections were immunostained as previously [25] for 3β-hydroxysteroid dehydrogenase (3βHSD) using a polyclonal antibody (generously provided by Dr. Anita Payne, Stanford University, Stanford, CA). Briefly, sections were rehydrated and boiled for 10 min in 10 mM sodium citrate, pH6.0 then treated for 10 min with 1 μg/ml proteinase K (Sigma). Endogenous peroxidase activity was quenched by 10 min of treament with 0.1% hydrogen peroxide followed by a PBS wash. Nonspecific antibody binding was blocked by incubation with 20% normal goat serum in PBS followed by incubation with the primary antibody diluted (1:300) in 10% normal goat serum for overnight at 4°. The following day, the slides were washed in PBS containing 0.025% Tween 20 and then incubated with secondary antibody (biotinylated goat anti rabbit antiserum; Vector Laboratories) for 1 h at room temperature. The sections were washed and incubated with streptavidin-conjugated horseradish peroxidase for 30 min, washed and reacted with the DAB substrate (3,3 diaminobenzidine)-substrate solution (Vector Laboratories) for 2 min, dehydrated, and mounted in permount.

Immunofluorescence. Ovaries were embedded in O.C.T. compound (Sakura Finetek USA Inc.) and stored at −70°C before the preparation of 5 micron sections, which were fixed overnight in PBS-buffered 4% paraformaldehyde at 4°C. Sections were then sequentially probed with primary anti-type IV collagen (Biogenesis, Inc.) or caspase 3 (Cell Signaling) and secondary Alexa Fluor 594- or 488-conjugated goat anti-rabbit IgG antibodies (Molecular Probes) as previously described [8]. Slides were mounted using VectaShield with DAPI (Vector Laboratories).

Statistical methods. Signal strengths for all PCR reaction products were normalized with corresponding L19 values before statistical analysis. One-way ANOVA was used to test for differences between groups, with $P < 0.05$ considered statistically significant. Tests were performed using Prism software Version 4.0a (GraphPad Software, Inc.). Prism software was also used to analyze serum levels of progesterone and prolactin.

RESULTS

Follicular Development is Normal in Fzd4$^{-/-}$ Mice

Mating pairs of *Fzd4*$^{+/-}$ mice were established to generate offspring null for *Fzd4*. When several (n = 8) *Fzd4*$^{-/-}$ female mice were mated to wild-type males, no offspring were observed, supporting the original observation that the mutant female mice are infertile (Y. Wang and J. Nathans, personal communication). To determine whether the *Fzd4*$^{-/-}$ gonads were abnormal, ovaries were collected from wild-type, heterozygous, and null mice at 4 and 8 wk of age and prepared for histological examination. As shown in Figure 1A, the ovaries of *Fzd4*$^{+/-}$ and *Fzd4*$^{-/-}$ mice at 4 wk of age appeared quite similar. All stages of follicular development up to the small antral stage are present. At 8 wk of age, the ovaries of *Fzd4*$^{+/-}$ mice contained corpora lutea with cells that exhibited the typical hypertrophic phenotype of luteal cells, evidence that these mice have begun to cycle (Fig. 1B). Of note, ovaries of some *Fzd4*$^{-/-}$ mice had large antral follicles but commonly lacked clear evidence of corpora lutea, whereas in other *Fzd4*$^{-/-}$ mice corpora lutea were evident, suggesting that formation or maintenance of corpora lutea might be one defect associated with their infertility. Genes known to be expressed in follicles (aromatase, *Cyp19* and LH receptor, *Lhcgr*) and corpora lutea or interstitial tissue (P450scc, *Cyp11a1*) [26] were expressed at similar levels in the immature (prepubertal) and adult mice. Furthermore, when immature *Fzd4* wild-type and null mice were treated with eCG to stimulate increased follicular growth, the expression of these genes was similar. These data combined with the morphological data indicate that *Fzd4*$^{-/-}$ follicles exhibit normal responses to gonadotropin stimulation during the development to the preovulatory stage.

Ovulation Occurs in Fzd4$^{-/-}$ Mice

To determine whether the *Fzd4*$^{-/-}$ mice could ovulate and whether the ovulated oocytes were normal, immature mice were treated with a superovulation regimen of hormones (eCG and hCG) and then placed with a wild-type adult male mouse. Females were checked the following morning for a vaginal plug, and on the next day oocytes were recovered from the oviducts, the total number of ovulated oocytes was counted, and the number of fertilized oocytes was scored. As shown in

TABLE 2. *Fzd4*$^{-/-}$ mice are infertile and exhibit impaired steroidogenesis.

Genotype	Day p.c.	Ovulated/ fertilized oocytes	Implantation sites	n[a]	Progesterone[b] (ng/ml)	n[a]	Prolactin[b] (ng/ml)	n[a]
Fzd4$^{+/+}$	1.5	yes			6.74 ± 2.06	4	32.0 ± 15.0	3
Fzd4$^{-/-}$	1.5	yes			3.18 ± 0.41	4	12.3 ± 4.0	3
Fzd4$^{+/+}$	5.5		7.75 ± 1.2	4	23.43 ± 4.45	5	100.0 ± 30.0	3
Fzd4$^{-/-}$	5.5		0	3	2.33 ± 0.09[c]	3	85.5 ± 30.0	3
Fzd4$^{+/+}$	7.5		8.00 ± 1.2	3	37.11 ± 15.00	3	159.0 ± 147.0	3
Fzd4$^{-/-}$	7.5		0	3	1.39 ± 0.21[c]	3	31.4 ± 13.0	3

[a]n = the number of animals in each group.
[b]Serum levels of progesterone and prolactin were determined by RIA and expressed as mean (ng/ml) ± SEM.
[c]Values are significantly different; $P < 0.05$.

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Table 1, *Fzd4*$^{-/-}$ female mice produced an equal number of fertilized oocytes as their wild-type and heterozygous littermates. These observations indicated that follicles of the *Fzd4*$^{-/-}$ mice could respond to hormones, ovulate, and release mature oocytes.

