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Generation of Mice Expressing Only the Long Form of the Prolactin Receptor Reveals That Both Isoforms of the Receptor Are Required for Normal Ovarian Function[1]

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ABSTRACT

Prolactin (PRL), a pleiotropic hormone essential for maintenance of corpus luteum (CL) function and pregnancy, transduces its signal through two types of receptors, a short form (PRLR-S) and a long form (PRLR-L). Both types of receptors are expressed in the CL, yet their individual roles are not well defined. We have shown previously that female transgenic mice expressing only PRLR-S display total infertility characterized by defective follicular development and early degeneration of CL, suggesting that expression of PRLR-L is a prerequisite for normal follicular development and maintenance of CL. To determine whether PRLR-L alone is the sole receptor required to maintain normal CL formation, differentiation, and progesterone secretion, we generated two transgenic mice which express only PRLR-L, either ubiquitously (Tg-RL) or in a CL-specific manner (CL-RL). To generate CL-specific expression, we used the HSD17B7 promoter. We found both transgenic mice models cycled normally, displayed no apparent defect in follicular development, and had normal ovulation rates. The STAT5 signaling pathway, considered essential for luteinization and progesterone production, was activated by PRL in both transgenic mice models. However, soon after mating, Tg-RL and CL-RL mice showed early regression of CL, lack of progesterone production, and implantation failure that rendered them totally infertile. Embryo transfer studies demonstrated no embryo abnormalities, and supplementation with progesterone rescued implantation failure in these mice. Close observation revealed lack of luteinization and reduced expression of proteins involved in progesterone biosynthesis despite normal levels of LHCGR (LH-R), ESR1 (ER-alpha), CEBPB (C/EBP-beta) and CDKN1B (p27), proteins essential for luteinization. However, we found VEGFA, a key regulator of angiogenesis and vascularization, to be dramatically reduced in both Tg-RL and CL-RL mice. We also found collagen IV, a marker for the basal lamina of endothelial cells, aberrantly expressed and a discordant organization of endothelial cells in CL. Although luteinization did not occur in vivo, granulosa cells isolated from these mice luteinized in culture. Taken together, these results suggest that a vascularization defect in the CL may be responsible for lack of luteinization, progesterone production, and infertility in mice expressing only PRLR-L. This investigation therefore demonstrates that in contrast to earlier presumptions that PRLR-L alone is able to support normal CL formation and function, both isoforms of the PRL receptor are required in the CL for normal female fertility.

corpus luteum, female infertility, ovary, prolactin, prolactin receptor, vascularization

INTRODUCTION

Embryo implantation and maintenance of pregnancy are dependent upon proper secretion of progesterone by the ovarian corpus luteum (CL), a transient endocrine gland formed from theca and granulosa cells following ovulation. In rodents, one of the primary hormonal stimuli necessary for CL formation and progesterone production is prolactin (PRL), a peptide hormone synthesized and secreted principally by the anterior pituitary [1–7]. Both PRL and PRL receptor (PRLR) null mice are infertile [8, 9]. CL in these mice ceases to grow, undergoes apoptosis, and rapidly regresses, leading to insufficient progesterone production [10–12]. Although several genes important for CL formation, survival, and function have been identified as direct targets of PRL [13–17], the signaling mechanism of PRL-mediated action in the CL is still unclear due in part to the fact that PRL can bind to two types of PRLR, a long (PRLR-L) and short (PRLR-S) form, derived by alternative splicing of the *Prlr* gene [12, 18]. These two receptors share homology in their extracellular and transmembrane domains and differ in length and composition in the intracellular domains. Although both PRLR-L and PRLR-S are expressed in the ovary, the function of each receptor for PRL-mediated action has not been elucidated [19, 20]. The generation of transgenic mice expressing only PRLR-S [21] allowed us to demonstrate [22–25] that PRL signaling through PRLR-S alone is unable to stimulate the canonical JAK2/STAT pathway in luteal cells or to sustain CL formation or function but causes repression of genes important for maintaining normal follicular development, causing premature ovarian failure. PRLR-S is unable to activate STAT5 upon PRL binding [25, 26], presumably due to the lack of specific tyrosine residues in its intracellular domain [27, 28]. In sharp contrast, the main pathway activated by PRL binding to PRLR-L in the ovary is the JAK2/STAT pathway [14, 17, 19, 29], and functional studies have revealed that activation of STAT5 proteins is essential for CL formation. Double-knockout of the STAT5A and STAT5B proteins led to infertility in female mice, characterized by few or no large CL in the ovary, emphasizing the importance of activation of the JAK2/STAT5 pathway by PRL for CL formation, survival, and function [30]. This led us and other investigators [6, 14, 31, 32] to suggest that PRL signaling through PRLR-L alone and activation of the JAK2/STAT5 pathway may be the sole factor required for CL formation and survival and thus for pregnancy. PRLR-L is ubiquitously expressed and found in the ovary, adrenal gland, kidney, mammary gland, decidua, small intestine, choroid plexus, and pancreas. Because of the broad distribution of

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PRLR-L and because PRLR-S is often coexpressed in the same tissue, it is difficult to examine the specific role of PRLR-L in the CL by using whole animals. In the present investigation, we generated two PRLR-L transgenic mouse lines on the PRLR null background, one line with ubiquitous expression and the second line with CL-specific expression of PRLR-L, and examined the role of PRL in ovarian development and function in these mice expressing only PRLR-L.

Generation of these transgenic mice did not support the contention that PRLR-L is the only essential receptor necessary for normal CL formation and function. PRL activation of this receptor leads to STAT5 phosphorylation and maintenance of key genes involved in luteinization and corpus luteum formation. However, this PRLR-L-mediated effect appears unable to sustain normal blood vessel formation and vascularization, a crucial event for luteal cell hypertrophy and steroidogenesis. The fact that PRLR-S is the predominant receptor expressed in endothelial cells derived from CL [33] suggests the intriguing possibility that PRLR-L expressed in luteal cells and PRLR-S expressed in the vasculature act in concert to allow normal CL formation and function, ultimately allowing the CL to sustain fertility and pregnancy.

MATERIALS AND METHODS

Generation of Transgenic Mice Expressing PRLR-L, Either Ubiquitously (Tg-RL Line) or in a CL-Specific Manner (CL-RL Line)

To create mice with ubiquitous expression of PRLR-L, a targeting construct was generated similar to the one used previously to generate mice expressing only PRLR-S [21]. The *Prlr*-L cDNA was generated by PCR and subsequently subcloned into the EF1A-pPolyIII vector (a gift from Dr. Nadine Binart at INSERM, Paris, France). This targeting vector (EF1-RL), used to generate mice with ubiquitous expression of PRLR-L, contains EF1A promoter, SRα enhancer region, *Prlr*-L cDNA, and a portion of human growth hormone (hGH) cDNA, a tag to identify transgenic mice.

To generate CL-targeted expression of PRLR-L, we cloned and isolated the promoter region of the CL-specific gene, *Hsd17b7*, by screening mouse 129/SvJ genomic library (Stratagene) with probes for exons 1 and 2. Of the 20 clones selected, the clone containing the 2.8-kb promoter region was subsequently subcloned, resequenced, and confirmed as being upstream of the mouse *Hsd17b7* gene by BLAST analysis. Subsequent 5′ truncations of the promoter identified the 1.2-kb region as having the highest promoter activity. Therefore, we replaced the EF1A promoter in the ubiquitous construct with the 1.2-kb HSD17B7 promoter. Additionally, to generate a fusion protein, the *Prlr*-L cDNA stop sequences were replaced by an enhanced green fluorescent protein (EGFP) sequence, isolated from pEGFP-N1 (Invitrogen).

Both transgenic constructs were tested in culture for their activity levels. Briefly, GG-CL cells, derived from rat CL generated in our laboratory [34], were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were cotransfected with empty vector and either EF1-RL or HSD17B7-RL-EGFP vector with equal amounts of either β-casein or LHRE promoter reporter constructs, respectively. Following transfection, cells were treated either with vehicle or 1 μg/ml ovine PRL (Protein Laboratories Rehovot, Ltd.) for 24 h.

Following confirmation of activity of the transgenic vectors, the constructs were linearized and injected into pronuclei of fertilized oocytes from FVBN mice by the Transgenic Production Services at the University of Illinois at Chicago. Transgenic mice were identified by PCR and Southern blotting using primers/probes to the hGH tag. Transgenic mice were backcrossed with PRLR null ($PRLR^{-/-}$) mice to generate mice expressing only PRLR-L, either ubiquitously (Tg-RL) or in a CL-specific manner (CL-RL).

