THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 290, NO. 5, pp. 2699–2714, January 30, 2015

Gonadotrope-specific Deletion of *Dicer* Results in Severely Suppressed Gonadotropins and Fertility Defects*

Received for publication, October 24, 2014, and in revised form, December 12, 2014 Published, JBC Papers in Press, December 18, 2014, DOI 10.1074/jbc.M114.621565

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Background: DICER mediates microRNA production, and its functional role in gonadotropes is not known.
Results: Deletion of *Dicer* in gonadotropes leads to suppression of gonadotropin synthesis and secretion and results in fertility defects.
Conclusion: DICER-dependent microRNAs are critical for gonadotropin homeostasis and fertility.
Significance: Understanding how microRNAs regulate gonadotropin homeostasis provides a new approach to enhance or block fertility in mammals.

Pituitary gonadotropins follicle-stimulating hormone and luteinizing hormone are heterodimeric glycoproteins expressed in gonadotropes. They act on gonads and promote their development and functions including steroidogenesis and gametogenesis. Although transcriptional regulation of gonadotropin subunits has been well studied, the post-transcriptional regulation of gonadotropin subunits is not well understood. To test if microRNAs regulate the hormone-specific gonadotropin β subunits *in vivo*, we deleted *Dicer* in gonadotropes by a *Cre-lox* genetic approach. We found that many of the DICER-dependent microRNAs, predicted *in silico* to bind gonadotropin β subunit mRNAs, were suppressed in purified gonadotropes of mutant mice. Loss of DICER-dependent microRNAs in gonadotropes resulted in profound suppression of gonadotropin-β subunit proteins and, consequently, the heterodimeric hormone secretion. In addition to suppression of basal levels, interestingly, the post-gonadectomy-induced rise in pituitary gonadotropin synthesis and secretion were both abolished in mutants, indicating a defective gonadal negative feedback control. Furthermore, mutants lacking *Dicer* in gonadotropes displayed severely reduced fertility and were rescued with exogenous hormones confirming that the fertility defects were secondary to suppressed gonadotropins. Our studies reveal that DICER-dependent microRNAs are essential for gonadotropin homeostasis and fertility in mice. Our studies also implicate microRNAs in gonadal feedback control of gonadotropin synthesis and secretion. Thus, DICER-dependent microRNAs confer a new layer of transcriptional and post-transcriptional regulation in gonadotropes to orchestrate the hypothalamus-pituitary-gonadal axis physiology.

Gonadotropes are the least abundant cell type, representing only 5–6% of all cells in the anterior pituitary (1, 2). They synthesize and secrete two heterodimeric glycoprotein hormones luteinizing hormone (LH)[2] and follicle-stimulating hormone (FSH) that are essential for gonadal growth, gametogenesis, and steroidogenesis (1, 2). Both these gonadotropic hormones consist of a common α- and a hormone-specific β-subunit that are encoded by distinct genes (1, 2). The development of the gonadotrope occurs via a combinatorial action of several transcription factors (3–8). In the embryonic mouse pituitary, the gene encoding the common α subunit (*Cga*) is expressed around E 11.5, and subsequently the *Lhb*- and *Fshb*-encoding genes are first detected between E 15.5 and E 17.5 (9).

Gonadotropin releasing hormone (GnRH) and gonadal steroids regulate the coordinated expression of the glycoprotein hormone subunits in the mature gonadotrope (1, 10, 11). GnRH activates the GnRH receptor in gonadotropes, a seven membrane-spanning G-protein-coupled receptor, to maintain the basal expression of gonadotropin subunit gene transcription (12). Activins, members of transforming growth factor-β superfamily, regulate *Fshb* transcription via SMAD- and FOXL2- dependent pathways (13). Another member of the same family, follistatin, is a locally produced factor that negatively regulates *Fshb* transcription by preventing activin action on gonadotropes (14).

A combinatorial action of several activators and repressors is required for transcription of gonadotrope subunit-encoding genes (10, 12, 15). Steroidogenic factor-1 (SF-1) is an orphan nuclear receptor protein activator that is required for transcription of gonadotropin subunits *in vivo* (16). Both *in vitro* and *in vivo* studies have identified that Msx1, a homeodomain protein, is a repressor for *Cga* and *Gnrhr* genes (17). Other studies iden-

* This work was supported, in whole or in part, by National Institutes of Health Grants HD056082, HD069751, AG029531, and CA166557 (to T. R. K.).
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[2] The abbreviations used are: LH, luteinizing hormone; cKO, conditional knockout; CL, corpora lutea; CV, coefficient of variation; GnRH, gonadotropin-releasing hormone; miRNA, microRNA; qPCR, quantitative PCR; *RT*, Rosa tomato; TSH, thyroid-stimulating hormone; SF-1, steroidogenic factor-1.

tified that FOXO1, a homeodomain protein, acts as a repressor for *Lhb* in immortalized gonadotrope tumor cells (18).

Although transcriptional regulation of gonadotropin subunits has been well studied (10, 12, 19–22) as described above, the post-transcriptional mechanisms that regulate gonadotropin subunits are unknown (23, 24). Of the three pituitary glycoprotein hormone subunit-encoding genes (*Cga*, *Lhb*, and *Fshb*), *Fshb* gene is unique in its organization. It encompasses a long 3′-untranslated region (UTR) whose functional significance is unknown (1). *In vitro* studies using primary pituitary cells indicate that activins, the gonadal- and pituitary-derived peptides, post-transcriptionally regulate *Fshb* mRNA stability (19, 25). Although regulation of *Lhb* and *Fshb* promoter activity in transformed gonadotrope cell lines has been extensively studied (26–35), post-transcriptional regulation and secretion of LH and FSH in these *in vitro* models is not clear.

DICER is an evolutionarily conserved endoribonuclease in the RNase III family that synthesizes microRNAs (miRNAs) and small interfering RNAs from double-stranded RNA (36–41). The miRNAs have recently emerged as critical regulators of tissue development and gene expression at multiple levels including post-transcription events (36–41). These include mRNA expression, turnover, stability, and translational control (36–41). Expression profiling studies have identified several miRNAs differentially regulated in whole pituitary (23, 24), various pituitary cell lineages (42), human pituitary tumors (43–45), and in an immortalized gonadotrope cell line in response to GnRH treatment (46–48). In addition, specific miRNAs regulating a repressor that selectively regulates LH levels in the female have also been identified (49). Although *in silico* analysis reveals clusters of miRNAs that are predicted to bind to 3′-UTRs of mouse *Lhb* and *Fshb* mRNAs, their functional significance *in vivo* is unknown.

Roles of the DICER-dependent miRNA biogenesis pathway in several mouse reproductive tissues have recently been identified (50). Studies in the female germ line confirm that although miRNA activity is suppressed in mouse oocytes, miRNAs are essential for zygotic development (51–54). Recent studies confirm the oocytes, unlike somatic cells, express high levels of endogenous-small interfering RNAs that directly target many maternal RNAs and regulate early embryo development (55). In contrast to data on oocytes, loss of miRNAs in granulosa cells, the somatic cells of ovary and uterus, causes variable phenotypes ranging from reduced number of ovulations, defects in oviduct and uterine morphogenesis, and embryo implantation (56–61). In the male germ line, miRNAs are important for the proliferation of primordial germ cells and spermatogonia but are dispensable for the repression of retrotransposons in developing germ cells (62, 63). Deletion of *Dicer* in Sertoli cells causes proliferation and maturation defects and eventually results in failure of germ cell maintenance (62, 64, 65). All of the above genetic models have illustrated the important roles of DICER-mediated miRNAs in various reproductive tissues. Here, we have deleted *Dicer* selectively in gonadotropes, the critical cell lineage in the hypothalamus-pituitary-gonadal (HPG) axis, and provide functional evidence that DICER-dependent miRNAs play key roles in gonadotropin homeostasis and fertility.

EXPERIMENTAL PROCEDURES

Generation of Gonadotrope-specific Dicer Knock-out Mice—To generate mice with a gonadotrope-specific deletion of *Dicer*, we set up a two-step breeding scheme (Fig. 1*A*). $Dicer^{flox/flox}$ males, a generous gift from Dr. Brian Harfe (66) were first crossed with *bLhb-Cre*$^{+}$ female mice (67) to obtain $Dicer^{f/+}$ Cre^{+} mice. These mice were subsequently intercrossed to finally generate $Dicer^{f/f}$ Cre^{+}, hereafter referred to as *Dicer* cKO mice. In the subsequent rounds of breeding, we also set up $Dicer^{f/f}$ Cre^{-} mice with $Dicer^{f/+}$ Cre^{+} mice to increase the frequency of generating the desired *Dicer* cKO mice. To genetically label gonadotropes *in vivo*, we set up $Dicer^{f/+}$ Cre^{+} males with *ROSATOMATO* (Gt(ROSA)26Sor<tm4-(ACTB-tdTomato,-EGFP) Luo>/J) mice (The Jackson Laboratory), referred to as *RT* mice, and eventually generated $Dicer^{f/f}$ Cre^{+} RT^{KI+} or $Dicer^{f/f}$ Cre^{+} $RT^{KI/KI}$ mice. Mice lacking either *Fshb* (68) or *Lhb* (69) or both were used as controls. $Esr1^{+/-}$ mice were purchased from The Jackson Laboratory, Bar Harbor, ME and intercrossed to generate $Esr1^{-/-}$ mice. All experiments with mice were done per National Institutes of Health guidelines and approved by the University of Kansas Medical Center Institutional Care and Use Committee Animal Protocol. Mice were maintained on standard dark:light (12:12) cycles and fed standard rodent chow, and water was provided *ad libitum*. All mouse strains were identified by genomic PCR reactions performed on tail DNA samples using primers that distinguish wild type and mutant alleles and detect the *Cre* transgene as described (67–69). The amplified products were separated on agarose gels and visualized by ethidium bromide staining.

Isolation of Gonadotropes by Fluorescence-activated Cell Sorting—Pools of pituitaries from 3–4 adult mice of identical genotypes were surgically resected and enzymatically dispersed using collagenase treatment at 37 °C as described (70). The final cell suspension was filtered through 100-μm nylon cap-containing tubes (BD Biosciences) to remove cell clumps. A 10-μl aliquot was checked for red/green fluorescence on an epifluorescence microscope (Zeiss), and the remaining cell fraction was subjected to FACS analysis using an AriaIIU flow sorter (BD Biosciences). A UV laser at 488 nm was used to excite GFP protein. The GFP^{+} fraction representing the gonadotropes and GFP^{-} non-gonadotrope cells was collected on ice, an aliquot was immediately checked for green fluorescence to confirm only GFP^{+} cells were present and was centrifuged at 3000 × *g* at 4 °C for 5 min, and the cell pellet was used for RNA isolation.