Corpus Luteum Formation is Impaired in Fzd4$^{-/-}$ *Mice*

These observations led us to hypothesize that the defect in ovarian function might reside in the formation or maintenance of the corpus luteum. To analyze this process, timed mating pairs were established. Females were checked daily for copulatory plugs and removed from the cage when plugs were present. Mice were killed at Day 1.5, 5.5, or 7.5 p.c., following cardiac punctures to collect blood. One ovary was fixed in paraformaldehyde and saved for histology, the other frozen for subsequent isolation of total RNA. All mice were checked for ovulation (Day 1.5) or implantation (Day 5.5 and Day 7.5). As shown in Table 2, serum levels of progesterone were 50% lower in the *Fzd4*$^{-/-}$ mice compared to the *Fzd4*$^{+/+}$ mice at Day 1.5 p.c. Furthermore, serum levels of progesterone in the Fz4 null mice decreased dramatically on Days 5.5 and 7.5 with no evidence of implantation, whereas levels of progesterone increased in the wild-type mice that were pregnant. No significant differences were observed in levels of serum prolactin between *Fzd4*$^{+/+}$ and *Fzd4*$^{-/-}$ mice (Table 2). On Day 5.5, levels of prolactin in *Fzd4*$^{+/+}$ and *Fzd4*$^{-/-}$ mice ranged from 39 to 139 ng/ml and 35 to 139 ng/ml, respectively, indicating that serum levels of this gonadotropin were within the physiological range for pregnant and pseudopregnant mice [27]. However, levels of prolactin were higher and more variable in *Fzd4*$^{+/+}$ mice on Day 7.5 that were clearly pregnant, most likely because of the twice-daily surges of prolactin that are known to occur during early pregnancy.

Histological analyses of ovaries obtained from mice at Days 1.5 and 5.5 p.c. revealed that corpora lutea were present at these early stages in ovaries of *Fzd4*$^{+/+}$ and *Fzd4*$^{-/-}$ mice (Fig. 2). Inspection at higher magnification indicated that at Day 1.5 cell morphology was similar in wild-type and *Fzd4*$^{-/-}$ mice. However, by Day 5.5 the cells within the corpora lutea of the

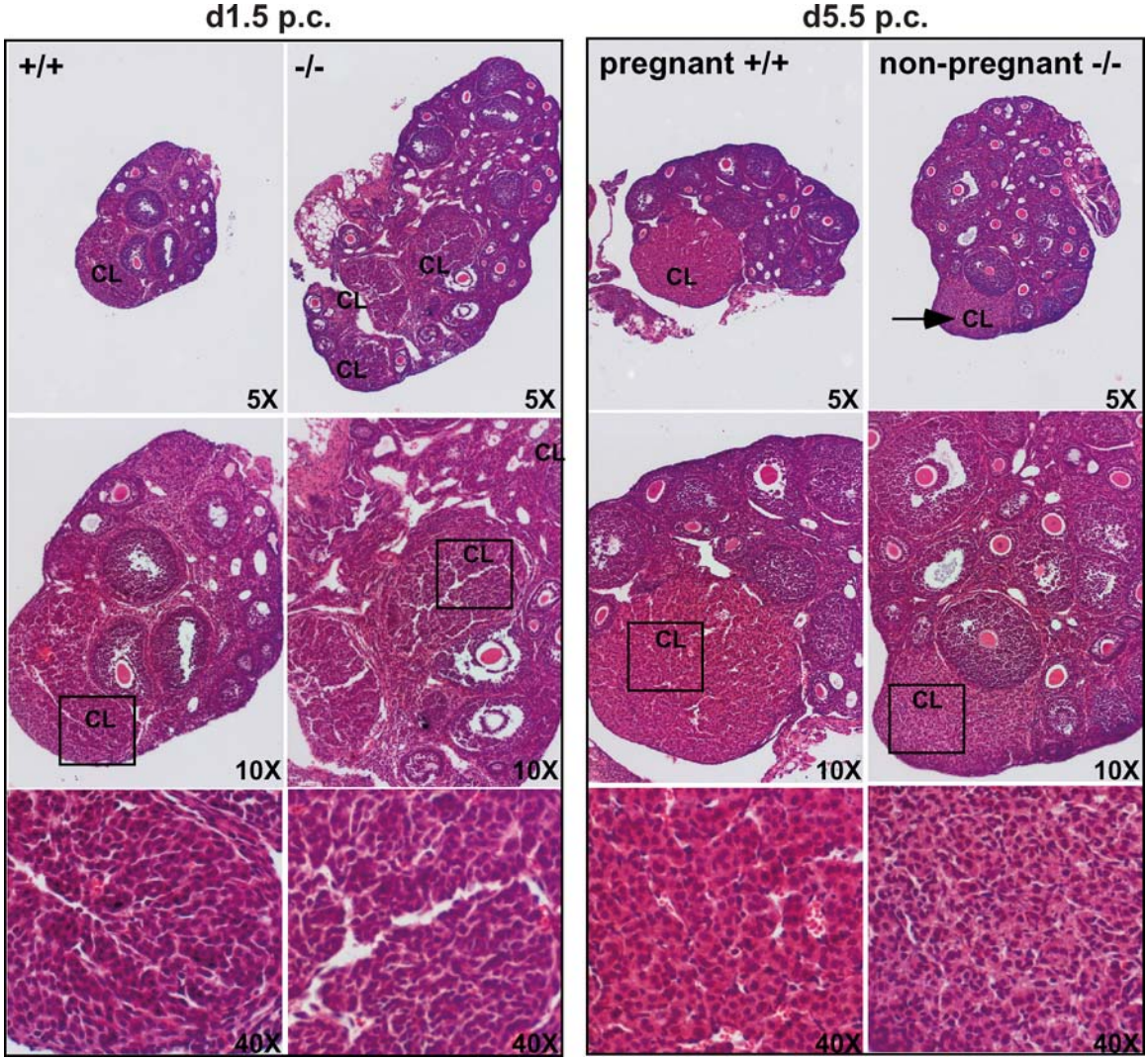

FIG. 2. Histological analysis of ovaries from Days 1.5 and 5.5 p.c. *Fzd4*$^{+/-}$ and *Fzd4*$^{-/-}$ mice. Tissue sections from ovaries of Days 1.5 and 5.5 p.c. wild-type (n = 3) and null (n = 3) mice were stained with hematoxylin-eosin. Stained sections are shown at increasing magnification in each column. Corpora lutea (arrows) are present in the ovaries of both *Fzd4*$^{+/+}$ and *Fzd4*$^{-/-}$ mice at Days 1.5 and 5.5 p.c. The luteal cells of the Day 1.5 p.c. *Fzd4*$^{-/-}$ mouse appear morphologically similar to the luteal cells of the Day 1.5 p.c. wild-type mouse. Luteal cells in the Day 5.5 pregnant wild-type ovary are marked by cellular hypertrophy, whereas luteal cells in the Day 5.5 nonpregnant *Fzd4* null ovary consistently exhibited a lower cytoplasmic to nuclear ratio. Images denoted by the boxes are shown at higher magnification in the lower panels.