Genotyping by PCR and Southern Blot Analysis

Genotyping was performed to identify transgenic mice as previously described [8]. The protocol used for Southern blot analysis has been previously described [35]. For Southern blot analysis, the hGH probe was generated by isolation and purification of hGH cDNA from the transgenic construct. The hGH probe was radiolabled with [^{32}P]deoxycytidine triphosphate by using Rediprime II random primer labeling kit (GE Healthcare). To determine copy number, genomic DNA from wild-type mice was spiked with appropriate amounts of transgenic DNA to generate a standard curve. Blots were visualized by autoradiography using Kodak Biomax MS film (Sigma) with an intensifier screen.

Experimental Animals

Mice were kept under conditions of controlled light (0700–1900 h) and temperature (22°C–24°C) with free access to standard rodent chow and water. All experimental procedures were performed in accordance with guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee. Transgene injections, collection of oocytes, and embryo transfer experiments were conducted with the help of Dr. Roberta Franks and Kimberly McLaughlin of the University of Illinois at Chicago Research Resources Center Transgenic Production Service.

To confirm PRL activation of PRLR-L and examine downstream targets, we injected mice s.c. with 200 μg of α-ergocryptine (Sigma) to block endogenous PRL secretion, followed by i.p. injection of 60 μg of ovine PRL (Protein Laboratories Rehovot, Ltd.) 4 h later. Mice were then euthanized at various time points post-PRL injection, and tissues were harvested and analyzed for protein expression.

To examine implantation sites, we allowed mice to mate and injected them with vehicle or 3 mg of progesterone daily to maintain pregnancy, beginning on the day the vaginal plug was present (pregnancy Day 0.5). On Day 5.5 of pregnancy, mice were injected with 0.1 ml of a 1% solution of Chicago SkyBlue 6B dye (ACROS Organics) by tail vein injection under isoflurane anesthesia, and the number of implantation sites in the uterus, stained with dye, was recorded. Mice were superovulated by i.p. injection of equine chorionic gonadotropin (eCG) (Sigma), followed 48 h with i.p. injection of human chorionic gonadotropin (hCG; Sigma), and ovaries were collected at various time points thereafter for either histology or protein. An n of 3–5 mice per group was used for all experiments.

RNA Isolation, Semiquantitative PCR, and Quantitative-PCR

RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's protocol. Total RNA was reversed transcribed to cDNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen) according to the manufacturer's protocol. L19 was used as an internal control for both RT-PCR and quantitative-PCR (Q-PCR). Primers for exon 5 of PRLR were adapted from reference [9]. Q-PCR protocol was adapted from Shehu et al. [35]. Briefly, standard curves were generated by a series of 1:5 to 1:500 dilutions prepared from reverse transcription (RT) products. Five-microliter aliquots of standards or diluted RT products were combined with 1× Fast SYBR Green PCR Master Mix (Applied Biosystems) and 50 nM forward and reverse primers. Reactions were carried out with a Prism 7700 sequence detection system (Applied Biosystems) for 40 cycles (95°C for 15 sec, 60°C for 1 min) after an initial incubation for 10 min at 95°C. Relative expression levels were calculated using the ΔΔCT method, with the *Rpl19* mRNA used as an internal control. The following primers were used for Q-PCR: *Esr1*, 5′-AACCGCCCATGATCTATTCTG-3′ and 5′-AGATTCAAGTCCCCAAAGCC-3′; *Cdkn1b*, 5′-TGGACCAAATGCCTGACTC-3′ and 5′-GGGAACCGTCTGAAACATTTTC-3′; and *Vegfa*, 5′-CATCTTCCAGGAGTACCCCGA-3′ and 5′-CACTCCAGGGCTTCATCGTT-3′. Q-PCR primers for *Lhcgr* (LH-R) and *Cebpb* (C/EBP/β) were adapted from references [36] and [37], respectively. Primers for *Cyp11a1*, *Hsd3b*, and *Star* were adapted from reference [11].

Immunohistochemistry

Ovaries were collected, processed, paraffin-embedded, and serially sectioned at 5 μm as previously described [22]. Paraffin sections were rehydrated and stained with hematoxylin and eosin by conventional procedures. Immunohistochemistry was performed as previously described [35]. The following antibodies from Santa Cruz Biotechnology were used for immunohistochemistry at the dilutions listed: PRLR M-170 (1:200 dilution), ESR1 (1:150 dilution), VEGF (1:200 dilution), and CDKN1B (1:100 dilution). HSD17B7 antibody was developed in our laboratory [38] and used at 1:250 dilution. The PRLR antibody recognizes the extracellular domain of the receptor. Because our mice express only *Prlr*-L cDNA, the staining corresponds only to PRLR-L in these mice. TUNEL assay (ApopTag Plus in situ detection kit; Chemicon International) was carried out according to the manufacturer's instructions.

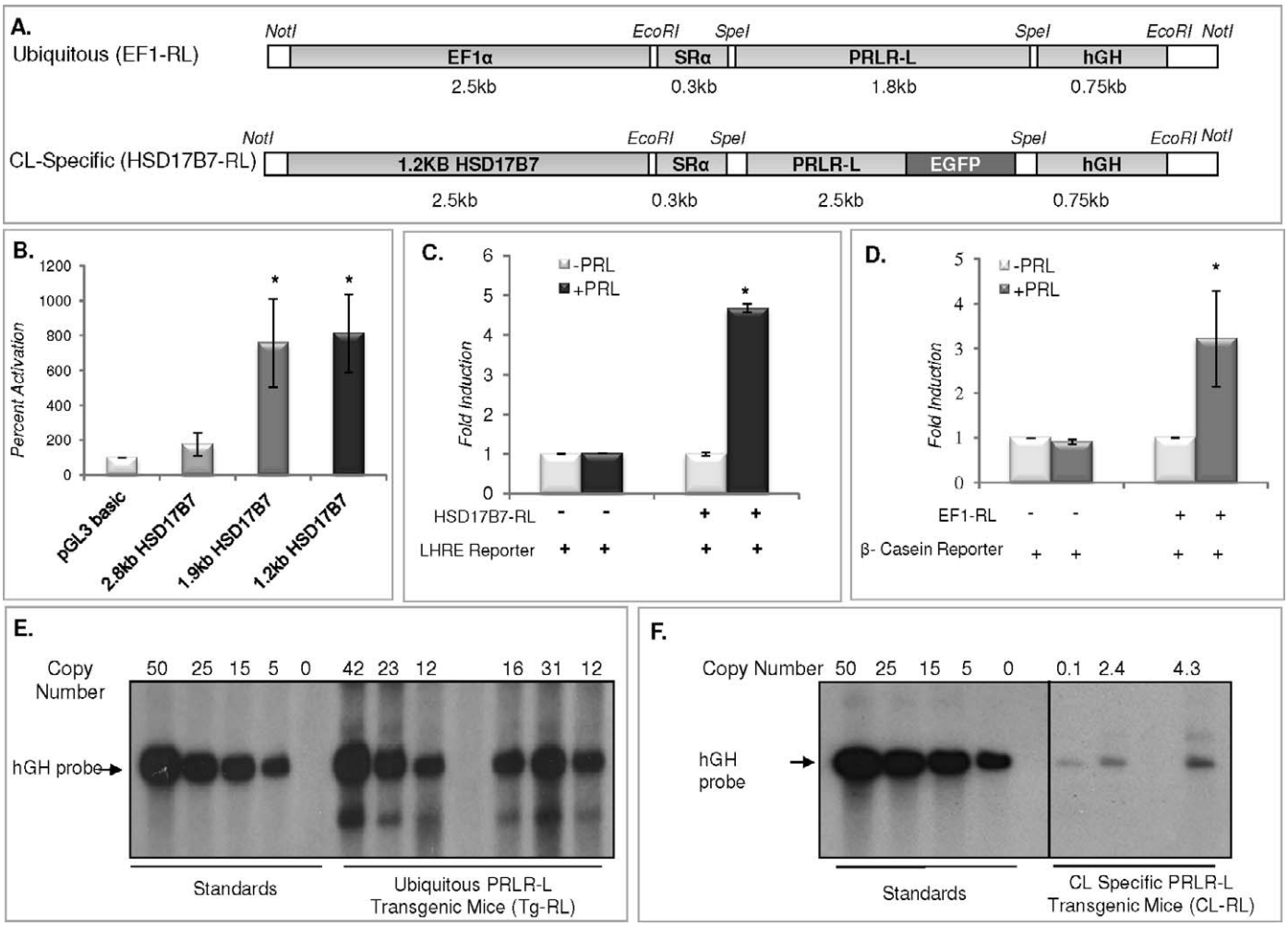

FIG. 1. Generation of transgenic mice expressing PRLR-L either ubiquitously (Tg-RL) or in a CL-specific manner (CL-RL). **A**) Schematic of the targeting construct used to generate mice with ubiquitous (EF1-RL) or CL-specific (HSD17B7-RL) expression of PRLR-L. **B**) Promoter activity of HSD17B7 was tested by transfecting GG-CL cells with 5′-truncated 2.8-kb (full length), 1.9-kb, and 1.2-kb promoters. To test the activity levels of the transgenic constructs, GG-CL cells were cotransfected with either the HSD17B7-RL (**C**) or EF1-RL (**D**) transgenic construct together with either the LHRE or β-casein promoter reporter plasmid, respectively, and treated with or without PRL. Promoter activity experiments are expressed as means ± SEM from three independent experiments performed in triplicate (*$P < 0.05$). Southern blot analysis was performed with genomic DNA isolated from mouse tail to determine the copy number for both Tg-RL (**E**) and CL-RL (**F**) mice, as described in *Materials and Methods*.