Isolation of mRNA, miRNA, and Real Time qPCR Assays—RNA and miRNA from individual mouse pituitaries or flow-sorted gonadotropes were isolated using either the RNAEasy mini kit or miRNA isolation kits (Qiagen), respectively, and DNase-treated on columns. After spectrophotometric quantitation, 200 ng to 1 μg of RNA was reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen) to measure gene expression by established protocols (71). Real time qPCR assays were done using Taqman primer/probe mixes (Applied Biosystems) using the absolute quantification method (71). For miRNA quantitation, specific forward primers were used to amplify the first strand of cDNA from single strand miRNAs, and the second strand was synthesized by the universal reverse

primer using the microScript II-RT kit. Quantitative RT-PCR was carried out using SYBR Green PCR method (Qiagen) according to the manufacturer's instructions on an Applied Biosystems HT7900 Sequence Detector. A standard curve was run in each assay, with an arbitrary value assigned to the highest standard and corresponding values to the subsequent dilutions. Each cDNA sample was run in triplicate, and the abundance of each mRNA was calculated by dividing that of internal control *Ppil1* to normalize for the starting quantity of cDNA. For miRNA quantification, 5 S rRNA and small nuclear RNA U19 were used as internal controls (72). For qPCR assay, RNA samples from at least three mice or three independent flow-sorting experiments were used.

Western Blot Analyses—Pituitary glands from adult mice were collected into chilled radioimmuno precipitation assay buffer (Santa Cruz Biotechnology) containing the protease and phosphatase inhibitors (Santa Cruz Biotechnology) and homogenized, and the supernatants were extracted by centrifugation as described (71). ~1 μg of prestained ready-to-load protein standards (Fermentas) or 20 μg of pituitary proteins were heat-denatured in SDS-PAGE sample buffer (final concentration = 32 mM Tris-HCl, pH 6.8, 12.5% glycerol (v/v), 1% SDS, and 31 μM β-mercaptoethanol) and separated on 12% polyacrylamide gels. The separated proteins were transferred onto PVDF membranes (Bio-Rad) and blocked in 5% nonfat dry milk for 2 h, and the membranes were incubated with primary antibodies for 2 h at room temperature, washed in TBST buffer, pH 7.6 (137 mM sodium chloride, 20 mM Tris-HCl, 0.1% (v/v) Tween 20), and incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology and Bio-Rad) at room temperature for 1 h. The immune complexes were visualized with an ECL kit (GE Healthcare) after exposure to a Biomax film (ISC Biosciences). The following antibodies (and dilutions) were used for Western blot analysis: goat anti-human LHβ (Santa Cruz Biotechnology, SC7824, 1:5000), goat anti-human FSHβ (Santa Cruz Biotechnology, SC7797, 1:5000), rabbit polyclonal anti-human β-tubulin (Santa Cruz Biotechnology, SC9104, 1:5000), and rabbit anti-FOXO1 (Cell Signaling Technologies, #2880, 1: 2000). Recombinant human FSH was a gift from Dr. Irving Boime. Highly purified bovine LH standard was obtained from Dr. A.F. Parlow, UCLA Medical Center.

Acute GnRH Response Assays—Adult mice were injected intraperitoneally with either 100 μl of PBS, pH 7.4, or the GnRH analog Buserelin (20 μg/ml). Blood was collected within 2 h and processed for serum LH and FSH measurements as described below.

Serum Hormone Assays—Mice were anesthetized under isoflurane anesthesia, and blood was collected by cardiac puncture and allowed to clot at room temperature. Serum was separated in MICROTAINER (BD Biosciences) tubes by centrifugation and stored frozen at −20 °C until assayed. Serum FSH, LH, and steroid hormones were measured at the University of Virginia Ligand Assay and Analysis Core Laboratory, Charlottesville, VA. Serum FSH was measured by a mouse FSH RIA (sensitivity = 2 ng/ml; intra assay CV = 6.1%; inter assay CV = 8.2%), LH was measured by a sandwich immunoradiometric assay (sensitivity = 0.07 ng/ml; intra assay CV = 3.9%; inter assay CV = 8.5%), testosterone was measured by RIA (sensitivity = 10 ng/dl; intra assay CV = 3.9%; inter assay CV = 7.8%, and estradiol was measured by an ELISA method (Calbiotech, Inc). The sensitivity of the estradiol assay was 3.94 pg/ml, the intra assay CV was 8.1%, and inter assay CV = 5.8% progesterone was measured by a RIA (sensitivity = 0.1 ng/ml; intra assay CV = 4.9%; inter assay CV = 6.9).

Gross and Histological Analyses—Gonads were collected from adult mice at different ages, gross images were photographed, and wet weights were recorded. The tissues were fixed in Bouin's reagent (Sigma) overnight at 4 °C, processed, and paraffin-embedded. Approximately 6-μm sections were cut and stained with periodic acid-Schiff's reagent/hematoxylin for routine histological analysis as described (71).

Immunofluorescence Assays—Adult mice were transcardially perfused under isoflurane (Piramal Critical Care) anesthesia first with 10 ml of cold 0.9% saline and followed by 60 ml of cold 4% paraformaldehyde, pH 7.2. The pituitaries were collected and post-fixed overnight in 10% sucrose at 4 °C. They were later embedded in Tissue-Tek OCT compound (Sakura FineTek) in cryomolds, and ~10-μm frozen sections were cut onto glass slides using a Leica CM 3050S model cryostat. The sections were rinsed in phosphate-buffered saline, pH 7.4 (PBS), blocked in ready-to-use normal goat or rabbit serum (Invitrogen), and incubated overnight with pairs of primary antibodies at 4 °C. The sections were rinsed in PBS incubated with the appropriate fluorochrome-conjugated secondary antibodies in a dark chamber for 1 h at room temperature and mounted in antifade (Invitrogen). The following primary antibodies were used at 1:500 dilution: mouse monoclonal anti-human FSH 4B (a gift from Dr. Irving Boime), rabbit polyclonal anti-human chorionic gonadotropin serum that recognizes mouse LH (generated in the Boime laboratory), rabbit polyclonal anti-SF-1 (a gift from Dr. Ken Morohashi), and all other pituitary hormone-specific antisera obtained from Dr. A. F. Parlow. All the conjugated secondary antibodies were used at a final dilution of 1:200.

Testes were harvested from adult mice, fixed in formalin, processed in graded alcohol series, and paraffin-embedded by standard methods (69, 71). ~6-μm-thick sections were cut, blocked in 10% normal goat serum (Invitrogen) for 2 h, incubated overnight at 4 °C with primary antibodies against SOX9 (1:1000, a gift from Dr. Ken Morohashi), GCNA1 (no dilution, a gift from Dr. George Enders), PLZF (1:200, Calbiochem OP128L), phospho-histone H3 (1:200, Santa Cruz, SC8656), and SP10 (1:200, a gift from Dr. Prabhakara Reddi), washed in PBS, incubated at room temperature for 2 h with Alexa 488-conjugated appropriate secondary antibodies, washed, and finally mounted in Antifade reagent (Invitrogen). Where necessary, the nuclei were stained with ethidium homodimer (Invitrogen) as described (69, 71). The sections were visualized with an epifluorescence microscope (Zeiss) and photographed.

Epididymal Sperm Quantification—Freshly isolated cauda epididymis from adult male mice were minced into small fragments in 1 ml of sterile M2 medium (Sigma) and incubated at 37 °C for 15 min to release the sperm. An aliquot containing the released sperm was diluted at 1:10 in PBS and counted with a hemocytometer as described (68, 71).

Fertility Assays—Adult control and cKO mice at 42 days of age were caged with proven fertile control mice (1 male with one female; a total of 5 pairs) over a period of 6 months. The number of litters and litter sizes was recorded and used to evaluate the breeding performance (68, 71).

Gonadectomy and Superovulation Assays—Adult mice were surgically gonadectomized under isoflurane anesthesia and maintained for 1 week to totally deplete the endogenous steroids as described (70, 73, 74). At this point, blood was collected for hormone assays; one-half pituitary was used for RNA and the other half for Western blot analyses as described above. Immature female mice at 21–23 days of age were superovulated using the standard protocol of equine and human chorionic gonadotropin hormone injection regimen and mated with proven fertile males as described (71). One-cell fertilized embryos were recovered in M-2 medium from oviducts, hyaluronidase-treated, counted, and photographed as described (71).

Statistical Analysis—Each experiment was performed at least three times and consisted of 5–6 mice per group. Statistical analysis was done by the PRISM program using Student's *t* test. When appropriate, one-way analysis of variance followed by Turkey's post hoc test was used. A p value <0.05 was considered statistically significant. Data are represented as the mean ± S.E.

RESULTS

Gonadotrope-specific Deletion of Dicer—Anterior pituitary consists of a heterogeneous population of hormone-producing cells (9). To delete *Dicer* selectively in gonadotropes, we used a well characterized *bLhb-Cre*$^+$ driver line in which ~700 bp of bovine *Lhb* promoter sequences direct *Cre* transgene expression in gonadotropes (67). CRE-mediated recombination should occur between the loxP sequences and remove the coding sequences of *Dicer*, thus resulting in loss of DICER enzyme (Fig. 1*A*). To test this, we generated *Dicer cKO* mice on an *RT* genetic background that allowed visualization of CRE-expressing gonadotrope cells in green (GFP$^+$) and their enrichment by flow sorting, whereas non-gonadotrope cells that did not express CRE remained red (Fig. 1*B*). Real time quantitative PCR assay showed that *Dicer* mRNA is totally suppressed in gonadotropes obtained from pituitaries of *Dicer cKO* mice when compared with that in gonadotropes obtained from pituitaries of *Ctrl* mice (Fig. 1*C*). Immunolabeling showed that flow-sorted GFP$^+$ gonadotropes expressed CRE but not DICER and further confirmed that *Dicer* was deleted specifically in the gonadotrope population (Fig. 1*D*). Furthermore, quantitative real time PCR indicated many of the miRNAs, computationally predicted to bind *Fshb*, *Lhb*, and *Cga* mRNAs, were significantly suppressed in gonadotropes of *Dicer* cKO mice (Fig. 2). Together, these data confirm that gonadotrope-specific deletion of *Dicer* results in loss of miRNAs that target gonadotropin β subunit-encoding mRNAs.