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Fzd4$^{-/-}$ mice exhibited less cytoplasm and appeared to be more disorganized.

To determine whether these apparent morphological differences were related to functional differences between the wild-type and null ovaries, semiquantitative RT-PCR and in situ hybridization assays were done to analyze the expression of genes that regulate the formation (LHR, *Lcgr*), maintenance (PRLR, *Prlr*), and function (P450scc, *Cyp11a1*, sFRP4, *Sfrp4*) of the corpus luteum [15, 16]. As shown in Figure 3, expression of message for LHR, PRLR, and P450scc was lower in the ovaries of the *Fzd4*$^{-/-}$ mice as early as Day 1.5 p.c., and remained low on Day 5.5 p.c. At Day 5.5 p.c., sFRP4

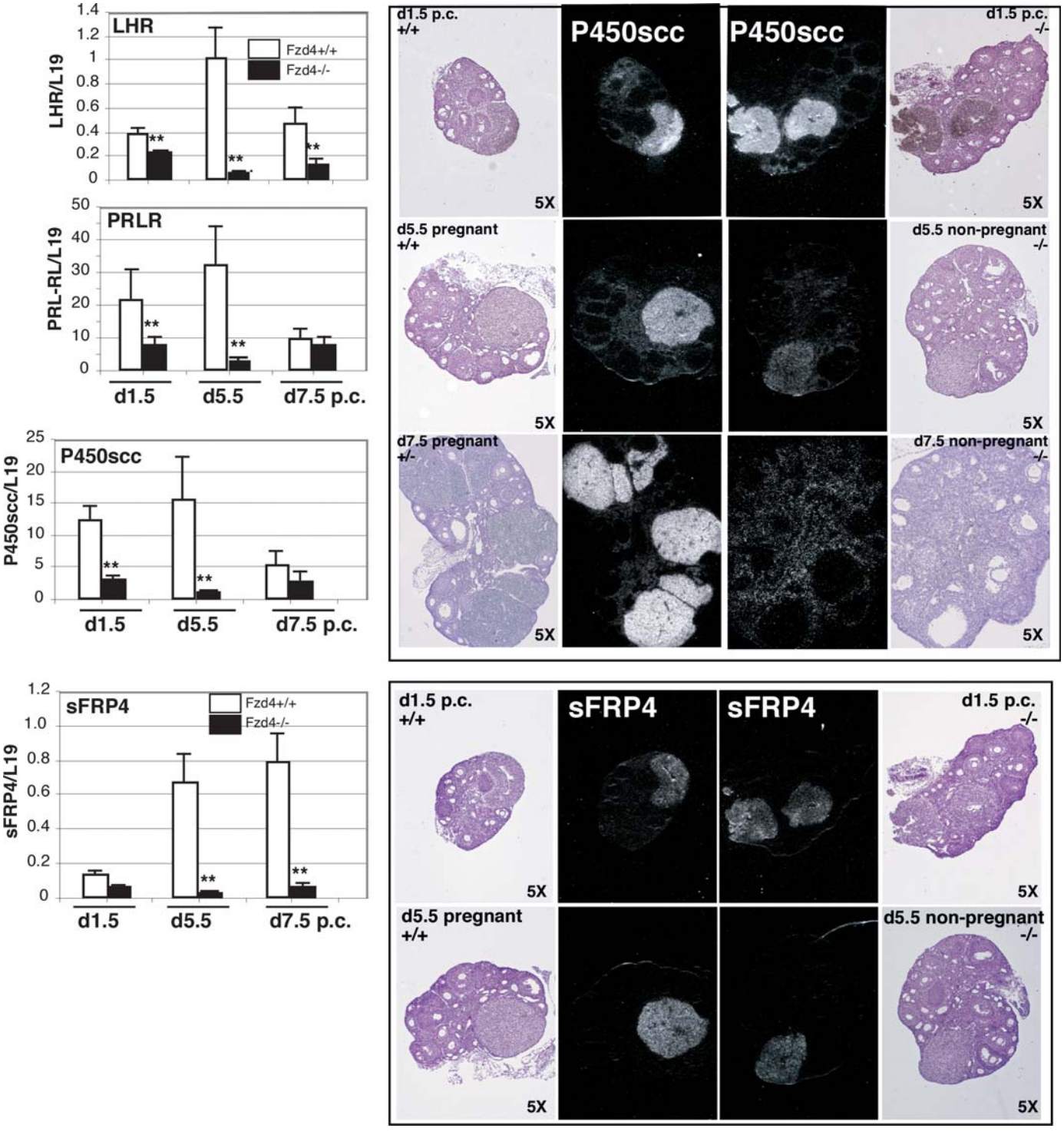

FIG. 3. Ovaries from *Fzd4*$^{-/-}$ mice exhibit impaired expression of genes associated with luteal cell function. Semiquantitative RT-PCR analyses of LHR, PRLR, P450scc and sFRP4 in ovaries of *Fzd4*$^{+/+}$ and *Fzd4*$^{-/-}$ mice at Days 1.5, 5.5, and 7.5 show that expression of these genes is reduced as early as Day 1.5. The prolactin-regulated genes, LHR and sFRP4, are also markedly lower in the *Fzd4*$^{-/-}$ compared to the *Fzd4*$^{+/+}$ mice at Day 7.5 p.c. Each point represents the mean ± SEM of three separate RNA samples (derived from three separate ovaries of three separate mice) run in triplicate. **Statistically significant difference ($P < 0.05$) between *Fzd4*$^{+/+}$ and *Fzd4*$^{-/-}$ samples at each time interval. In situ hybridization shows the localization of P450scc and sFRP4 to corpora lutea and the reduced levels of expression in corpora lutea at d5.5.

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and similarly LHR, both of which are specific targets of prolactin in the mouse corpus luteum, were reduced, and remained low at Day 7.5p.c. [15, 28]. In situ hybridization analyses confirmed the tissue-specific expression of P450scc and sFRP4 in luteal cells and the reduced expression of these genes in ovaries of the *Fzd4*$^{-/-}$ mice, especially on Day 5.5 (Fig. 3). It should also be noted that, by Day 7.5 p.c., corpora lutea were not obvious in the ovaries of the *Fzd4*$^{-/-}$ mice, indicating further that the formation of functional corpora lutea was impaired.