To visualize endogenous PRLR-L-EGFP fusion protein expression in CL-RL mice, we harvested and fixed ovaries for 2 h in a solution of 4% formalin, 7% picric acid, and 10% sucrose. Tissues were placed in Tissue-Tek OCT embedding compound, flash frozen over liquid nitrogen, and sectioned at 5-μm thickness.

Western Blotting

Whole ovarian extracts were prepared by homogenization of tissue in radioimmunoprecipitation assay buffer (Boston BioProducts) containing 1× protease inhibitor cocktail (Calbiochem) and 1 mM sodium orthovanadate. Western blotting was performed as previously described [38]. Antibodies used for immunoblots were PRLR (M-170; Santa Cruz Biotechnology), anti-phospho-STAT5A/B (Millipore), mouse anti-STAT5 (Pan; Invitrogen), and CEBPB (C/EBPβ; Santa Cruz Biotechnology).

Primary Luteinized Granulosa Cells

Immature (21- to 26-day-old) mice were treated with 8 IU of eCG (Sigma Aldrich) for 48 h to stimulate follicular development and then treated with 8 IU of hCG (Sigma) to induce ovulation. Primary luteinized granulosa cells were harvested 12 h post-hCG injection and incubated sequentially in 6 mM ethylene glycol tetraacetic acid (EGTA) in Dulbecco modified Eagle medium (DMEM)/F-12 medium and 0.5 M sucrose in DMEM/F-12. A 30-G needle was used to puncture follicles to release luteinized granulosa cells into medium. Cells were plated at 150 000 cells/well in a 12-well plate precoated with mouse laminin (BD Biosciences) and incubated at 37°C at 5% CO_2. Conditioned medium was collected and changed every 24 h for up to 5 days.

Statistical Analysis

Data were examined by *t*-test, two-way ANOVA, and one-way ANOVA, followed by the Tukey test using Prism software (GraphPad Software Inc.). All error bars represent ±SEM. Values are considered statistically significant at *P* values of <0.05*, <0.01**, and <0.001*** (asterisks refer to figure legends).

RESULTS

Generation of Ubiquitous (Tg-RL) and CL-Specific (CL-RL) PRLR-L Transgenic Mice

Targeting constructs used to generate mice with either ubiquitous (EF1-RL) or CL-specific (HSD17B7-RL) expression of PRLR-L are shown in Figure 1A. The EF1A promoter, active in all cells, was used to drive ubiquitous expression of PRLR-L, whereas the HSD17B7 promoter was used to drive CL-specific expression of PRLR-L. Both constructs contained

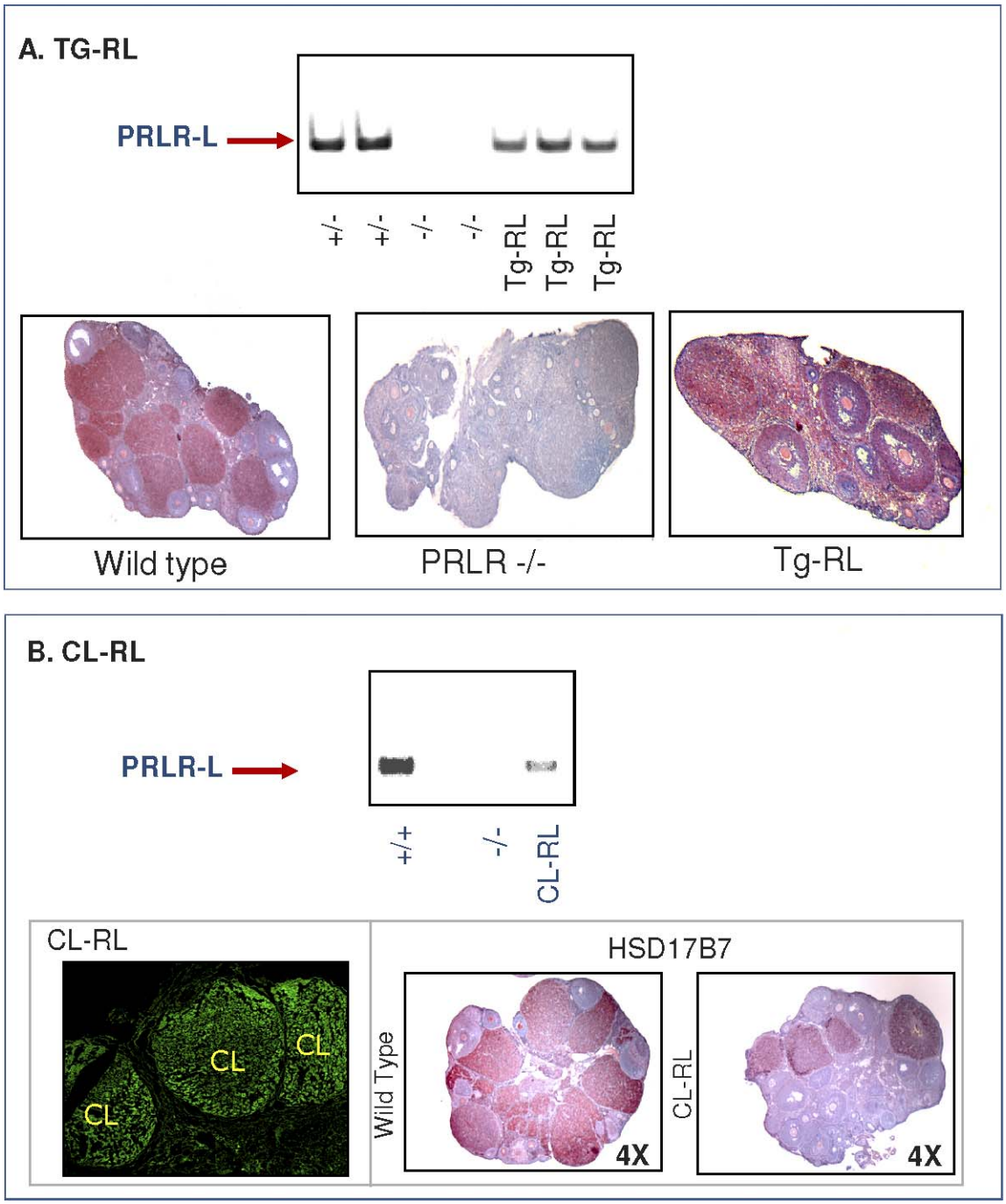

FIG. 2. Ovarian expression of PRLR-L in Tg-RL and CL-RL mice. Transcript levels of PRLR (exon 5) were examined by RT-PCR in ovaries from wild-type/heterozygote, PRLR null, and Tg-RL mice (**A**, upper panel) or CL-RL mice (**B**, upper panel). PRLR-L expression was also detected by immunohistochemistry (shown in red) in ovaries of wild-type, PRLR-null, and Tg-RL mice (**A**, lower panel). **B**) Confocal imaging of an ovary from CL-RL mice shows CL-specific expression of the PRLR-L-EGFP fusion protein (lower left panel). Ovaries from wild-type and CL-RL mice were subjected to immunohistochemistry using HSD17B7 polyclonal antibody to confirm CL-specific HSD17B7 expression (**B**, lower right panels). Reactivity is shown in red, and hematoxylin counterstain is shown in blue.

a portion of hGH cDNA, which functioned as a tag. The promoter activity of HSD17B7 was tested (Fig. 1B) by transfecting cells with 2.8-kb (full length), 1.9-kb, and 1.2-kb 5′-truncated promoters. The 1.2-kb HSD17B7 promoter had the highest activity and therefore was used to drive CL-specific expression of PRLR-L. To test the activity of the HSD17B7-RL construct (Fig. 1C), GG-CL cells, a luteum-derived cell line generated in our laboratory [34], were cotransfected with the transgenic vector and the LHRE promoter-reporter and subsequently treated with vehicle or PRL. The activity of EF1-RL was examined in cells cotransfected with the β-casein promoter-reporter (Fig. 1D). Both the LHRE and β-casein promoter-reporters are known to be activated by PRL through the PRLR-L form [39–42]. Following PRL treatment, there was a robust stimulation in reporter activity, indicating that both EF1-RL and HSD17B7-RL transgenic receptor constructs are functional.