Loss of Dicer in Gonadotropes Results in Suppressed Gonadotropin Synthesis and Secretion in cKO Male Mice—Loss of microRNAs could result in up- or down-regulation of mRNAs and the corresponding proteins depending on cell type and developmental status and for other reasons (75–78). To test, how loss of DICER-dependent miRNAs affects gonadotropin

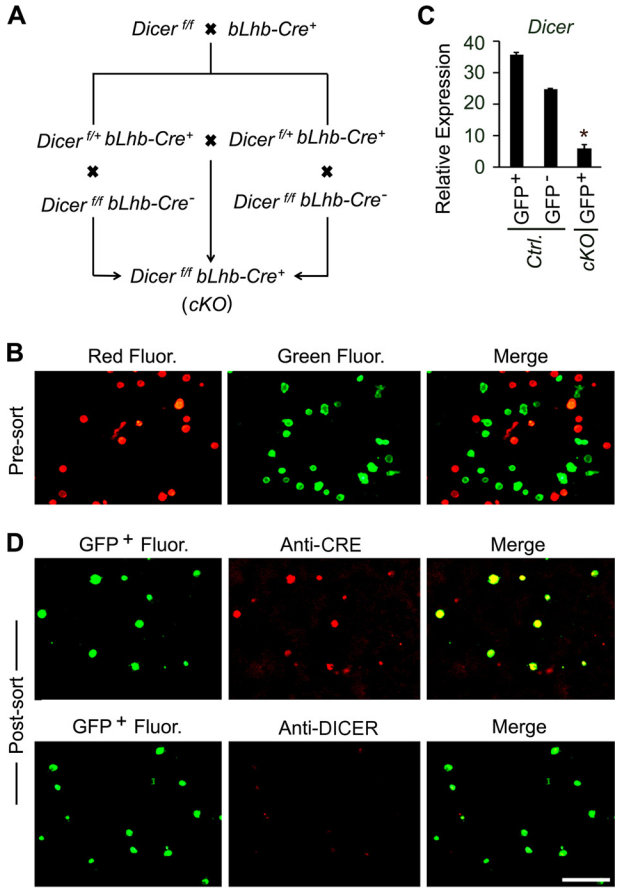


FIGURE 1. **Generation of *Dicer* cKO mice.** *A*, *Dicer*$^{f/f}$ male mice were first intercrossed with *bLhb-Cre*$^+$ female mice to generate *Dicer*$^{f/+}$ *bLhb-Cre*$^+$ mice. These mice were intercrossed in a second step to generate the desired *Dicer*$^{f/f}$ *bLHb-Cre*$^+$ (*Dicer* cKO) mice. In addition, we also intercrossed *Dicer*$^{f/+}$ *bLhb-Cre*$^+$ male mice with *Dicer*$^{f/f}$ *bLHb-Cre*$^-$ females and vice versa to increase the frequency of generating *Dicer* cKO mice. Pituitaries from adult *Dicer* cKO mice on a Rosa tomato genetic background were harvested, and an aliquot of enzyme-dispersed cell fraction was visualized before sorting. In *B*, the merged image shows that *Cre* is expressed selectively in gonadotropes (GFP$^+$ green fluorescent cells); the non-gonadotropes exhibit red fluorescence (GFP$^-$ *Cre*$^-$). *C* shows *Dicer* mRNA expression by Taqman real time qPCR assay using FACS-sorted cells. Note that *Dicer* mRNA was significantly suppressed in GFP-expressing gonadotropes obtained from pituitaries of *Dicer* cKO mice compared with that in GFP$^-$ non-gonadotrope cells and GFP-expressing gonadotropes obtained from age-matched control (*Ctrl*) mice. *, $p < 0.01$ *versus Ctrl* (5 mice per genotype; each sample was run in triplicate). Immunofluorescence visualization post-sort (*D*) GFP$^+$ gonadotrope cells were immunoreactive with an antibody against CRE (*yellow cells in the merge image*) but not DICER (*presence of only green fluorescence but no yellow in merge image*). The *white bar* represents 50 μm.

synthesis in pituitaries of male mice, we first quantified gonadotropin subunit mRNAs by real time qPCR assays. All the three subunit-encoding mRNAs, namely, *Fshb*, *Lhb*, and *Cga*, were significantly suppressed in purified gonadotropes of *Dicer* cKO mice compared with those in control mice (Fig. 3*A*). As expected, the expression of gonadotropin subunit mRNAs was nearly undetectable in the GFP$^-$ negative non-gonadotrope population of cells (Fig. 3*A*) and thus further validated the flow sorting strategy to purify gonadotropes. Similarly, direct visualization of immunolabeled gonadotropes in the pituitaries of control and *Dicer* cKO mice using antibodies against FSHβ and LHβ (Fig. 3*B*) revealed that expression of only gonadotropin subunits, *i.e.* FSHβ and LHβ, but not other non-gonadotrope markers, *i.e.* TSHβ (thyrotropes), adrenocorticotropic hor-

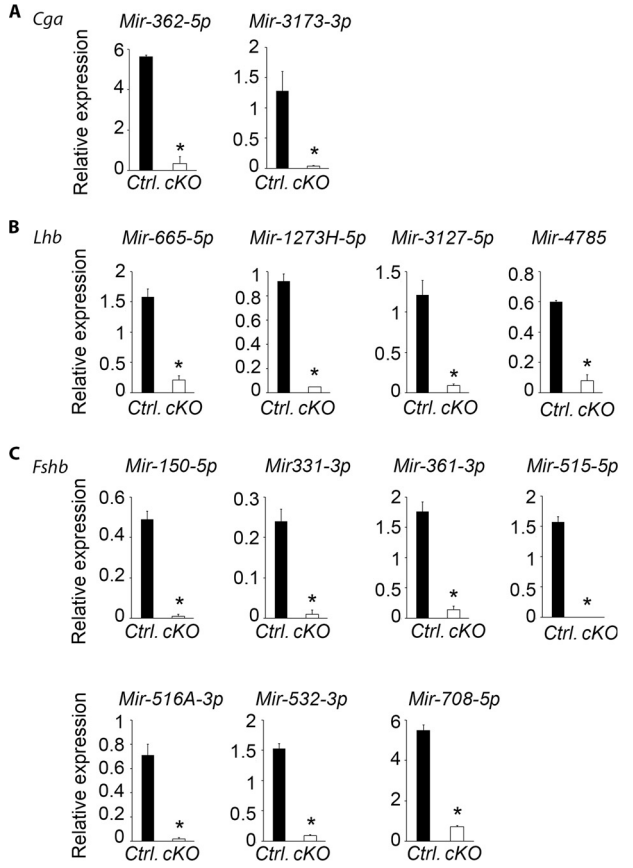


FIGURE 2. **Expression of miRNAs in pituitary gonadotropes of *Dicer* cKO mice.** Specific primers for each of the computationally predicted miRNAs that bind gonadotropin subunits were designed and tested in real time qPCR assays using gonadotrope cells purified from control (*Ctrl*) and *Dicer* cKO mice. Loss of DICER resulted in suppression of all these predicted miRNAs in the pituitaries of *Dicer* cKO mice compared with those in controls (*, $p < 0.01$). FACS-sorted GFP^+ gonadotropes were obtained from age-matched adult males (three mice per genotype), and cDNA samples were run in triplicate. Expression of 5 S rRNA was used as an internal control.

mone (corticotropes), prolactin (lactotropes), and growth hormone (somatotropes) was significantly reduced in *Dicer* cKO mice. The immunolocalization data were further confirmed by Western blot analysis. These data indicated that expression of both FSHβ and LHβ proteins was significantly suppressed in pituitaries of *Dicer cKO* males compared with that in controls (Fig. 3*C*). Loss of one allele of *Dicer* (*Dicer*$^{f/+}$ *Cre*$^+$ or *Dicer*$^{\Delta/+}$) did not significantly affect the expression of gonadotropin subunits compared with that in controls. Recombinant human FSH (*rhFSH*) and bovine LH (*bLH*) standards served as positive controls, whereas pituitary extracts prepared from *Fshb Lhb* double null mice served as negative controls and confirmed the specificity of the antibodies used for immunoblots (Fig. 3*C*). Collectively, these results indicate that loss of *Dicer* in gonadotropes leads to suppression of gonadotropin synthesis in male mice.

Testosterone and gonadal peptides exert a negative feedback control to regulate gonadotropin synthesis and secretion in the male (1, 10, 11). We next tested whether castration, which removes gonadal negative feedback, affects gonadotropin synthesis and secretion in the absence of DICER-dependent miRNAs in gonadotropes. Compared with the levels in the intact group of control mice, castration resulted in a significant increase in expression of gonadotropin mRNAs encoding α- and FSHβ but not LHβ subunits when assayed by real time qPCR (Fig. 3*D*). In contrast, castration did not significantly affect expression of mRNAs encoding gonadotropin α and β subunits compared with those in the corresponding intact group of *Dicer* cKO male mice (Fig. 3*D*). Similarly, Western blot analysis showed castration resulted in a significant decrease in FSHβ and LHβ protein expression compared with that in intact group of control mice (Fig. 3*E*). However, no significant change in expression of FSHβ and LHβ proteins was detected when intact and castrated groups of *Dicer* cKO mice were compared (Fig. 3*E*). Quantification by specific RIAs further confirmed that gonadectomy-induced rise in serum FSH and LH was observed only in control but not *Dicer* cKO mice (Fig. 3*F*). Although *Dicer* cKO mice demonstrated suppressed basal levels of serum LH, acute GnRH response to release LH after a single bolus of buserelin injection was maintained similar to that seen in control male mice (Fig. 3*G*). Thus, these data reveal that DICER-dependent miRNAs in gonadotropes are critical for post-castration rise in gonadotropin synthesis and secretion in male mice.

Altered Expression of Transcriptional Activators and Repressors in Pituitaries of Dicer cKO Mutant Male Mice—If DICER-dependent miRNAs directly bind to and post-transcriptionally regulate gonadotropin subunit mRNAs, loss of DICER should result in up-regulation of the corresponding gonadotropin subunit proteins. In contrast, we observed suppression of gonadotropin proteins in the pituitaries of *Dicer* cKO male mice (Fig. 3). We, therefore, next investigated two possibilities. First, we computationally identified miRNAs that were predicted to bind some of the known mRNAs encoding transcriptional repressors. In the absence or severe suppression of a set of these miRNAs in purified gonadotropes (Fig. 4*A*), we found that some of the repressor-encoding mRNAs including *Foxo1*, *Sox2*, and *Fst* and at least one repressor protein, FOXO1, were up-regulated (Fig. 4*C*) in the pituitaries of *Dicer* cKO mice compared with those in control mice. Other known repressor-encoding mRNAs such as *Msx1*, *Esr1*, and *Zeb1* did not show any significant differences in *Dicer* cKO mutant pituitaries (data not shown). Immunolocalization studies also failed to detect any significant baseline expression of other repressor proteins including RGS3, ATF3, and ZEB1 in the pituitaries of control mice (data not shown). Second, we found that *Gnrhr* and *Acvr2* but not *Foxl2* mRNAs (Fig. 4*D* and data not shown) and SF-1 (Fig. 4*E*) were also significantly suppressed in the pituitaries of cKO mutants. Together, these data indicate that loss of DICER-dependent miRNAs results in an increase in gonadotropin subunit transcriptional suppressors and suppression of gonadotropin subunit transcriptional activators with a net effect of severely suppressed gonadotropin subunit proteins in the pituitaries of *Dicer* cKO male mice.