Vascular Defects Are Associated with Abnormal Corpus Luteum Formation in Fzd4$^{-/-}$ *Mice*

Because *Fzd4*$^{-/-}$ mice have vascular defects in the retina and inner ear [8], we sought to determine whether the *Fzd4*$^{-/-}$ mice might exhibit impaired vascular development of the corpora lutea. To determine if specific defects in angiogenesis occurred, immunofluorescent and RT-PCR analyses of known markers and regulators of vascular formation were performed. Ovaries from *Fzd4*$^{+/+}$ and *Fzd4*$^{-/-}$ mice were isolated at Day 1.5 p.c. and prepared for immunofluorescent analyses using type IV collagen as a marker of the basal lamina of endothelial cells as well as other cell layers [8]. The granulosa cell layer of growing and antral follicles was devoid of collagen IV staining (Fig. 4, upper panel), supporting previous studies that have shown the granulosa cell layer to be avascular [12]. Rather, collagen IV was restricted to the basal lamina surrounding the outer layer of granulosa cells, vascular elements of the stroma, and the surface epithelium. However, during the formation of the corpus luteum, an elaborate vascular network is established within 24 h following the LH surge (Fig. 4, lower panels) [29–31]. In ovaries of *Fzd4*$^{+/+}$ and *Fzd4*$^{-/-}$ mice at Day 1.5 p.c.,

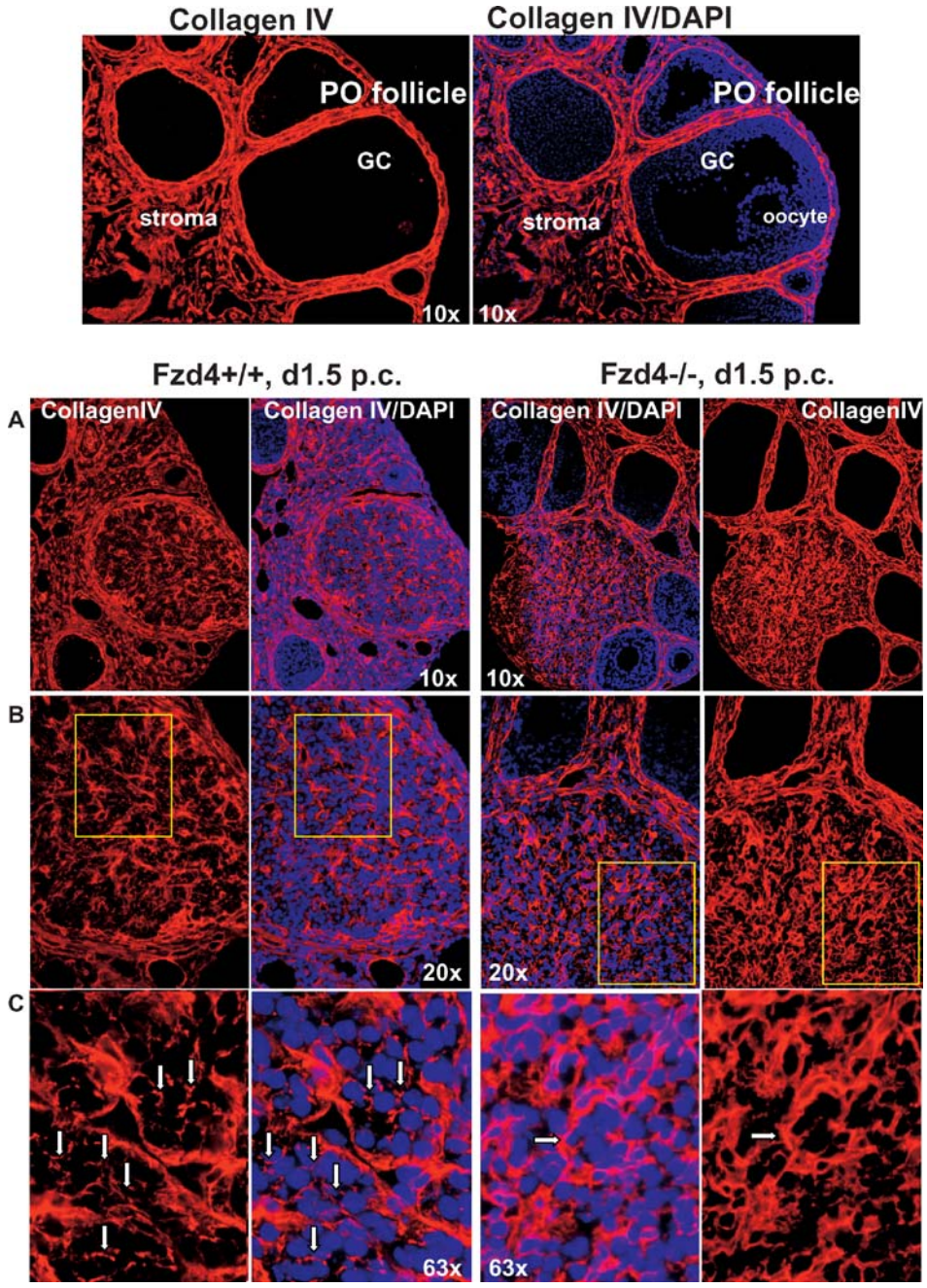

FIG. 4. Vascular changes that occur during luteinization appear abnormal in the *Fzd4*$^{-/-}$ mice. Upper panels: immunofluorescence analysis of ovaries of eCG primed *Fzd4*$^{+/+}$ mice (n = 3) localizes type IV collagen (red) to the basal lamina of blood vessels within the stroma and theca layer. No signal for type IV collagen is observed in the avascular granulosa cell layer of preovulatory (PO), antral, or small antral follicles. DAPI (blue) was used to stain nuclei. Lower panels: localization of type IV collagen (red) and nuclei (DAPI) in ovaries of *Fzd4*$^{+/+}$ mice (n = 3) and *Fzd4*$^{-/-}$ mice (n = 3) at Day 1.5 p.c. shows the presence of corpora lutea, as indicated by the network of collagen IV staining in luteal structures. Close examination of the vascular network present in the *Fzd4*$^{+/+}$ corpora lutea shows a fine,, filamentous pattern punctuated with small endbuds (multiple arrows). This pattern is not observed in the *Fzd4*$^{-/-}$ corpora lutea. Rather the corpora lutea of the *Fzd4*$^{-/-}$ mice exhibit a thick, dense collagen IV network (single arrows). Magnification ×10 (**A**), ×20 (**B** and **C**), and ×63 (boxed area shown in **B**).