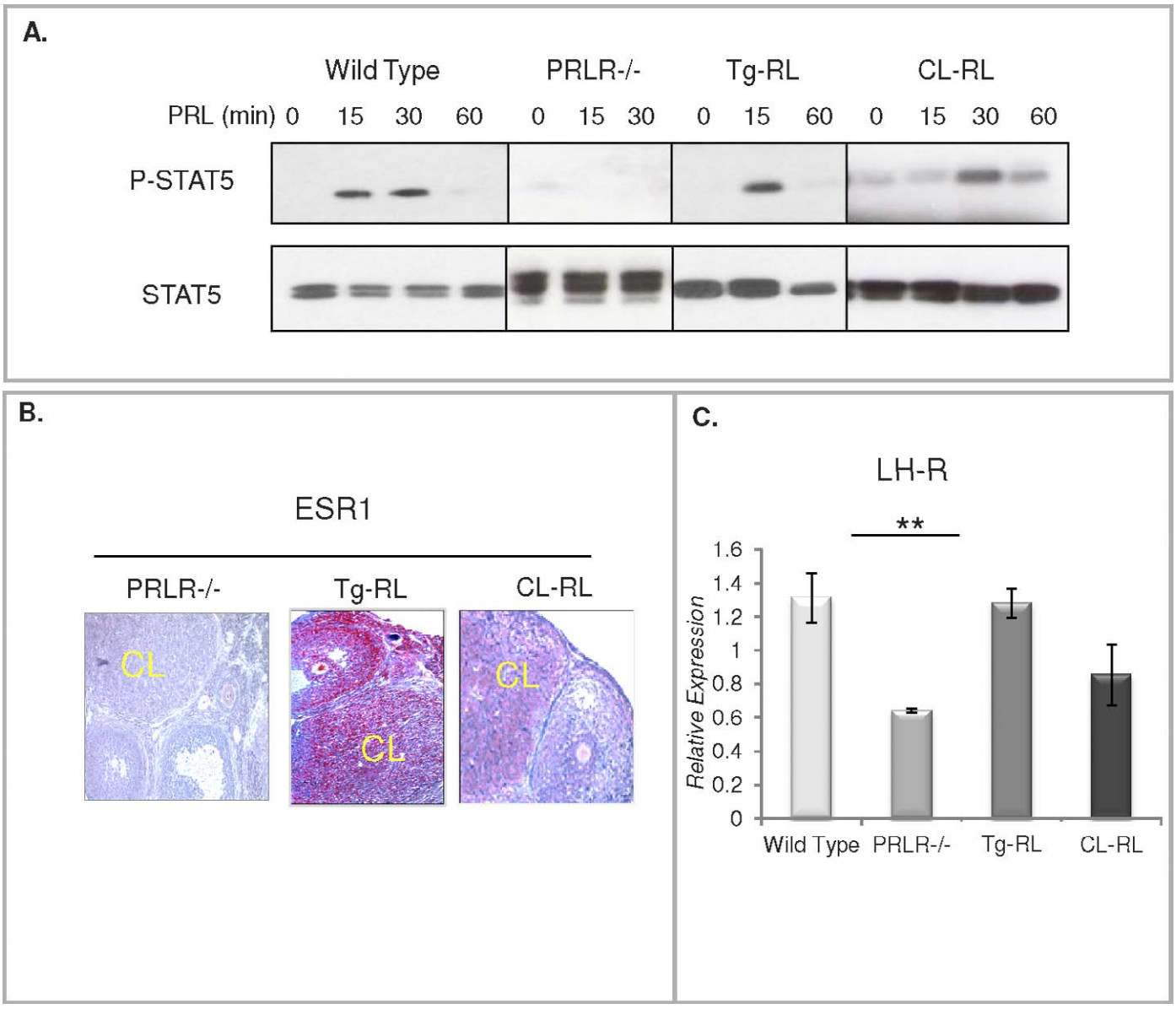

FIG. 3. Activation of PRLR-L in Tg-RL and CL-RL mice. **A**) Western blot analysis of STAT5 phosphorylation from ovaries of wild-type, PRLR-null, Tg-RL, and CL-RL mice treated with ergocryptine for 4 h, followed by PRL treatment for various time points. **B**) Immunohistochemical examination of ESR1 expression in ovaries of PRLR null, Tg-RL, and CL-RL mice (shown in red) at Day 1.5 of pregnancy. **C**) Q-PCR expression of LHCGR in the ovaries of wild-type, PRLR-null, Tg-RL, and CL-RL mice (**$P < 0.01$).

As shown in Figure 1, E and F, several mouse lines carrying either the ubiquitous PRLR-L transgene (Tg-RL) or the CL-specific PRLR-L transgene (CL-RL) were generated. The Tg-RL mouse carrying 31 copies of PRLR-L (Fig. 1E) was chosen to expand the colony, and all Tg-RL mice subsequently used were derived from that mouse. Integration of the CL-RL transgene had a lower copy number (Fig. 1F); therefore, mice with the highest copies were intercrossed. Both strains of transgenic mice were backcrossed to the PRLR null background such that both lines expressed only PRLR-L, either in a ubiquitous or CL-targeted manner.

Expression and Activation of PRLR-L in Tg-RL and CL-RL Female Mice

We examined the expression of PRLR-L in the ovaries of transgenic mice by using both RT-PCR and immunochemistry (Fig. 2A). Ovaries were obtained from mice 1.5 days after they mated. As previously reported [43], PRLR-L was detected in both CL and follicles of wild-type mice (Fig. 2A, lower panel). No receptor was found in the ovary of PRLR null mice, whereas it was readily detectable in Tg-RL mice by both RT-PCR (Fig. 2A, upper panel) and immunohistochemistry (Fig. 2A, lower panel). In the Tg-RL mice, the receptor was clearly expressed in both CL and follicles. PRLR-L was also detected by RT-PCR in the ovaries of CL-RL mice expressing the HSD17B7-RL vector (Fig. 2B, upper panel). Green fluorescence, indicative of PRLR-L-EGFP fusion protein expression, was seen only in the CL and not in follicles of CL-RL mice (Fig. 2B, lower left panel), and immunohistochemical analysis confirmed the specific expression of HSD17B7 in the CL (Fig. 2B, lower right panel).

We ascertained that the transgenic PRLR-L is functional in vivo in Tg-RL and CL-RL female mice by examining the