Dicer cKO Male Mice Display Fertility Defects—Gonadotropins serve as key trophic hormones for male fertility. FSH regulates Sertoli cell number and consequently the germ cell-carrying capacity and testis size (68, 79, 80). *Fshb* null male mice, hence lacking FSH dimer, are fertile despite reduced testis size, Sertoli and germ cell number, and sperm (68). LH is required

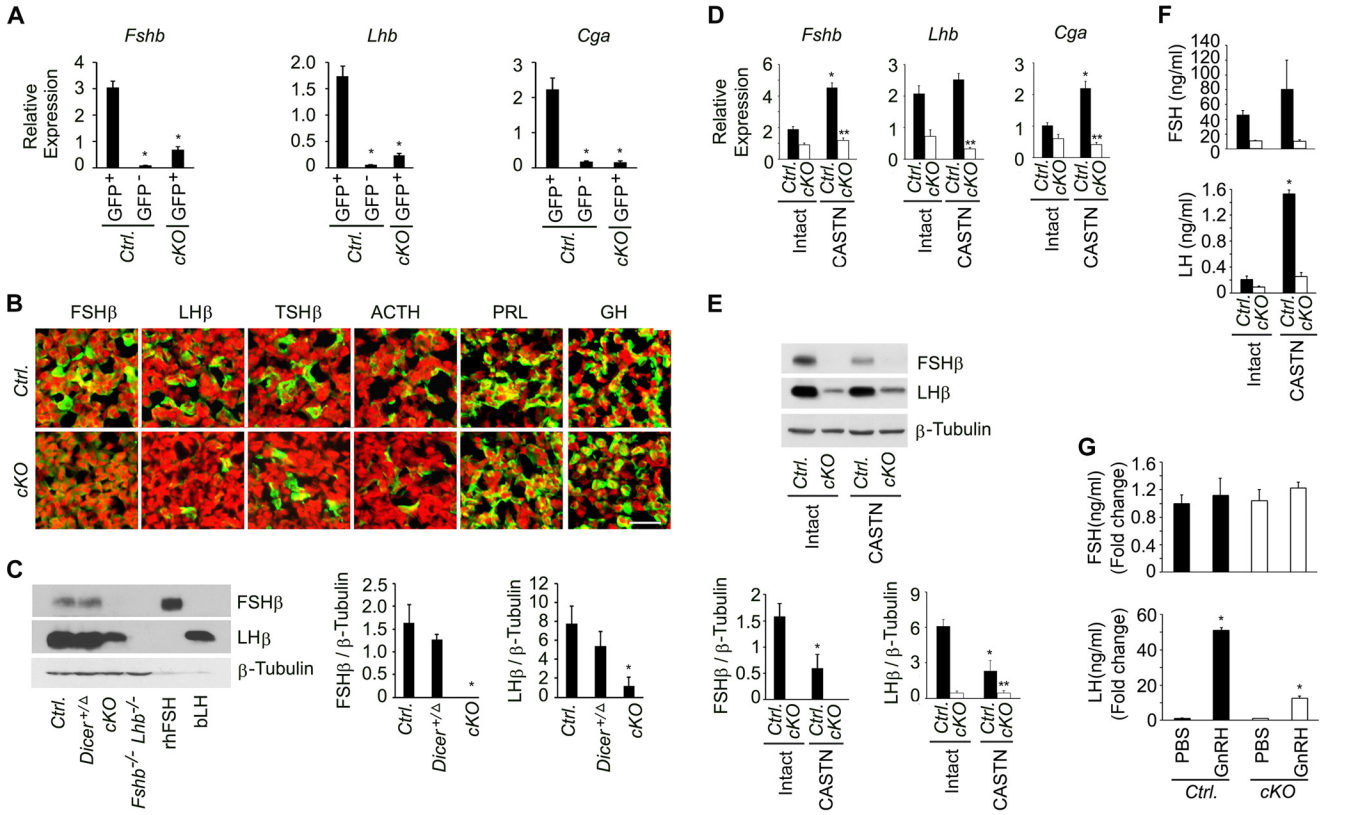


FIGURE 3. **Regulation of gonadotropins in *Dicer* cKO male mice.** In *A*, expression of the gonadotropin subunit encoding mRNAs (*Fshb*, *Lhb*, and *Cga*) was measured by Taqman real time qPCR assays. Purified gonadotrope (GFP^+) and non-gonadotrope (GFP^-) cells were obtained from the pituitaries of adult male control and *Dicer* cKO mice. Loss of DICER in gonadotropes led to severe suppression of all three gonadotropin subunit mRNAs compared with that in corresponding controls ($p < 0.01$). Note that GFP^- non-gonadotrope cells do not express gonadotropin subunits, confirming the validity of the flow sorting to enrich GFP^+ gonadotropes. In *B*, anterior pituitary markers were evaluated in frozen tissue sections from control and *Dicer* cKO adult male mice. Immunofluorescence was performed using specific antisera against each of the hormones or hormone subunits as indicated and visualized by appropriate fluorochrome-conjugated secondary antibodies. Loss of DICER in gonadotropes resulted in marked suppression of only FSHβ and LHβ but not non-gonadotrope markers including thyroid stimulating hormone β-subunit (*TSH*β), corticotropin (adrenocorticotropic hormone (*ACTH*)), prolactin (*PRL*), and growth hormone (*GH*). Hormone expression was indicated by green fluorescence, whereas the nuclei were stained red. The *white bar* represents 20 μm. Immunofluorescence data were further confirmed by Western blot analysis (*C*) using total pituitary extracts from control (*Ctrl*), *Dicer* heterozygous ($Dicer^{+/\Delta}$), and homozygous (cKO) mice. Pituitary extracts from double null mice lacking *Fshb* and *Lhb*, recombinant human FSH (*rhFSH*), and purified pituitary bovine LH (*bLH*) were used, respectively, as negative and positive controls for specificity of the antibodies used. Densitometry quantification showed that both gonadotropins were suppressed in the pituitaries of male mice lacking *Dicer* in gonadotropes compared with those in controls or heterozygous *Dicer* cKO mice ($p < 0.01$). *Panels D* and *E* show RNA (*D*) and protein (*E*) expression, respectively, by Taqman real time qPCR assays and Western blot analysis of gonadotropin subunits in the pituitaries of control (*Ctrl*) and *Dicer* cKO mice after castration (*CASTN*). Castration resulted in significant up-regulation (*panel D*; *, $p < 0.01$) of *Cga* and *Fshb* gonadotropin subunit mRNAs in the pituitaries of control (*Ctrl*) but not *Dicer* cKO mice (*panel D*; **, $p > 0.05$). Densitometry data of Western blots show that castration caused a significant reduction in gonadotropin β subunits in control (*panel E*; *, $p < 0.01$) but not *Dicer* cKO (*panel E*; **, $p > 0.05$) mouse pituitaries. In *F*, serum gonadotropins in adult intact and castrated *Ctrl* and *Dicer* cKO male mice were measured by RIAs. Castration resulted in significantly elevated serum gonadotropin levels in *Ctrl* but not *Dicer* cKO male mice, indicating that DICER-dependent miRNAs are critical regulators of post-gonadectomy rise in gonadotropins. Adult mice were injected with PBS or 2 μg of buserelin, a GnRH agonist. After 2 h, serum gonadotropins were measured by RIAs to quantify the acute GnRH response. The -fold changes of GnRH response with respect to PBS-injected values were presented. Although FSH was not released in control or *Dicer* cKO mice (*G*, *top panel*), LH was robustly released (*G*, *bottom panel*) in *Ctrl* mice in response to acute GnRH. Although LH release was not of the same magnitude as in *Ctrl* mice (5 *versus* 40-fold), GnRH did cause a significant LH release in *Dicer* cKO mice (*, $p < 0.05$ *versus* corresponding PBS group). For Taqman real time qPCR assays, expression of *Ppil1* was used as an internal control. For Western blot experiments, antibodies specific to gonadotropin β subunits were used at a 1:5000 dilution, and expression of β-tubulin was used as an internal control. A representative Western blot was shown, and the densitometry data represent the mean ± S.E. of three independent experiments.

for Leydig cell development and testosterone production, and *Lhb* null male mice have hypogonadism and defects in steroid biosynthesis and display infertility (69). To determine how suppressed gonadotropins affect fertility in *Dicer* cKO males, we first evaluated both morphology and histology of testes collected from reproductively mature adult males at 42 days of age. Testis size was significantly reduced in *Dicer* cKO mice compared with age-matched controls (Fig. 5, *A* and *B*). Histological analyses of periodic acid/Schiff's reagent-hematoxylin-stained testis sections from *Dicer* cKO mice indicated decreased tubule size with apparently heterogeneous tubule architecture including some tubules with grossly normal spermatogenesis but several abnormal tubules containing very few or no germ cells (Fig. 5*F*). The majority (23/26 = 88%) of the *Dicer* cKO male mice displayed this abnormal testis tubule histology. Consistent with testis histology, epididymal sperm number (Fig. 5*C*) and histology of cauda epididymis showed a significantly lower number of sperm in mutants (Fig. 5*F*). These testicular defects did not affect mating performance *per se* of mutant males because they sired similar number of litters compared with controls over a period of 6 months (Fig. 5*D*). However, the litter size, *i.e.* the number of pups produced per litter during this time period was significantly reduced when *Dicer* cKO mutants were compared with control males (Fig. 5*E*).

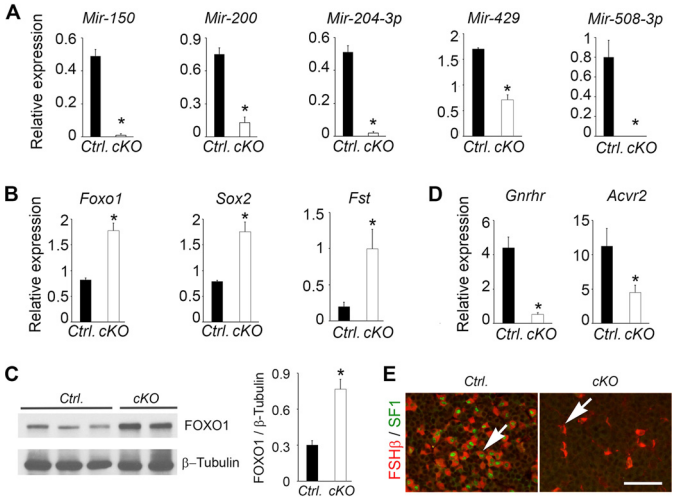
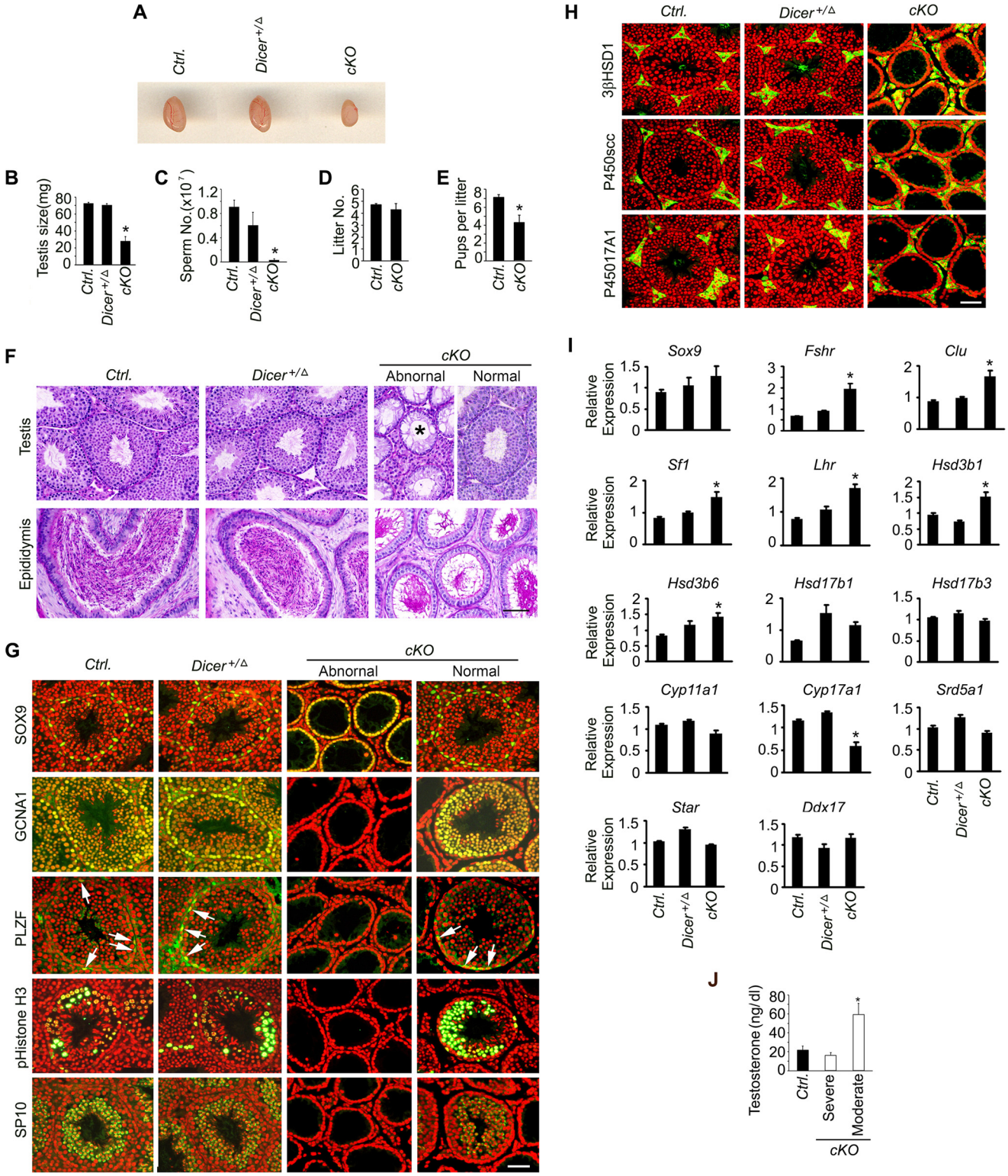