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corpora lutea are clearly distinguished from surrounding follicles by a complex network of collagen IV staining. However, even at low magnification, and more obviously at higher magnification, the pattern of the collagen matrix is visibly different in the *Fzd4*$^{-/-}$ corpora lutea compared to the wild-type ovary. Specifically, in the *Fzd4*$^{+/+}$ corpora lutea, the collagen IV staining is more filamentous and punctate (suggesting greater arborization of the vessels) than that observed in the *Fzd4*$^{-/-}$ corpora lutea. Although the differences may appear subtle, they were consistently observed in each tissue examined.

Formation and maintenance of the vascular system is complex, tissue-specific, and dependent on many factors [32], among which are the newly identified FZD4 ligand NDP [8] and EFNB2 and EPH4 [33], as well as the VEGF family of growth factors [32]. Because mutations in the *NDP* and *FZD4* genes present a similar but highly variable phenotype in humans, characterized by incomplete vascularization of the peripheral retina up to complete blindness, and because mice null for *Fzd4* also exhibit impaired retinal development and vascular formation [8], we first sought to determine whether message encoding the murine homolog *Ndph* was expressed in the ovary and during the formation of the corpus luteum. As shown in Figure 5A, *Ndph* and *Fzd4* transcripts overlap in the ovary as well as in other mouse tissues, especially the brain and uterus. In the ovary, *Ndph* message was detected in isolated granulosa cells as well as in the residual tissue remaining after removal of most granulosa cells via needle puncture. *Ndph* message was expressed in the ovary at multiple stages of follicular development and during ovulation and luteinization, but was not markedly regulated by hormones and was not elevated in luteal tissue (Fig. 5B). Additionally, *Ndph* mRNA was expressed equally well in *Fzd4*$^{+/+}$ and *Fzd4*$^{-/-}$ mice at Day 5.5 p.c. (Fig. 6). Histological analyses of ovaries from adult 8-wk-old wild-type and null NDP mice revealed an abundance of corpora lutea in each genotype (Fig. 5C) unlike that observed in the 8-wk-old *Fzd4*$^{-/-}$ mice (Fig. 1); corpora lutea appeared normal in the *Ndph*$^{-/-}$ mice. That the corpora lutea of the *Ndph*$^{+/+}$ as well as the *Ndph*$^{-/-}$ mice exhibited intense staining for the steroidogenic