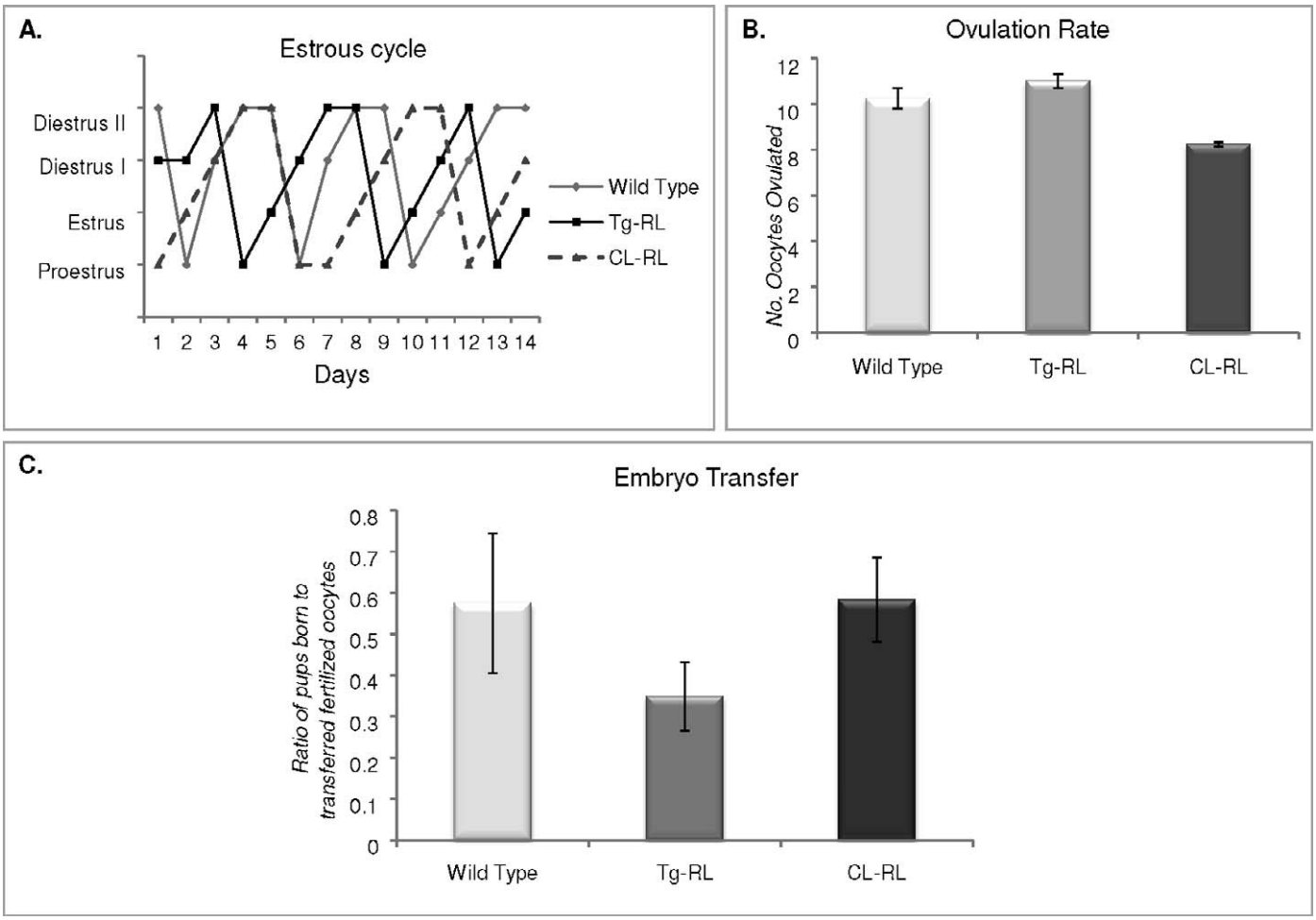

FIG. 4. Tg-RL and CL-RL mice have normal estrous cycles and ovulation. **A**) Vaginal smear assays were conducted daily for 14 days to monitor the stage of the estrous cycles of the mice and were graphed to demonstrate the cycling patterns of wild-type, Tg-RL, and CL-RL mice. The graph shows representative cycles monitored from mice (n = 3–5 per group). **B**) Ovulation rates of wild-type, Tg-RL, and CL-RL mice were determined by collecting and counting the number of oocytes ovulated postmating. **C**) Fertilized oocytes from wild-type, Tg-RL, or CL-RL mice were transferred to wild-type surrogate mothers. The ratio of the number of pups born to the number of fertilized oocytes implanted per mother was plotted to compare whether the transgenic embryos were able to implant normally compared to wild-type embryos (n = 3–5 mice per group for each experiment).

ability of PRL to stimulate STAT5 activity. As shown in Figure 3A, a rapid increase in STAT5 phosphorylation was observed in the ovaries of both transgenic mice after in vivo treatment with PRL. Because ESR1, as we have previously shown [20], is normally expressed in the CL, is up regulated by PRL, and is a downstream target of the PRLR-L/STAT5 pathway [29, 44], we examined its ovarian expression in both of the PRLR-L transgenic mice lines. As shown in Figure 3B, ESR1 is nonexistent in PRLR null mice, providing further support to previous reports of PRL-mediated stimulation of ESR1. Interestingly, we found that ubiquitous expression of PRLR-L in Tg-RL mice drives expression of ESR1 in the follicles as well as in the CL (Fig. 3B), whereas the CL-specific transgene induces the expression of ESR1 in CL only. To further establish the activity of these receptors, we used Q-PCR to examine ovarian LHCGR expression, another downstream target of PRL [39, 45]. We found a marked decrease in ovarian LHCGR levels in the PRLR null mice and a significant stimulation in both transgenic mice compared to that in null mice (Fig. 3C). Taken together, these results indicate that the PRLR-L transgenic receptor is functional in vivo in both Tg-RL and CL-RL mice.

Luteinization and Progesterone Production Are Defective in Tg-RL and CL-RL Mice

Tg-RL and CL-RL mice have normal estrous cycles (Fig. 4A) and mate with fertile males (as confirmed by a vaginal plug). However, surprisingly, no pregnancy was observed in either transgenic mice line (Table 1). This was not due to a defect in ovulation, because, as shown in Figure 4B, fertilized eggs were found in the oviduct and the ovulation rate in Tg-RL and CL-RL mice appeared to be similar to that of wild-type mice.

We examined whether infertility was due to an intrinsic defect in the Tg-RL and CL-RL embryo by transplanting fertilized oocytes from transgenic mice into wild-type surrogate mothers. As shown in Figure 4C, the number of Tg-RL and CL-RL pups born in relation to the number of fertilized oocytes implanted was similar to that in wild-type controls, indicating that Tg-RL and CL-RL fertilized oocytes are indeed able to implant in wild-type mice. However, as shown in Figure 5A, upper panel, no implantation sites were detected in Day 5.5 postmated Tg-RL and CL-RL females. Treatment with progesterone rescued the implantation defect seen in both Tg-RL and CL-RL female mice, indicating that the lack of

TABLE 1. Infertility in Tg-RL and CL-RL mice is rescued with progesterone treatment.*

Females	No. of vaginal plugs found	No. of pregnancies	Avg. no. of implantation sites on Day 5.5		Avg. no. of embryos lost by Day 11.5
			−Progesterone	+Progesterone	
Wild-type	8	8	10 ± 0.445	N/A	0.4 ± 0.4
Tg-RL	22	0	0	8.5 ± 1.848	2.5 ± 0.288
CL-RL	13	0	0	10 ± 1	0.67 ± 0.33

*$P < 0.01$.

implantation and pregnancy in Tg-RL and CL-RL female mice was due to an ovarian shortcoming and insufficient progesterone production.

Interestingly, continued progesterone treatment completely rescued pregnancy in CL-RL mice but not in Tg-RL mice (Fig. 5B) because of significant fetal death by Day 11.5 (Table 1). These findings suggest that ubiquitous expression of PRLR-L may modulate other confounding factors that contribute to infertility in these mice. In addition to the ovary, the uterus was shown to be a target tissue of PRL, where it affects decidual expression of cytokines and enzymes [46]. To determine whether a uterine defect in Tg-RL mice was the reason for such a fetal loss, we conducted a reverse embryo transfer, where fertilized wild-type embryos were transplanted into either a