FIGURE 4. **Regulation of repressors and activators in pituitaries of *Dicer* cKO male mice.** In *A*, real time qPCR analyses indicate that several miRNAs computationally predicted to bind some of the repressors (that normally inhibit gonadotropin subunit gene transcription) were significantly suppressed in purified gonadotropes of *Dicer* cKO male mice. Consequently, mRNAs encoding the repressors were significantly up-regulated in pituitaries of cKO males (*B*). In *C*, Western blot analysis (*left*) followed by densitometry quantification (*right*) shows that FOXO1, a repressor for *Lhb* expression, was significantly up-regulated in the pituitaries of *Dicer* cKO compared with that in control (*Ctrl*) male mice. In *D*, real time qPCR assays indicate that *Gnrhr* and *Acvr2* mRNAs that encode two major signaling pathway receptors required for activation of gonadotrope subunit gene transcription were significantly suppressed in gonadotropes of *Dicer* cKO male mice. In *E*, dual label immunofluorescence localization confirms that SF-1, a gonadotrope-specific transcriptional activator, was severely suppressed in a pituitary section obtained from an adult *Dicer* cKO male mouse pituitary. *, $p < 0.05$ *versus* control (*Ctrl*) group; $n = 4$ mice; for real time qPCR assays each sample was run in triplicate. For immunofluorescence, formalin-fixed pituitaries were obtained from adult mice ($n = 3$), incubated with a rabbit antiserum raised against SF-1 and an FSHβ-specific mouse monoclonal antibody, and visualized by appropriate secondary antibodies conjugated with fluorochromes. The *white arrow* in *E* indicates SF-1 nuclear localization in a FSHβ+ gonadotrope that was suppressed in a section obtained from *Dicer* cKO male mouse pituitary. Multiple sections were observed per pituitary and per genotype. The *white bar* in *E* represents 100 μm.

To further evaluate the male fertility defects in *Dicer* cKO mutants, we assessed by immunofluorescence cell-specific expression of functional markers in the testes of reproductively mature adult mice (Fig. 5*G*). The transcription factor SOX9 is expressed downstream of SRY, the testis determining factor, and defines the Sertoli cell lineage in the testis (81–83). The expression of SOX9 did not change irrespective of whether the male fertility was moderately or severely affected in *Dicer* cKO mutant mice (Fig. 5*G*). Whereas the expression of other germ cell markers including GCNA1 (spermatogonia), PLZF (stem cells), phospho-histone H3 (mitotically diving germ cells), and SP10 (round spermatids) was similar in moderately affected mutants (Fig. 5*G*), these markers were undetectable in testes of severely affected mutants. Thus, there were mostly Sertoli cell-only tubules in the testes of severely affected mutants (Fig. 5*G*). We next tested whether the defects in tubules manifest as a result of defects in Leydig cells that produce the androgen testosterone. Immunofluorescence analysis of three testosterone biosynthetic pathway enzymes, namely, 3β-HSD1, SCC, and CYP17, that are adult mature Leydig cell markers showed identical expression pattern in the testes of mutants and control mice (Fig. 5*H*). Consistent with the Leydig cell marker expression, serum testosterone levels were not significantly different in *Dicer* cKO mutants compared with those in controls (Fig. 5*J*). However, 50% of mutant males in which testes size was moderately affected showed elevated serum testosterone (Fig. 5*J*). Similarly, real time qPCR assays of Sertoli, Leydig, and germ cell markers did not reveal any significant differences between *Dicer* cKO mutants and controls (Fig. 5*I*). Interestingly, immunofluorescence of gross testis from mutants on an *RT* genetic background did confirm that *Cre* was active locally within the testis (Fig. 6*A*), and expression of *Dicer* was significantly reduced in the testes of *Dicer* cKO male mutants (Fig. 6*B*).

Loss of Dicer in Gonadotropes Results in Suppressed Gonadotropin Synthesis and Secretion in Female Mice—Gender-specific regulation of gonadotropins has been well studied (68, 70, 74). To determine if loss of *Dicer* in gonadotropes affects gonadotropin synthesis and secretion in females similar to males, we first analyzed gonadotropin subunit mRNAs by real time qPCR assays using purified gonadotropes (GFP^+) prepared from the pituitaries of adult female mice. Unlike *Lhb* and *Cga* subunit mRNAs, which were both suppressed (Fig. 7*A*, *middle* and *right panels*), *Fshb* mRNA was unaffected in pituitary in the absence of *Dicer* (Fig. 7*A*, *left panel*) compared with controls. As expected, gonadotropin subunit-encoding mRNAs were undetectable in GFP^- non-gonadotropes (Fig. 7*A*). Immunofluorescence was used to further evaluate and visualize how gonadotropin protein expression was affected in the absence of DICER. Consistent with RNA expression data, we observed that LH immunostaining was mostly undetectable and, even when present, less intense in only few gonadotropes in pituitary sections obtained from *Dicer* cKO female mice compared with that in control sections (Fig. 7*B*). In contrast to LH expression, although *Fshb* RNA levels remain unchanged, the corresponding protein was undetectable in pituitary sections obtained from *Dicer* cKO mice compared with that in control sections (Fig. 7*B*). Loss of DICER in gonadotropes did not affect the expression of non-gonadotrope markers such as adrenocorticotropic hormone, prolactin, and growth hormone whose staining in *Dicer* cKO pituitary sections was similar to that in controls. Western blot analysis followed by densitometry was used to further quantify gonadotropin subunit expression normalized to that of an internal control β-tubulin (Fig. 7*C*). These values closely paralleled the qualitative data obtained with immunofluorescence, *i.e.* whereas expression of RNA and protein correlated to each other in the case of LH, there was a discordant correlation between the two in case of FSH in pituitaries of *Dicer cKO* mice. Pituitary extracts from double null mice lacking both *Fshb* and *Lhb* and (hence FSH and LH) and FSH (recombinant human FSH) and LH (bovine LH) standards served as negative and positive controls, respectively (Fig. 7*C*), and validated the specificity of the antibodies used in Western blot analysis. The acute GnRH response to release a bolus of LH was unaffected in *Dicer* cKO females similar to that seen in males (Fig. 7*G*). Together, these data indicate that gonadotrope-specific loss of DICER affects gonadotropins in a sexually dimorphic pattern and selectively regulates the expression of *Fshb* mRNA at the post-transcriptional level within the female pituitary.

Both ovarian steroids (progesterone and estrogen) and peptides (inhibins and follistatin) act at the level of hypothalamus and pituitary (gonadotropes) and regulate gonadotropins in the female (1, 10, 11). We next tested if DICER is required for ovariectomy-induced changes in gonadotropins at the level of pituitary. Ovariectomy caused a significant up-regulation of three gonadotropin subunit mRNAs in pituitaries of control mice as assessed by real time qPCR (Fig. 7*D*), an increase in the corresponding gonadotropin β-subunit proteins in pituitaries determined by Western blot analysis (Fig. 7*E*), and resulted in elevated serum gonadotropin levels controls (Fig. 7*F*). In contrast, ovariectomy failed to up-regulate gonadotropin expression in *Dicer* cKO mice at all levels analyzed including RNA and protein in the pituitary and circulating hormones in serum (Fig.



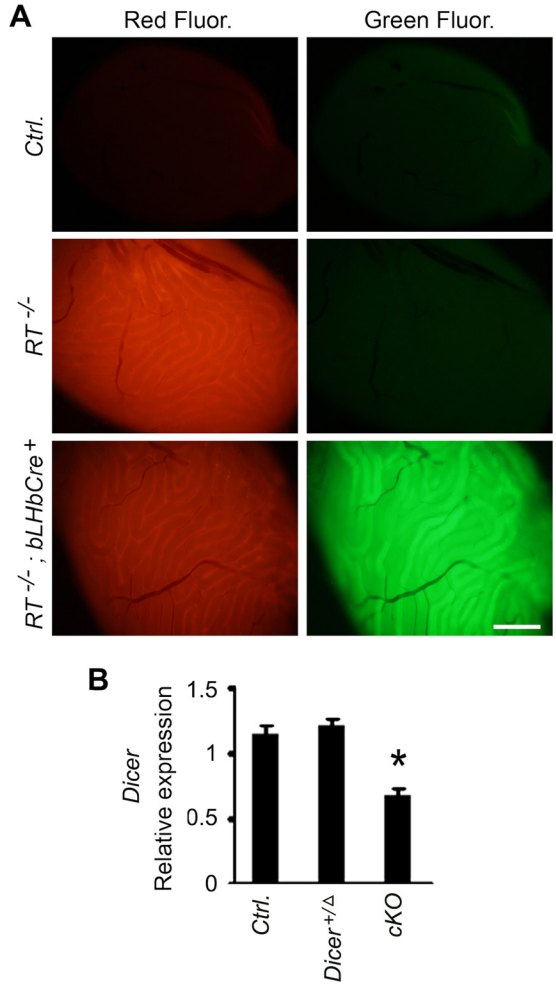


FIGURE 6. **Ectopic expression of the *bLhb-Cre* transgene and expression of *Dicer* mRNA in testis.** Testes were harvested from adult male mice, and whole testes were imaged in cold PBS under an epifluorescence microscope. Note the green fluorescence in testis (*A*) obtained from only *bLhb-Cre*$^{+}$ mice but not in controls. Rosa tomato ($RT^{-/-}$) mice show only red fluorescence in testes. This ectopic expression of *Cre* caused a local reduction in *Dicer* mRNA (*B*) and likely contributed to aberrant testis tubule histology in *Dicer* cKO male mutants. The Taqman assay was performed on triplicate samples of the testes from four adult male mice. *, $p < 0.05$ *versus* both control and heterozygous mice. The *white bar* in *panel A* represents 2 mm.