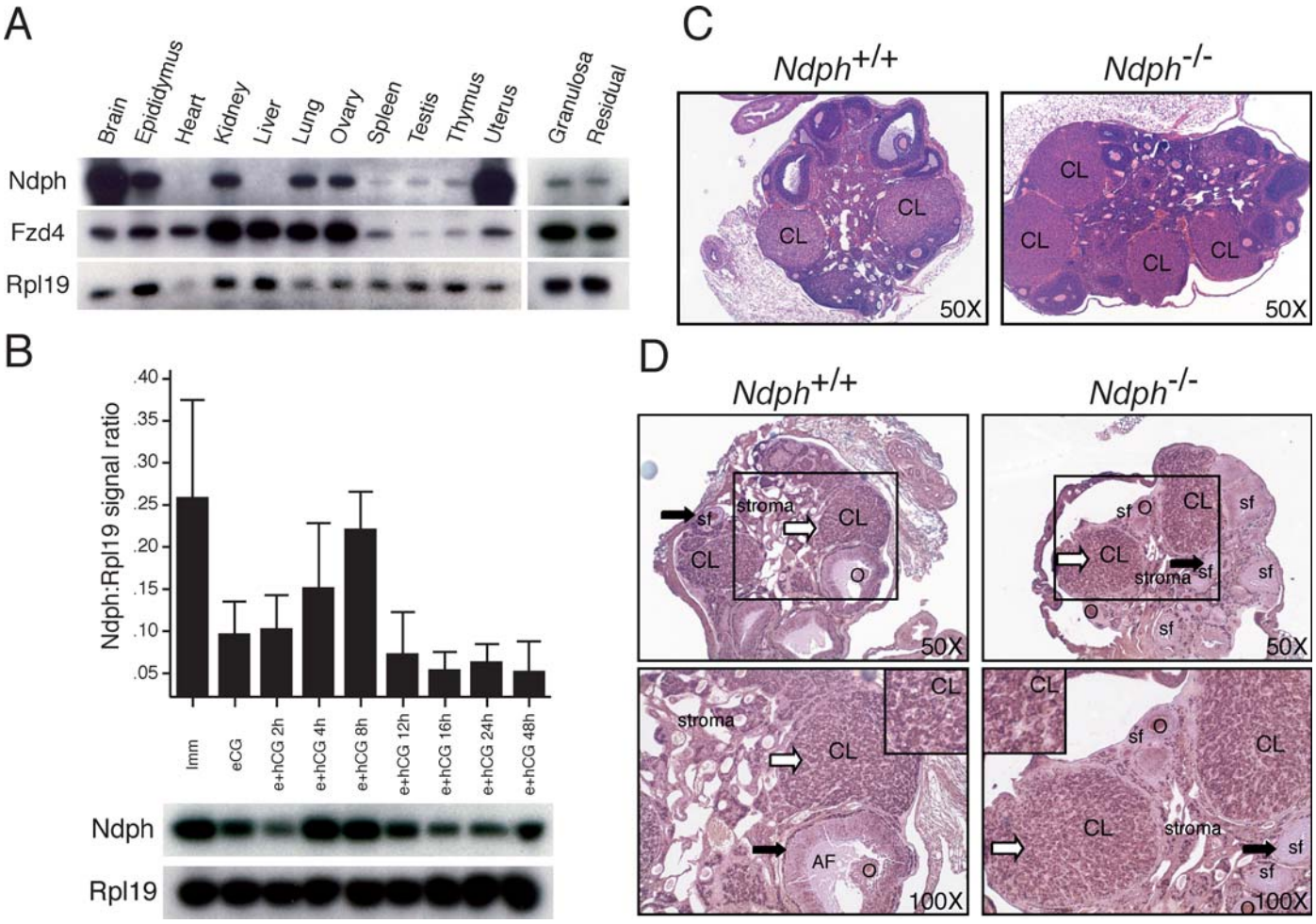

FIG. 5. NDP (*Ndph*) and Fzd4 (*Fzd4*) transcripts are expressed in various mouse tissues, including the ovary. **A**) Semiquantitative RT-PCR analyses were done to determine the relative expression of *Ndph* and *Fzd4* in various mouse tissues. Significant expression of both *Ndph* and *Fzd4* mRNA is observed in the ovary. However, highest expression of *Ndp*h is observed in the uterus, brain, and epididymus. *Ndph* was expressed in granulosa cells and residual tissue. **B**) Semiquantitative RT-PCR analyses of *Ndph* at different stages of follicular development and luteinization. Each point represents the mean ± SEM of three separate RNA samples (derived from three ovaries of three separate mice) run in triplicate. Expression of *Ndph* was not regulated significantly ($P > 0.05$) by hormones. **C**) Histological analyses of ovaries from adult 8-wk-old *Ndph*$^{+/-}$ *and Ndph*$^{-/-}$ mice indicate that multiple corpora lutea (CL) are present. **D**) Corpora lutea of the *Ndph*$^{+/-}$ *and Ndph*$^{-/-}$ mice are immunopositive for the steroidogenic marker HSD3β, which is present in the cytoplasm of the luteal cells (insert in lower panels). The stromal tissue is also immunopositive. Granulosa cells of small follicles (sf) are not stained; granulosa cells of AF show weak staining. Oocyte (o) staining is nonspecific.

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marker 3βHSD (HSD3β) provided additional evidence that these corpora lutea were functional (Fig. 5D). Thus, despite its expression in the mouse ovary, NDP does not appear to be the key mediator of FZD4 signaling in this tissue, because *Ndph*$^{-/-}$ mice do not phenocopy the *Fzd4*$^{-/-}$ mice.

The WNT signaling pathway has been shown to impact the Ephrin/Eph signaling pathway in several tissues [33]. In addition, expression transcripts encoding *Efnb1*, *Efnb2*, and *Efnb4* have been linked to arterial venous junction formation in tissues including the corpus luteum [33, 34]. Therefore, we next analyzed the expression of these factors in ovaries of *Fzd4* wild-type and null mice at Days 1.5, 5.5, and 7.5 p.c. As shown in Figure 6, expression of *Efnb1*, *Efnb2*, and *Efnb4* message was significantly lower in *Fzd4*$^{-/-}$ ovaries at Days 1.5 and 5.5 pc but not at Day 7.5 pc, indicating that these regulators of vascular development were reduced as early as Day 1.5 p.c. Because the VEGF family of regulators impacts both angiogenesis and lymphangiogenesis, we also examined two transcript encoding members of this family, *Vegfa* and *Vegfc* [12, 29, 30, 35]. As shown, the expression of *Vegfa* and *Vegfc* mRNA was significantly reduced in the *Fzd4*$^{-/-}$ compared to the *Fzd4*$^{+/+}$ ovaries on all days analyzed. These data support the immunofluorescent analyses indicating that markers of vascular cell function are reduced in ovaries of Fzd4-null female mice following natural mating.

Finally, activated caspase 3 activity, a known marker of apoptotic cells, was analyzed in ovaries from the *Fzd4*$^{+/+}$ and *Fzd4*$^{-/-}$mice by indirect immunofluorescence using a specific antibody against activated caspase 3. As shown in Figure 7, no signal was detected in corpora lutea of *Fzd4*$^{+/+}$ mice, whereas intense signals were observed in corpora lutea of *Fzd4*$^{-/-}$ mice at Day 1.5 p.c. These data clearly indicate that cells of the corpora lutea of the *Fzd4*$^{-/-}$ mice undergo apoptosis already at this early time point and therefore do not form appropriately.

DISCUSSION

These studies show that female *Fzd4*$^{-/-}$ mice present a defective reproductive phenotype that is characterized by relatively normal ovarian follicular development and responses to eCG/FSH but exhibits impaired formation and function of corpora lutea. Specifically, the histological appearance of ovaries of immature *Fzd4*$^{-/-}$ mice was similar to that of their heterozygous and wild-type littermates. Molecular markers of follicular development, i.e., aromatase, LH receptor, and P450scc mRNAs, are expressed at similar levels in ovaries of immature *Fzd4*$^{-/-}$ and *Fzd4*$^{+/+}$ mice. Furthermore, when immature *Fzd4*$^{-/-}$ and *Fzd4*$^{+/+}$ mice were treated with eCG to enhance follicular development, ovarian levels of aromatase, LH receptor, and P450scc message were induced equally. These results indicate that early stages of follicular development and the growth of follicles to the preovulatory stage are similar in the *Fzd4*$^{-/-}$ and *Fzd4*$^{+/+}$ mice. These functional characteristics differ dramatically from the phenotype of the *Wnt4*$^{-/-}$ mice that at birth exhibited altered ovarian development in which few oocytes were present and testicular-like structures expressing testis-specific genes were evident [3]. Thus, FZD4 is not likely to be the receptor for WNT4 in the embryonic gonad.

Adult *Fzd4*$^{-/-}$ females exhibit normal mating behavior and ovulate mature oocytes that can be fertilized. Thus, not only do preovulatory follicles mature, but by inference the positive feedback control to the pituitary is also operative to stimulate an endogenous LH surge sufficient for ovulation. However, implantation was never observed, suggesting that although corpora lutea were present initially following natural matings,