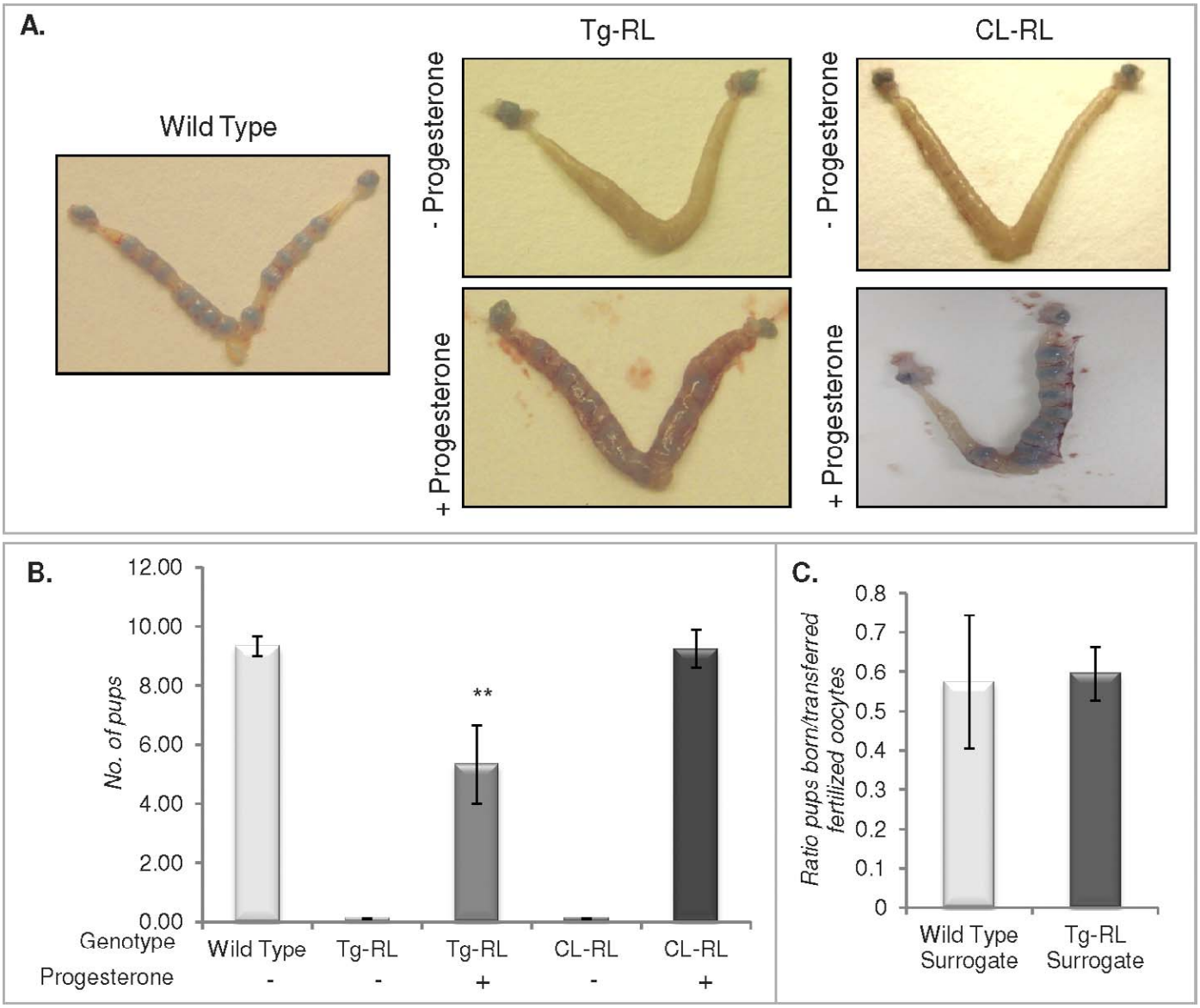

FIG. 5. Progesterone supplementation cannot completely rescue pregnancy. **A**) Female mice were mated with fertile males, treated with vehicle or progesterone daily beginning on Day 0.5, and subsequently injected with Chicago Sky Blue dye on Day 5.5 of pregnancy to examine implantation sites. **B**) At Day 16.5 of pregnancy, following daily administration of vehicle or progesterone, the number of pups present in the uterus was recorded. **C**) A reverse embryo transfer experiment, where fertilized wild-type oocytes were transferred to wild-type or progesterone-treated Tg-RL surrogate mothers, was conducted.

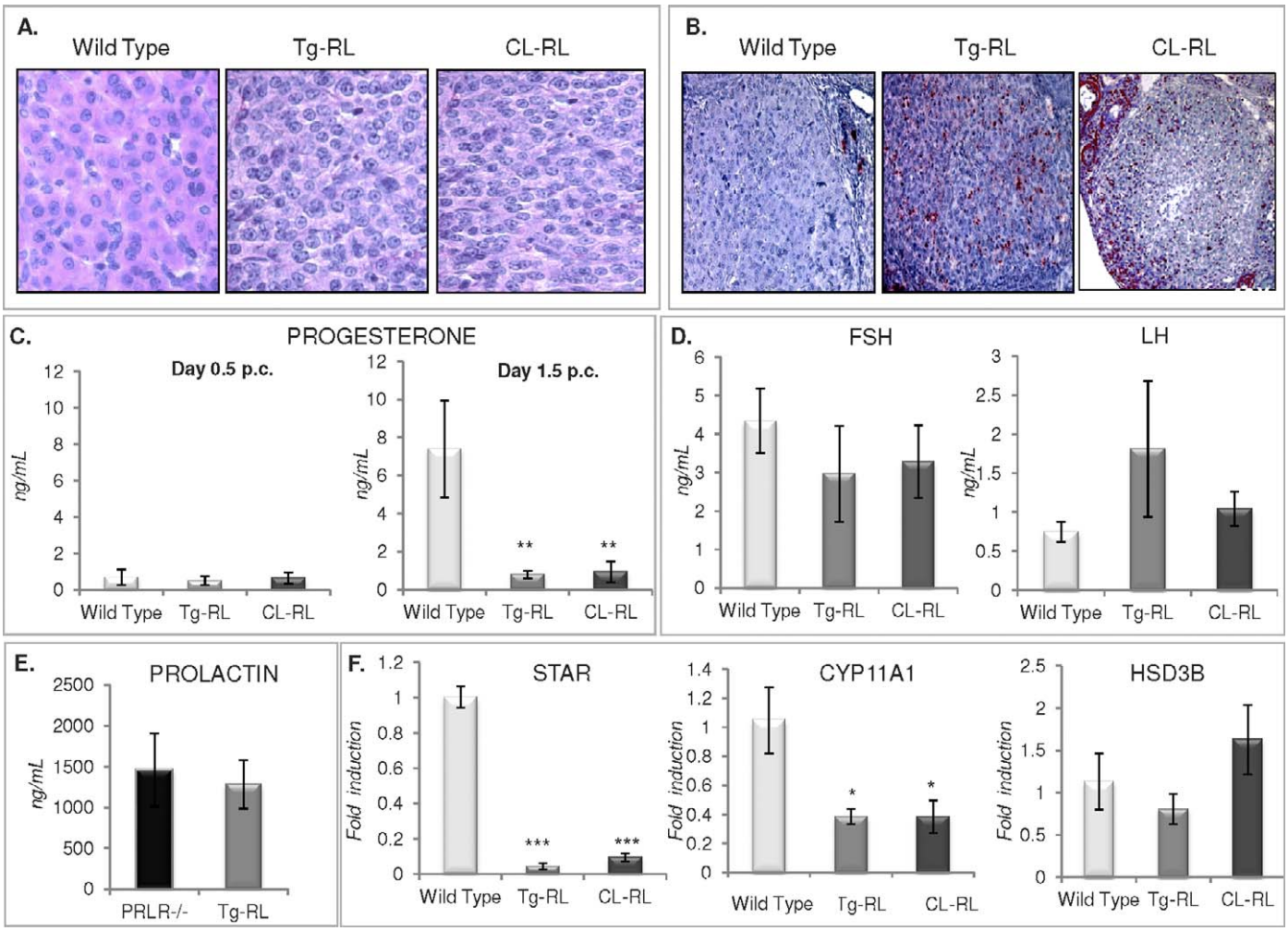

FIG. 6. Luteinization and impaired progesterone biosynthesis in Tg-RL and CL-RL mice. **A**) Photomicrographs of CL from hematoxylin-eosin-stained ovaries of wild-type, Tg-RL, and CL-RL mice at Day 1.5 of pregnancy (original magnification ×100). **B**) TUNEL assay with paraffin-embedded ovarian sections was performed at Day 3.5 of pregnancy, revealing reactivity (seen in red) in the CL of wild-type, Tg-RL, and CL-RL mice (original magnification ×40). **C**) Serum progesterone levels were measured on Days 0.5 (left panel) and 1.5 (right panel) postmating. **D**) Serum FSH and LH levels were measured at proestrus phase in wild-type, Tg-RL, and CL-RL mice. **E**) Serum PRL levels were measured using the NB2 assay from PRLR null and Tg-RL mice. **F**) Q-PCR analysis of the expression of STAR (left panel), CP11A1 (middle panel), and HSD3B (right panel) in ovaries from wild-type, Tg-RL, and CL-RL mice at Day 1.5 of pregnancy. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

wild-type surrogate mother or a PRLR-L female surrogate treated with progesterone. As shown in Figure 5C, there was no significant embryo loss in PRLR-L surrogates compared to that in wild-type surrogates, indicating that the uterine environment in PRLR-L female mice is capable of maintaining normal pregnancy if supplemented with progesterone. This finding confirmed the fact that the major defect is in the ovaries of both transgenic mice lines.

Examination of the ovaries of Tg-RL and CL-RL female mice at 1.5 days after they mated revealed the presence of CL. However, cell hypertrophy, a typical characteristic of luteinization in wild-type mice (Fig. 6A, left panel), was absent in CL of both Tg-RL and CL-RL mice, indicating a defect in the luteinization process in these mice. In addition to defective luteinization, extensive cell death was detected by TUNEL assay in CL of Tg-RL and CL-RL mice but not in wild-type mice (Fig. 6B) by Day 3.5 of pregnancy. Early regression of the CL can lead to a defect in progesterone synthesis, and as expected, serum progesterone levels were low on Day 0.5 postmating in all groups of mice (Fig. 6C, left panel). On Day 1.5 of pregnancy, the characteristic rise in serum progesterone levels seen in wild-type mice was not observed in either Tg-RL or CL-RL mice (Fig. 6C, right panel). No significant difference were observed between FSH and LH levels in wild-type mice and those in transgenic mice (Fig. 6D). PRL levels were elevated in Tg-RL mice (Fig. 6E), similar to levels found in PRLR null and PRLR-S transgenic mice, as previously reported [22]. Not surprisingly, mRNA expression levels of steroidogenic acute regulatory protein (STAR) and P450 side chain cleavage (P450scc [CYP11A1 gene]), two proteins involved in progesterone biosynthesis [41, 42], were significantly reduced in Tg-RL and CL-RL ovaries compared to those in the wild type (Fig. 6F). However, no significant differences in 3-beta-hydroxysteroid dehydrogenase (HSD3B) were observed (Fig. 6F, right panel).