7*F*). Thus, DICER-dependent miRNAs in gonadotropes are essential for ovariectomy-induced up-regulation of gonadotropins in female mice.

Altered Expression of Transcriptional Activators and Repressors in Pituitaries of Dicer cKO Female Mice—Loss of DICER-dependent miRNAs in the pituitaries of *Dicer* cKO female mice also caused suppression of gonadotropin subunit proteins similar to that in males. To check if transcriptional repressors and activators were, respectively, up and down-regulated similarly in pituitaries of female cKO mutants, we first assayed computationally predicted miRNAs that target repressors by Taqman real time qPCR. We found that these miRNAs were significantly suppressed in purified gonadotropes of *Dicer* cKO females compared with those in controls (Fig. 8*A*). Unlike in *Dicer* cKO males, only *Esr1* mRNA, but not other repressors, was significantly up-regulated in the pituitaries of female *Dicer* cKO mice (Fig. 8*B*). Moreover, in pituitaries of female $Esr1^{-/-}$ mice, *Cga* and *Lhb*, but not *Fshb* mRNAs, were significantly up-regulated by real time qPCR (Fig. 8*C*) as has been shown by others (84). In addition to up-regulation of repressors, either suppression or loss of activators could also result in suppression of gonadotropin subunit proteins. To address this, we tested the expression of activators for gonadotropins and found that *Gnrhr* and *Nr5a1* (encodes SF-1) mRNAs were both significantly suppressed (Fig. 8*D*). Immunolocalization data further confirmed that SF-1 protein levels in FSHβ-stained gonadotropes were significantly suppressed in the pituitaries of *Dicer* cKO female mice (Fig. 8*E*). Collectively these studies suggest an indirect mechanism likely involving regulation of transcriptional activators and ESR1-mediated repression contributed to the observed defects in gonadotropin subunit protein expression in pituitaries of *Dicer* cKO female mice.

Dicer cKO Female Mice Display Fertility Defects—Loss of gonadotropins and their cognate receptors results in impairment of ovarian folliculogenesis at distinct phases of ovarian development (85, 86). *Fshb*/*Fshr* null females mostly phenocopy each other and demonstrate a pre-antral stage block in ovarian folliculogenesis (85, 86). *Lhb*/*Lhr* null females also phenocopy each other and display a pre-ovulatory stage block in ovarian folliculogenesis (69, 87, 88). To determine how severely suppressed gonadotropins affect female fertility of *Dicer* cKO mice, first, we analyzed grossly the female reproductive tracts. At 9 weeks of age, both uteri and ovaries were grossly hypoplastic in *Dicer* cKO mice compared with controls, and the phenotype was more severely affected by 18 weeks of age (Fig. 9*A*). Mating trials between *Dicer* cKO adult females with proven fertile males over a period of 6 months resulted in severely reduced number of litters (Fig. 9*B*) and litter size, *i.e.* number of pups per litter (Fig. 9*C*). During the same time period, one of the three mutant "test" females did not produce a single litter. The remaining two females delivered only 1–2 litters and then

FIGURE 5. **Male reproductive phenotypes in *Dicer* cKO mice.** Gross testis morphology (*A*) in adult male mice at 42 days of age is shown. Testis size (*B*) and cauda epididymal sperm (*C*) were significantly reduced in *Dicer* cKO mice compared with control (*Ctrl*) or heterozygous mice lacking one *Dicer* allele (*Dicer*$^{+/\Delta}$) in gonadotropes. Although both control and *Dicer* cKO male mice sired the same number of litters (*D*) in a 6-month period, the litter size (pups per litter) was significantly reduced in mutants compared with controls (*E*). Periodic acid/Schiff's reagent-hematoxylin-stained testis histology (*F*) appears grossly normal in some tubules in *Dicer* cKO mice, similar to that compared in *Ctrl* and *Dicer*$^{+/\Delta}$ mice. Abnormal histology with mostly Sertoli cell-only like phenotype (*black star*) was also apparent in some tubules within the same testis sections. Consistent with the testis phenotype, very few sperm were present in the epididymis of mutant males compared with control groups of mice (*F*, *lower panels*). *G*, expression of testis markers was shown. Immunolocalization was done on formalin-fixed testis sections using antibodies against Sertoli (*SOX9*)-, PAN germ cell (*GCNA1*)-, undifferentiated spermatogonia (*PLZF*; *white arrows*)-, mitotic germ cell (*pHistone H3*)-, and round spermatid (*SP10*)-specific markers. Note that histologically normal tubules in testis of *Dicer* cKO mouse express all the testis cell markers similar to those in control sections. However, abnormal tubules in *Dicer* cKO mouse testis expressed only SOX9 but none of the germ cell markers confirming that this is truly a Sertoli cell-only tubule phenotype. Specific marker expression was shown in *green*, and nuclei are stained *red*. Although defects in tubules were apparent, Leydig cell markers were all normally expressed in *Dicer* cKO mutant testes (*H*). Taqman real time qPCR assays (*I*) indicate that Sertoli and Leydig cell mRNAs were aberrantly regulated in the testes of *Dicer* cKO mice compared with those in control mice (indicated by the *). Serum testosterone levels were elevated in moderately affected but not severely affected *Dicer* cKO (*open bars* in *J*) male mice compared with controls (*black bar* in *J*). *, $p < 0.05$ *versus* corresponding *Ctrl* group. Groups of 5–6 adult male mice per genotype were used. *White* and *black bars* in *F–H panels* represent 20 μm.

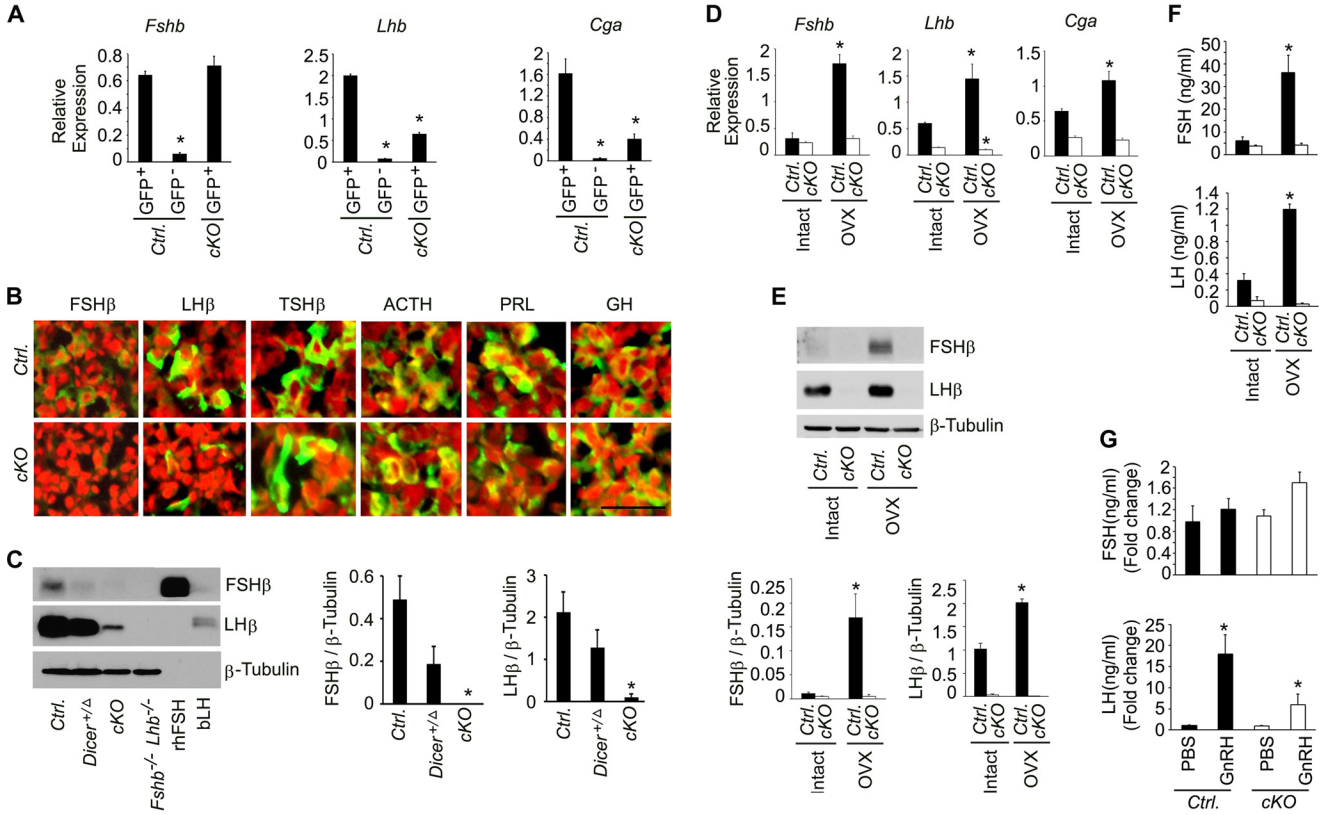


FIGURE 7. **Regulation of gonadotropins in *Dicer* cKO female mice.** Taqman real time qPCR assays (*A*) confirm that although *Lhb* and *Cga* mRNAs were significantly suppressed, *Fshb* mRNA was not affected in the absence of DICER in gonadotropes in pituitaries of female mice. Immunofluorescence localization (*B*) and Western blot followed by densitometry analyses (*C*) indicate that both FSHβ and LHβ were significantly suppressed in the pituitaries of *Dicer* cKO mice. In *B*, expression of non-gonadotrope cell markers was not affected and similar to that in control mouse pituitaries. Ovariectomy (*OVX*) caused a marked up-regulation of gonadotropin subunit mRNAs (*D*) and FSHβ and LHβ proteins (*E*) in the pituitaries of intact control (*Ctrl*) but not *Dicer* cKO mice. The pituitary expression data closely matched that of serum gonadotropin levels shown in *F*. In *G*, in an acute GnRH response assay, female mice responded (*G*) similarly to male mice (Fig. 3*G*). LH release response to buserelin, although not at the same magnitude as in control (*Ctrl*) group (5 *versus* 15-fold), there was a significant increase in LH release response in *Dicer* cKO female mice compared with the PBS-injected group. Other experimental details were identical to those described in Fig. 3 legend. The *white bar* in *B* represents 20 μm.