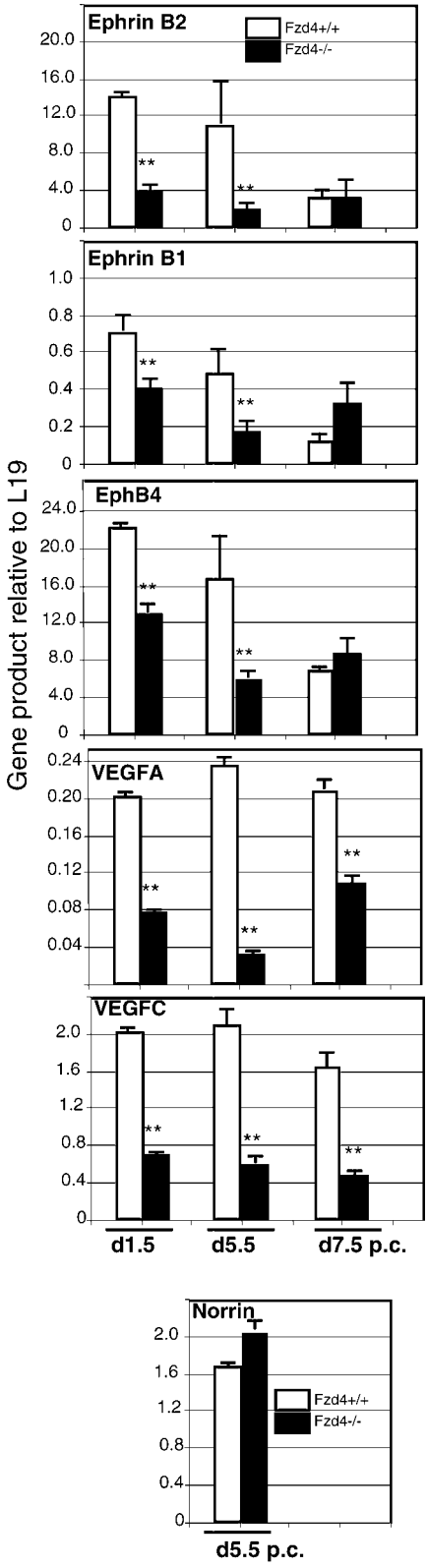

FIG. 6. Markers of vascular endothelial cell function are reduced in the *Fzd4*$^{-/-}$ ovaries. Semiquantitative RT-PCR analyses of Ephrin B2 (*Efnb2*), Ephrin B1(*Efnb1*), Eph4 receptor (*Eph4*), *Vegfa*, and *Vegfc* show significantly lower expression of each gene at Days 1.5 and 5.5 p.c. In addition, *Efnb2* and *Vegfc* are also markedly reduced on Day 7.5 p.c. In contrast, expression of NDP (*Ndph*) is similar on Day 5.5 p.c. Each point represents the mean ± SEM of three separate RNA samples (derived from three ovaries of three separate mice) run in triplicate. **Statistically significant difference ($P < 0.05$) between *Fzd4*$^{+/+}$ and *Fzd4*$^{-/-}$ at that time point.

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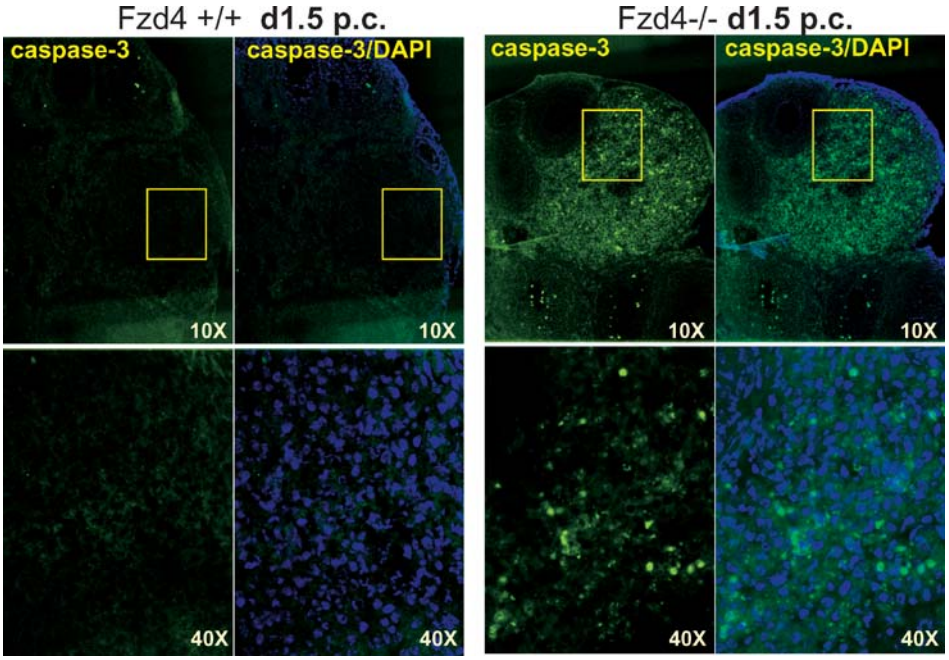

FIG. 7. Corpora lutea of *Fzd4*$^{-/-}$ mice at Day 1.5 p.c. exhibit signs of apoptosis, as indicated by the presence of activated caspase 3. Frozen sections of ovaries from *Fzd4*$^{+/+}$ mice (n = 3) and *Fzd4*$^{-/-}$ (n = 3) mice were prepared for immunofluorescence using an anti-caspase 3 antibody that recognizes activated caspase 3. As shown, no signal was observed in corpora lutea of wild-type mice at Day 1.5 p.c., whereas corpora lutea from *Fzd4*$^{-/-}$ mice gave an intense signal.

they might be functionally defective in these mice. Indeed, *Fzd4*$^{-/-}$ ovaries collected at Days 1.5, 5.5, and 7.5 p.c. exhibited an altered histological appearance and reduced expression of genes that are known to be associated with luteinization. These include the LH receptor (*Lhcgr*) [14], PRLR (*Prlr*) [13, 16, 28], P450scc (*Cyp11a1*) [36], and sFRP4 (*Sfrp4*) [15]. The reduced expression of P450scc is associated with the reduced levels of progesterone that is likely one major factor in implantation failure.

Because the most dramatic decreases in gene expression occurred in those genes highly regulated by prolactin, namely the LH receptor [14] and sFRP4 [15], these data suggested to us that prolactin signaling might be defective and/or not established in the newly forming corpora lutea of the *Fzd4*$^{-/-}$ mice. In support of this, the phenotype of the *Fzd4*$^{-/-}$ null mice is similar to that of mice null for the prolactin receptor (*Prlr*$^{-/-}$) [16]. Although corpora lutea form in the *Prlr*$^{-/-}$ mice, the morphological appearance of the luteal cells was altered and the cells were highly disorganized. Further, in the *Prlr*$^{-/-}$ mice, expression of LH receptor was significantly reduced by Day 1.5 p.c., and P450scc by Day 3.5 p.c. Thus, in both the *Fzd4*$^{-/-}$ and *Prlr*$^{-/-}$ mice, corpora lutea form following natural matings but luteal cell function is impaired.