Defective CL Vascularization in Tg-RL and CL-RL Mice

To further investigate the mechanism of defective luteinization in the transgenic mice, we looked at the expression levels of CDKN1B and CCAAT-enhancer binding protein (C/EBP) β, CEBPB, two key proteins that are critical for CL formation. CDKN1B, an inhibitor of cell cycle progression

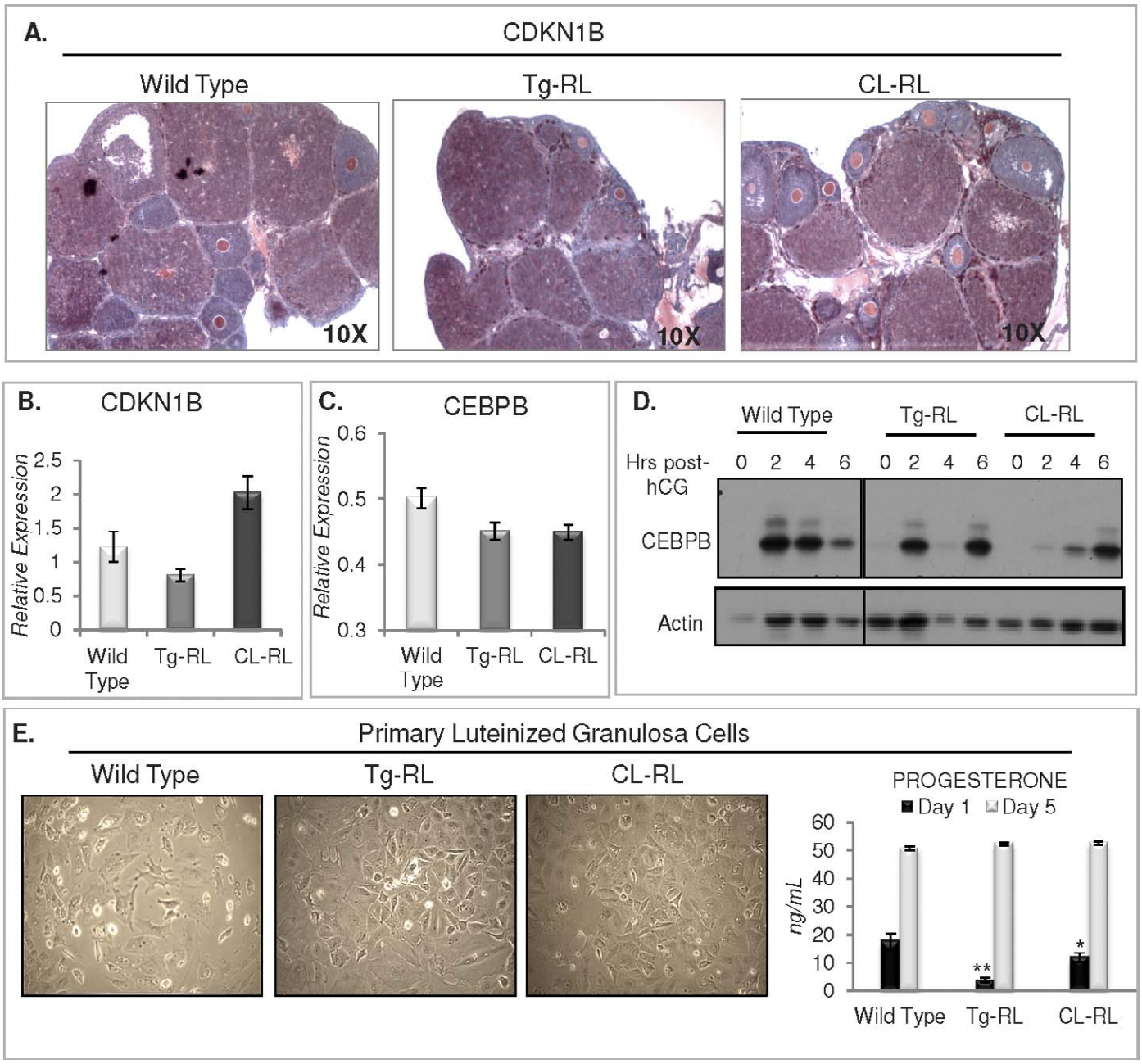

FIG. 7. Examination of markers of luteinization in vivo and of primary luteinized granulosa cell hypertrophy and progesterone production in culture. **A**) Immunohistochemical expression of CDKN1B (shown in red) in the CL of wild-type, Tg-RL, and CL-RL mice following superovulation. Transcript levels of CDKN1B (**B**) and CEBPB (**C**) were detected by Q-PCR from cDNA of ovaries from wild-type, Tg-RL, and CL-RL mice at early pregnancy. **D**) Western blot analysis of ovarian CEBPB protein induction following hCG injection in wild-type, Tg-RL, and CL-RL mice. **E**) View of primary luteinized granulosa cells isolated from wild-type, Tg-RL, and CL-RL mice following 5 days in culture (left) and respective progesterone levels in conditioned medium from primary cells at Days 1 and 5 in culture (right).

required for cells to exit the cycle and initiate CL formation, was expressed in both transgenic mice lines in response to superovulatory doses of eCG and hCG, similar to that in the wild type as determined by both immunohistochemistry and Q-PCR (Fig. 7, A and B). Transcript levels of CEBPB, a transcription factor induced following the LH surge, was similar in ovaries from wild-type and both Tg-RL and CL-RL transgenic mice (Fig. 7C), and its protein expression was induced following hCG administration (Fig. 7D), indicating that the ovaries of both transgenic mice are responsive to LH. Indeed, we found that after an LH surge, granulosa cells of mice harvested from Tg-RL, and CL-RL luteinize in culture similar to wild-type cells (Fig. 7E), suggesting that these cells have the intrinsic capacity to undergo hypertrophy. We also measured progesterone levels in conditioned medium collected from primary luteinized cells after 1 and 5 days in culture (Fig. 7E, right panel). Luteinized granulosa cells isolated from Tg-RL and CL-RL ovaries produced significantly lower levels of progesterone after 1 day in culture. However, by Day 5, there were no differences in progesterone secretion between cell types, indicating that the defect in CL formation in the ovaries of Tg-RL and CL-RL mice might have been caused by a defect not necessarily intrinsic to luteal cells as they can undergo hypertrophy and produce progesterone in culture. Indeed most