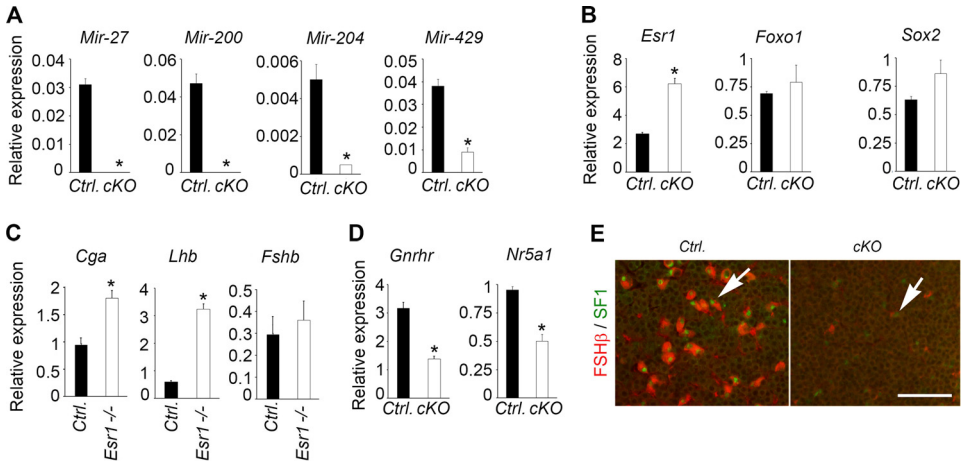


FIGURE 8. **Regulation of repressors and activators in pituitaries of *Dicer* cKO female mice.** In *A*, real time qPCR assay data confirm that computationally predicted miRNAs that bind repressor-encoding mRNAs were significantly suppressed in gonadotropes of *Dicer* cKO female mice. However, only *Esr1* expression, but not other repressor encoding mRNAs (*Foxo1*, *Foxo3*, and *Sox2* in B), was found significantly up-regulated in cKO female mutants. Loss of *Esr1* resulted in significant up-regulation of *Cga* and *Lhb* mRNAs but not *Fshb* mRNA in pituitaries of cKO mutants compared with those in control (*C*) as measured by real time qPCR assays. In *D*, expression of *Gnrhr* and *Nr5a1* mRNAs encoding two critical activators were reduced in gonadotropes of cKO mutants. *, $p < 0.05$; $n = 3–4$ mice; each sample was run in triplicate for real time qPCR assays. Dual immunolabeling (*E*) confirmed that SF-1 protein expression was severely reduced in a pituitary section obtained from an adult female cKO mouse (*E*, *right*) compared with that in control mouse (*E*, *left*). Gonadotropes were labeled *red*, and SF-1 was localized to nuclei and visualized *green* (*E*, *white arrows*). Multiple pituitary sections from three mice were analyzed. The *white bar* represents 100 μm.

stopped breeding. We next analyzed ovarian histology to identify if folliculogenesis was defective and could explain the observed fertility defects in *Dicer* cKO mutant females. The presence of corporal lutea (CL) indicating ovulations and normal estrus cycles was readily apparent in PAS/hematoxylin-stained ovarian sections from control mice at 9 weeks of age

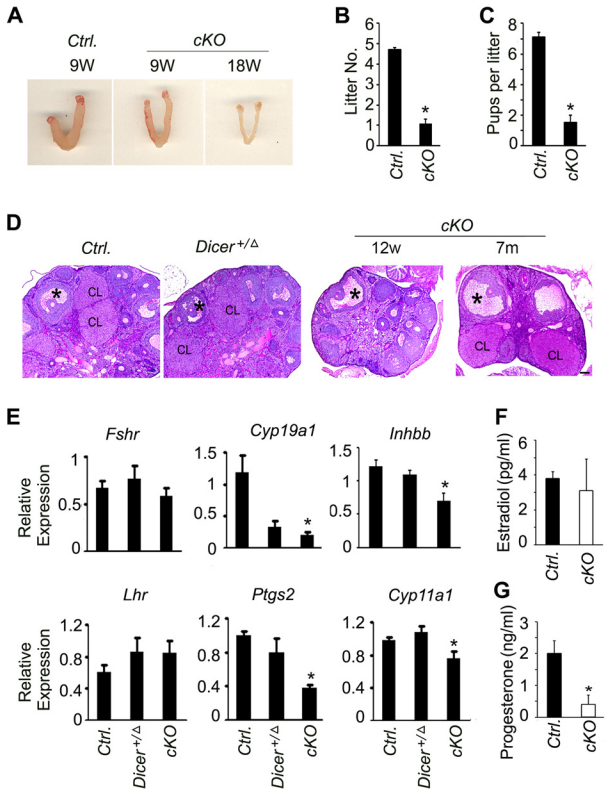


FIGURE 9. **Reproductive phenotypes in *Dicer* cKO female mice.** Gross morphology of the female reproductive tract (*A*) shows hypoplastic ovaries and uteri at two different ages in *Dicer* cKO mice. Fertility analysis over a 6-month period indicated severely reduced litter number (*B*) and litter size (*C*) in female *Dicer* cKO mice compared with those in age-matched controls. Ovarian histology (*D*) in control mice shows CL; they were rare and apparent only in 1 of the 20 *Dicer* cKO female mice analyzed. *Black stars* indicate antral follicles, and the *black bar* represents 20 μm in *D*. Ovarian gene expression by Taqman real time qPCR assays (*E*) indicated that FSH-responsive *Cyp19a*, *Inhbb*, and LH-responsive *Ptgs2* were significantly suppressed in *Dicer* cKO mutants ($p < 0.05$ *versus* controls) consistent with suppressed serum FSH and LH in these cKO mice. Serum estradiol showed a trend toward reduction (*F*), whereas progesterone was suppressed (*G*) in *Dicer* cKO female mice compared with controls. Adult mice at 6–9 weeks of age were used. *, $p < 0.05$ *versus* corresponding *Ctrl* group. Groups of 5–6 adult mice per genotype were used.

(Fig. 9*D*). However, we rarely found CL in ovarian sections obtained from 9 of 10 (90%) age-matched *Dicer* cKO females (Fig. 9*D*). Only 1–2 CL (compared with 6–7 CL in control ovary) were present in ovarian sections obtained from 1 of 10 (10%) mutant mice analyzed at ~6 months of age. Although CL were rarely present, both antral and pre-ovulatory follicles were apparent in the ovaries of *Dicer* cKO mutants (Fig. 9*D*). Consistent with ovarian histology, some (*Cyp19a1* and *Inhbb*) but not other (*Fshr* and *Lhr*) ovarian marker genes regulated by FSH and LH (*Ptgs2* and *Cyp11a1*) were suppressed in adult mutant ovaries (Fig. 9*E*). Serum estradiol levels were not significantly different (Fig. 9*F*), but progesterone levels were suppressed in *Dicer* cKO mutants compared with those in control mice (Fig. 9*G*). Collectively these data indicate that *Dicer* cKO female mice display a hypoplastic reproductive tract and severely suppressed fertility.

Superovulation Rescues the Ovulation Defect in Dicer cKO Female Mice—Female mice null for *Fshb* or *Lhb* lack either FSH or LH, respectively, but retain the responsiveness to exogenous gonadotropins (68, 69). Thus, these null mice can be pharmacologically rescued in a superovulation assay. To test if reduced number of ovulations in cKO mice is secondary to suppressed gonadotropins, we injected immature female mice with equine chorionic gonadotropin (analog containing both FSH- and LH-like activities)/human chorionic gonadotropin (analog containing LH-like activity) and counted the number of eggs released into oviducts. *Dicer* cKO mice responded better than double null mice lacking both LH and FSH but less efficiently than control mice in this superovulation assay (Fig. 10, *A* and *B*). Histological analyses revealed CL were readily detectable in ovarian sections obtained from all three genotypes of hormone-treated mice (Fig. 10, *C–E*). The ovarian histology correlated well with induction of most of the gonadotropin-induced marker genes that showed a response in *Dicer* cKO mice nearly identical to those in PBS-injected control mice and better than that in gonadotropin-deficient double null mice (Fig. 10*F*). Together, these data confirm that *Dicer* cKO female mice retain the gonadotropin responsiveness and some of the ovarian defects are secondary to suppressed gonadotropins in these mutant mice.

DISCUSSION

Transcriptional regulation of pituitary gonadotropin subunits has been extensively studied using both *in vitro* and *in vivo* models. However, post-transcriptional regulation of these subunit genes is not well understood. DICER is an essential enzyme for biogenesis of mature miRNAs that are key players in post-transcriptional regulation and various other events in gene expression (75). Although several cell-specific *Dicer* loss-of-function mouse models with defective reproductive function have been generated (75), functional analysis of DICER specifically within pituitary gonadotrope lineage has not been tested. Here, we used a Cre-lox genetic inactivation strategy using the *bLhb-Cre* driver line and successfully deleted *Dicer* specifically within the gonadotrope lineage. Loss of DICER beginning around embryonic day (E) 16.5, when *bLhb-Cre* is first activated (67) in the mouse pituitary, did not affect gonadotropes *per se*, because gonadotropes could be routinely purified from *Dicer* cKO mouse pituitaries by flow sorting. Whether pituitary lineage specification would be affected by deleting *Dicer* using other gonadotrope-specific Cre lines in which Cre recombinase is expressed earlier than E16.5 (89, 90) remains to be tested.

Loss of DICER selectively in gonadotropes resulted in suppression of all of the miRNAs computationally predicted to target gonadotropin subunit-encoding mRNAs. It is interesting to note that many of the predicted miRNAs target the *Fshb* mRNA 3′-UTR region that is strikingly longer compared with that of *Cga* and *Lhb* mRNAs. The observation that this long 3′-UTR of *Fshb* is the target for binding of many miRNAs and consequently post-transcriptional regulation is consistent with our previous *in vivo* studies in which we expressed human *FSHB* transgenes carrying different deletions within the 3′ region and analyzed their regulation within mouse gonadotropes (91). It is likely that miRNAs respond to multiple signaling networks (GnRH, activins, bone morphogenetic proteins) and orchestrate the coordinated regulation of gonadotropin subunits within gonadotropes. Delineating which sets of miRNAs respond to each of these signaling pathways will be