Importantly, in both mutant mouse models the altered function of the corpora lutea appears to be associated with distinct structural abnormalities within the vascular network. Whether or not the cause for abnormal vascularization is the same remains to be determined. In the *Prlr*$^{-/-}$ mice the altered vascular morphology was visualized by a reduced amount of immunostaining of the tissue with CD31/PECAM-1, a marker of endothelial cells [16]. In the *Fzd4*$^{-/-}$ mice, the immunofluorescent pattern of type IV collagen, a marker of the basal lamina of developing vessels, was distinctly altered as early as Day 1.5 p.c. Specifically, the filamentous type IV collagen network visible in corpora of the *Fzd4*$^{+/+}$ mice was lacking in the *Fzd4*$^{-/-}$ mice. By Day 5.5 p.c., altered morphology (Fig. 2) was also clearly evident using standard histological staining. Thus, in the *Fzd4*$^{-/-}$ mice the establishment of the vascular network was impaired as early as Day 1.5 p.c., and impairment was more dramatic at Day 5.5 p.c.

The gene product of the Norrie disease gene, NDP, has recently been found to be a specific high-affinity ligand for FZD4 in neuronal cells [8]. Furthermore, NDP was shown to activate the FZD4 receptor only in the presence of the coreceptor LRP5, thus mimicking the activation by Wnt ligands [8]. These data suggested that NDPH might be the ligand for FZD4 in the ovary and might control a specific step in vascularization. Herein we document for the first time that *Ndph* mRNA is expressed in the ovary (both in granulosa cells and in residual and luteinized ovarian tissue) as well as the testis, albeit at lower levels than in the brain and uterus. Importantly, expression of *Ndph* mRNA was not hormonally regulated or altered in the *Fzd4*$^{-/-}$ mice, indicating that *Ndph* is not a target of *Fzd4*. Follicular development as well as corpus luteum formation appear to be normal in the *Ndph*$^{-/-}$ mice, because follicles of many sizes and corpora lutea are present in ovaries of adult *Ndph*$^{-/-}$ mice at 8 wk of age. Furthermore, the corpora lutea express 3βHSD (Fig. 5) and P450scc (not shown) protein, which is indicative of normal function. Thus, contrary to our original hypothesis, NDPH may not be the critical activating ligand for FZD4 in murine corpora lutea, and therefore does not appear to be a factor controlling vascular formation in the corpus luteum. This may be related in part to the very specific sites and types of vessels that NDPH has been shown to regulate, namely the formation of the hyaloid vasculature of the retina and inner ear [8]. However, because *Ndph* mRNA is expressed at high levels in the uterus, the ligand may activate FZD4 to impact the function of uterine events, including implantation. Although the functional FZD4 ligand remains to be determined, *Wnt4* is expressed in the mouse corpus luteum and therefore may be the FZD4 ligand in this tissue [7]. However, the role of *Wnt4* at this stage of ovarian function in adult mice awaits the conditional ovarian knockout of the *Wnt4* gene.

Vascular development is complex, tissue-specific, and controlled by many factors, including members of the VEGF family and the Eph receptor/Ephrin ligand family [30, 32, 37]. Angiogenesis in the ovary, and more specifically during luteinization, has been studied by several groups [12, 29, 30, 38]. Although in most mouse models in which angiogenic

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factors have been genetically targeted for deletion, the mice die in utero, precluding careful analyses in adult tissues [30, 32]. Studies have shown that VEGFA, which regulates endothelial cell proliferation, migration, and permeability, is expressed and regulated during the ovulation process [12, 30] and is essential for corpus luteum formation [29]. The vascular endothelial cell-specific adhesion molecule VE-cadherin and VEGF receptor 2 are also obligatory for corpus luteum formation and survival, respectively [31, 39]. That VEGFC, a factor regulating development of lymphatic vessels, is also expressed and regulated during the formation of corpora lutea represents a novel observation. Furthermore, expression of both *Vegfa* and *Vegfc* mRNAs is reduced in ovaries of the *Fzd4*$^{-/-}$ mice compared to wild-type mice on Days 1.5, 5.5, and 7.5 p.c. These data indicate that vascular development is impaired in the *Fzd4*$^{-/-}$ corpora lutea and may contribute to luteal cell malfunction or be a reflection of luteal cell demise.

Recently, *Efnb2* has been shown to be expressed in theca cells and corpora lutea of mice genetically engineered to express LacZ driven by the *Efnb2* promoter [33]. In addition, *Efnb1* and *Efnb2* as well as *Efnb4* receptor mRNAs are expressed on luteinizing granulosa cells in human corpora lutea during the early luteal phase [40]. Our results show that both *Efnb2* and *Efnb1* as well as *Ephb4* are expressed at high levels in newly forming corpora lutea on Days 1.5 and 5.5 p.c. of *Fzd4*$^{+/+}$ mice but are markedly reduced at these times in the *Fzd4*$^{-/-}$ mice ovaries. Because the Ephrin signaling through the Eph receptors involves direct cell-cell interactions and because ENFB and EPHB receptor pairs exert effects on adjacent cells in a bidirectional manner [33, 37], our results suggest that these factors may aid in establishing the arterial-venous system in the corpus luteum. The reason or reasons for the decrease in Eph4 on Day 7.5 in ovaries of *Fzd4*$^{+/+}$ mice are not known, but may relate the to more potent role of this system in establishing vascular tissue than in maintaining it. Because EPH receptors signal through tyrosine kinases and because their expression has been shown in some tissues to be controlled by the β-catenin/TCF pathway [41], it is also possible that they are targets of FZD4 in the ovary.

Collectively, our results indicate that ovarian follicular development and ovulation can occur in the *Fzd4*$^{-/-}$ mice but that development of functional corpora lutea is impaired. Defective luteal cell function appears to be associated with impaired angiogenesis, in which the rich vascular network that normally develops in the early stages of the developing corpus luteum is not appropriately established. Because disruption of vascular development impairs luteal cell formation [29–31] and because WNT/FZD signaling has been linked to vascular development in other tissues [4, 8], abnormal angiogenesis may contribute to defective luteal cell formation in the *Fzd4*$^{-/-}$ mice. Alternatively, because *Prlr* mRNA levels were low in *Fzd4*$^{-/-}$ mice at Days 1.5 and 5.5 p.c., and because the *Fzd4*$^{-/-}$ mice phenocopy closely the structural and functional abnormalities observed in the *Prlr*$^{-/-}$ mice, including apoptosis, defective PRLR signaling may contribute not only to the reduced expression of steroidogenic genes and Wnt signaling molecules such as *Sfpr4* but also to the reduced expression of specific vascular regulatory factors during corpus luteum formation.

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