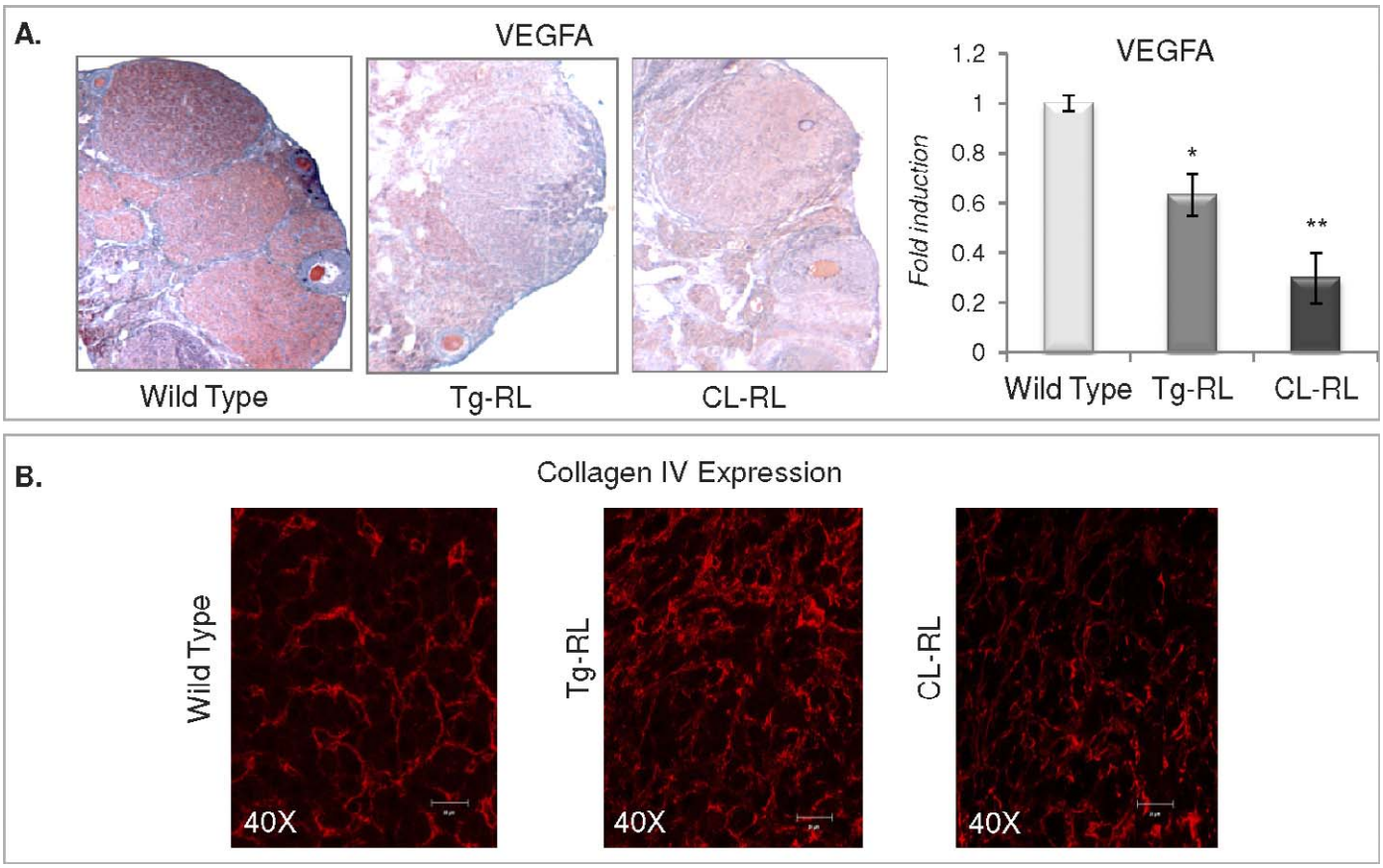

FIG. 8. Vascularization is defective in the ovaries of Tg-RL and CL-RL mice. **A**) Paraffin-embedded ovarian sections from wild-type, Tg-RL, and CL-RL mice at Day 1.5 of pregnancy were probed with VEGFA antibody (left panels). Reactivity is shown in red and hematoxylin counterstain in blue (original magnification ×10). *Vegfa* mRNA levels were detected by Q-PCR in ovaries from wild-type, Tg-RL, and CL-RL mice (right). **B**) Immunofluorescence shows collagen IV staining (red) of the basal lamina of endothelial cells in CL of wild-type, Tg-RL, and CL-RL mice at Day 1.5 of pregnancy.

of the cells found in the CL were from a vascular origin. The CL has the highest blood flow of any tissue, and neovascularization is essential for CL formation and survival [47]. Vascularization provides a transport network for nutrients and hormones, most importantly progesterone in CL. We found vascular endothelial growth factor A (VEGFA), a key player in CL vascularization [6], was highly expressed in wild-type CL but almost undetectable in CL of both Tg-RL and CL-RL mice (Fig. 8A). Collagen IV is the primary extracellular matrix component of the subendothelial basal lamina [48] and has been shown to modulate angiogenesis [49, 50] and appears as a continuous network in later stages of angiogenesis [51]. Collagen IV staining, a marker for basal lamina of endothelial cells, is aberrantly expressed and reveals discordant organization of endothelial cells in CL of transgenic mice at Day 1.5 of pregnancy, further suggesting that angiogenesis in the CL of these mice is disrupted (Fig. 8B).

DISCUSSION

While PRL has long been known to be essential for CL formation and function in rodents, the generation of mice lacking either the *Prl* or *Prlr* gene made apparent the need for this hormone in the transformation of CL of the cycle to that of pregnancy [8, 9]. In the absence of PRL or PRLR, CL forms after ovulation but secretes very low levels of progesterone and involutes rapidly. Although PRL binds to both PRLR types, it was well accepted but never proven that PRL sustains CL formation and steroidogenic activity acting through the PRLR-L form [29, 44, 52–54]. The generation of transgenic mice expressing only PRLR-S [22], which showed no rescue of the CL, further substantiated this possibility. Similar to PRLR null mice, transgenic mice expressing only PRLR-S have CL that rapidly degenerates and fails to produce progesterone [22]. Moreover, these mice exhibited a severe ovarian pathology characterized by massive follicular recruitment followed by granulosa cell and oocyte death, leading to premature ovarian failure [22]. The findings that CL expresses both PRLR types [19, 20] and that PRLR-S is unable to rescue CL formation or its steroidogenic capacity independently led us to rigorously test the hypothesis that PRLR-L is the sole receptor necessary for CL formation and function.

In the present study, we report that mice expressing PRLR-L, either ubiquitously or in a CL-specific manner, have a severe ovarian impairment because of a defect in normal CL development. Interestingly, the presence of PRLR-L alone in the ovary does indeed allow for PRL stimulation of STAT5 and expression of genes known to be regulated by PRL activation of the PRLR-L, such as ESR1 [29, 44, 55] and LHCGR [10, 39, 40, 45]. Yet, it does not allow for cell survival and CL development, establishing for the first time that PRL activation of STAT5 through PRLR-L is insufficient for the proper formation and maintenance of CL of pregnancy. Unlike transgenic mice, which express only PRLR-S, both Tg-RL and CL-RL mice have normal follicular development and ovulation rates. We have previously shown that both types of

PRLR cross-talk in the follicle and that expression of PRLR-S alone leads to severe inhibition in several transcription factors including FOXO3A [22] and SP1 [25] and to downregulation of galactose-1-phosphate uridyl transferase (GALT), an enzyme essential for follicular survival [22]. Coexpression of PRLR-S and PRLR-L in either the follicle or in cultured cells rescues the deleterious suppression by PRL activation of PRLR-S alone on GALT expression and restores normal follicular development [22]. This finding implies that PRLR-L can inhibit PRLR-S-mediated cellular death signaling in the follicle. The mechanism of cross-talk between these two types of receptors remains largely unknown. Recent investigations suggest that both types of PRLR can homo- and heterodimerize in the absence of ligand [56, 57], which may be one means of cross-talk between the receptors.

Another novelty of this investigation is the generation of transgenic mice with CL-specific expression of PRLR-L by using the HSD17B7 promoter. The HSD17B7 enzyme was originally discovered as a 32-kDa protein most abundantly and specifically expressed in the CL [58, 59]. It was not found in any other tissue [58]. During pregnancy, HSD17B7 expression becomes detectable, although at much lower levels, in the decidua and trophoblast [35]. However, because this expression in the conceptus occurs only around Day 9 of pregnancy, long after CL of pregnancy forms and becomes highly active, the HSD17B7 promoter can be used to generate a CL-specific transgene for either early pregnancy study or in nonpregnant mice. To our knowledge, this is the first CL-specific transgenic mouse model ever generated.

Results of the present investigation together with our previous data generated with mice expressing only PRLR-S indicate that neither PRLR subtype alone can transduce the repertoire of signals that prevent cell death and those that are necessary for luteinization and vascularization, two events that occur simultaneously after ovulation that are absolutely necessary for CL formation. With early degeneration of CL as shown by a marked increase in apoptotic cells in CL, it was not surprising that transcript levels of steroidogenic enzymes were low in both Tg-RL and CL-RL mice, contributing to the lack of progesterone production. The facts that CL of PRLR-L-expressing mice express abnormally low levels of VEGFA and have impaired vascularization, as shown by discordant organization of endothelial cells in CL, and that granulosa cells isolated from these mice after ovulation luteinize normally in culture suggest that the apparent reason for defective CL formation is improper vascularization.

Neovascularization is a critical requirement for proper CL formation and progesterone production because it provides an import/export system for nutrients, hormones, cholesterol, and most importantly, progesterone [47]. Staining of collagen IV, the primary extracellular membrane component of the subendothelial basal lamina and a factor involved in angiogenesis (Fig. 8B), revealed a disrupted network of endothelial cells in ovaries of both Tg-RL and CL-RL mice. Of the angiogenic factors present in the ovary, VEGF is the key player in angiogenesis in CL [60]. Among the five different isoforms of VEGF that are expressed in the CL [61], VEGFA is the most important one because disruption of VEGFA signaling [62] or inhibition of VEGFA [60] results in a lack of vascularization of CL and inhibition progesterone production. Our finding that VEGFA expression is markedly reduced in both Tg-RL and CL-RL mice provides the primary explanation for impaired progesterone production in these mice. Indeed, luteal cells respond to PRL activation of PRLR-L with STAT5 activation, and primary luteinized granulosa cells isolated from Tg-RL and CL-RL ovaries can undergo hypertrophy and produce progesterone in culture, suggesting that the lack of nutrient access in vivo due to defective vascularization appears to be causal for defective luteal cell hypertrophy and CL function. The fact that PRLR-S is the predominant receptor expressed in endothelial cells derived from CL, as well as other vasculature sources, and that treatment of anti-PRL antibody leads to a significant reduction of endothelial cell growth [42, 63, 64] implies that the lack of PRLR-S in Tg-RL and CL-RL mice may result in the observed decrease in vascular formation. It remains to be determined whether PRLR-S activation induces VEGFA expression in both luteal and endothelial cells, given that PRLR null [11] and PRLR-L-expressing mice both have a luteal vascularization defect. This suggests the intriguing possibility that PRLR-S expressed in the vasculature and PRLR-L expressed in luteal cells act in concert to permit normal CL formation and function, ultimately allowing CL to sustain fertility and pregnancy.

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