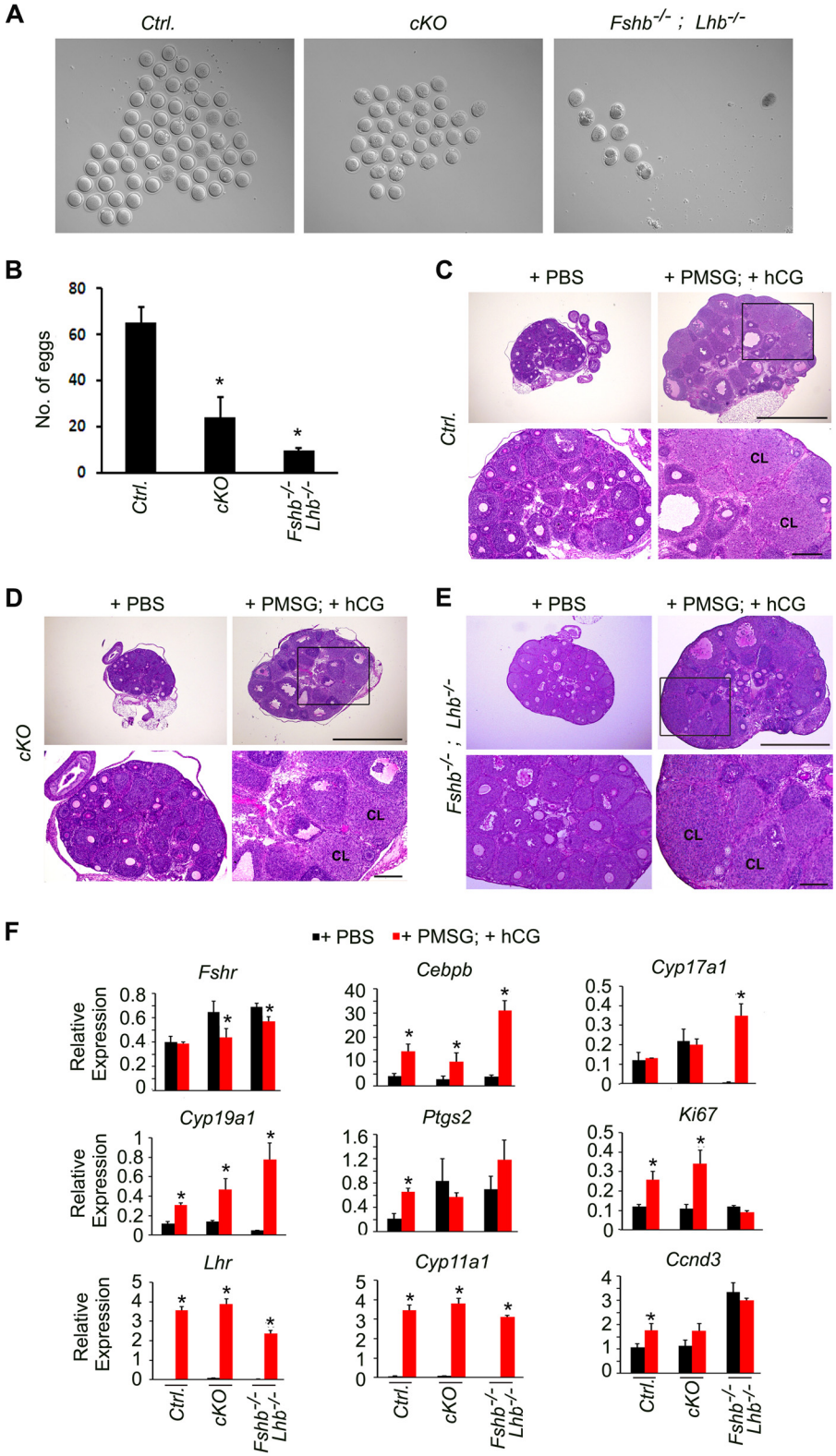


FIGURE 10. **Pharmacological rescue of *Dicer* cKO female mice.** Superovulation of immature female mice shows *Dicer* cKO mice respond to exogenous hormones (*A*). The response, quantified by number of eggs released (*B*), was better than in mice lacking both FSH and LH but not as efficient as that in control mice. In (*C–E*), ovarian histology confirmed presence of CL in superovulated compared with PBS-injected mice belonging to all genotypes, *Ctrl*, *Dicer* cKO, and *Fshb Lhb* double null mice. The *black bar* represents 50 μm. Ovarian gene expression analysis by Taqman real time PCR assays (*F*) provided further evidence that all genotypes of mice similarly responded to exogenous hormones compared with the corresponding PBS-injected controls. The only exception was *Ki67*, which was not significantly induced by hormone treatment in *Fshb Lhb* double null mice. *, $p < 0.05$ *versus* PBS-injected group.

critical for future understanding of gonadotropin secretion dynamics that plays a fundamental role in gonad development and gametogenesis.

We found gender-specific differences in transcriptional regulation of gonadotropin β subunits in the absence of DICER in gonadotropes. In the cKO male, basal levels of all gonadotropin subunit mRNAs were suppressed, whereas in the cKO female basal levels of only *Cga* and *Lhb* but not *Fshb* mRNA were suppressed. This suggests a female-specific post-transcriptional mechanism of *Fshb* gene regulation. This gender-specific regulation could also be due to differences in either the local growth factor signaling pathways within gonadotropes of male *versus* female or differences in negative feedback control because of the presence of estrous cycles in the female.

Our expression data on transcriptional repressors and activators is consistent with a model wherein a balance between DICER-dependent miRNAs and repressors/activators is critical for gonadotropin subunit protein synthesis (Fig. 11). This model based on our data suggests an indirect mechanism in which simultaneous up-regulation of repressors and loss of activators contributes to suppression of *Cga*, *Lhb*, and *Fshb* mRNAs and the corresponding subunit proteins in pituitaries of *Dicer* cKO male mice. This could be the net affect, although it cannot be ruled out that DICER-dependent miRNAs could also directly bind to individual gonadotropin subunit miRNAs. A similar mechanism could be invoked to explain the suppression of common α and LHβ subunit proteins in the pituitaries of female *Dicer* cKO mice. Interestingly, *Fshb* mRNA levels unlike *Cga* and *Lhb* mRNAs were not suppressed in female cKO mutants nor they were suppressed in *Esr1*$^{-/-}$ mice. These observations suggest it is likely that yet unknown DICER-independent miRNAs (76) could at least in part contribute to suppression of FSHβ synthesis in female mice (Fig. 11). This mechanism needs to be tested further in the future.

Irrespective of the gender-specific differences in *Fshb* gene regulation at the transcriptional or post-transcriptional level, the post-gonadectomy increase in serum gonadotropins was completely abolished in the absence of DICER in gonadotropes of both sexes. Similarly, acute GnRH treatment that normally results in robust LH release in control mice was abolished in *Dicer* cKO mice. Thus, our studies uncover that DICER-dependent miRNAs play an important role in post-gonadectomy rise in gonadotropins. Whether DICER-dependent miRNAs also play critical roles in temporal regulation of gonadotropin synthesis and secretion throughout the estrous cycle in the female will need to be investigated further. Our future studies will examine how other local signaling pathways regulated by TGF-β members, particularly activins and their signaling components, were affected in *Dicer* cKO mice.

Loss of DICER in gonadotropes resulted in suppressed gonadotropins and altered reproductive function of *Dicer* cKO male mice. The male reproductive phenotypes of *Dicer* cKO mice are reminiscent of those in *Fshb* null males (68) but less severe than those in *Lhb* null mice (69). The majority of *Dicer* cKO mutant males demonstrated a moderate reduction in testis size, sperm number, litter size, and thus a sub-fertility phenotype. In addition, testes in the majority of the mutant males (18 of 20; 90%) contained atypical tubules with germ cell

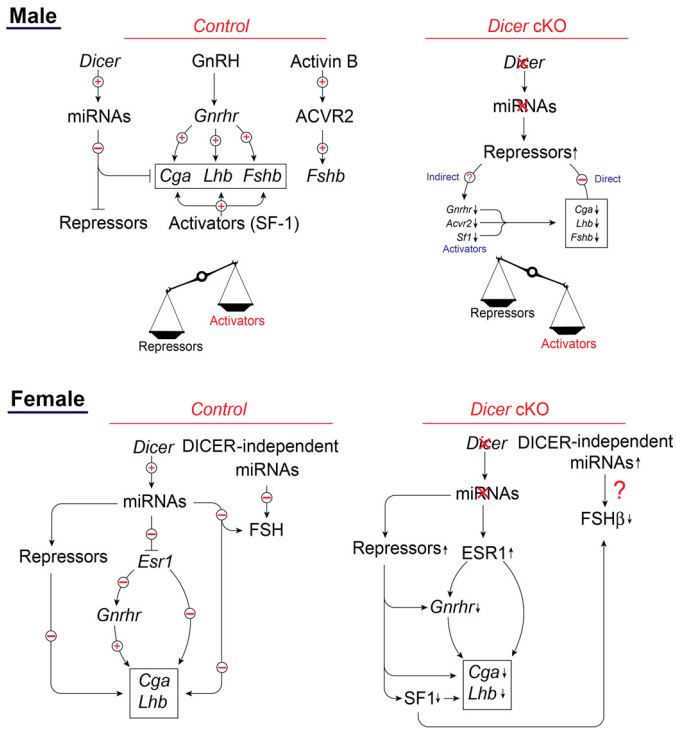


FIGURE 11. **A model for miRNA- mediated regulation of gonadotropin subunit expression.** In pituitaries of control male mice, DICER-dependent miRNAs negatively regulate repressors that normally suppress gonadotropin subunit expression. MicroRNAs can also directly bind to the 3′-UTR regions of gonadotropin subunit mRNAs and regulate them directly. GnRH acts via GnRH receptors on gonadotropes and controls expression of gonadotropin subunits. Locally produced activins, primarily activin B, acts via activin receptor type 2 (ACVR2) expressed on gonadotropes and regulates *Fshb* expression. SF-1 is one of the key transcriptional activators that regulates all gonadotropin subunit genes. A balance between activators and repressors ultimately dictates the coordinated regulation of gonadotropin subunit expression. In *Dicer* cKO mutants, in the absence of DICER-dependent miRNAs, repressors get up-regulated in pituitary gonadotropes and down-regulate expression of gonadotropins in two ways. They can bind *Cga*, *Lhbv* and *Fshb* directly and suppress their transcription. Alternatively, they can down-regulate the expression of activator pathways (*Gnrhr*, *Acvr2*, and *Sf1*), which in turn results in suppression of gonadotropin subunit expression. Thus, in the absence of DICER-dependent miRNAs the balance shifts toward up-regulation of repressors resulting in suppression of gonadotropins. In pituitaries of control female mice, DICER-dependent miRNAs directly bind gonadotropin subunit mRNAs or suppress expression of various repressors including *Esr1*. ESR1 binds to promoters of *Gnrhr*, *Cga*, and *Lhb* and negatively regulates their expression. *Fshb* could also be negatively regulated by DICER-independent miRNAs. In the absence of DICER-dependent miRNAs, repressors including ESR1 get up-regulated. Repressors can act at multiple levels including down-regulation of *Gnrhr*, *Sf1*, *Cga*, and *Lhb* by direct binding or indirectly via suppression of only activators such as *Sf1* and *Gnrhr*, which in turn cause a reduction in gonadotropin subunit expression. Loss of DICER-dependent miRNAs did not cause a reduction in *Fshb* mRNA in *Dicer* cKO female mice. Hence, an alternate mechanism to explain suppressed FSHβ protein could be that there are yet uncharacterized DICER-independent miRNAs (that bind *Fshb* mRNA) that get up-regulated and suppress FSHβ protein expression in female mutants. This proposed mechanism remains to be experimentally tested.

aplasia, confirmed by a lack of germ cell marker protein expression. Although Leydig cell marker expression and serum testosterone levels were unaffected in the majority of mutant males, only 50% of mutant males with moderate testis weight reduction showed elevated levels of testosterone. Suppression or the absence of gonadotropins alone may not account for these abnormal testes phenotypes, as male mice lacking either FSH (68) or LH (69) or both (92, 93) do not display this severe germ cell aplasia phenotype. Because *bLhb-Cre* transgene is residu-

ally active in mouse testes (67), we suspect that the above abnormal testis phenotypes could have emerged as a result of recombination events locally within testes. We are currently testing this possibility by generating *Dicer* conditional mutants using a more tightly regulated gonadotrope-specific *Cre* expressing mouse line in which *Cre* is not ectopically expressed in testis.

In contrast to variable reproductive phenotypes in *Dicer* cKO male mice, *Dicer* cKO female mice demonstrated hypoplastic uteri and ovaries. Their fertility was more severely suppressed with significantly reduced litter numbers and pups produced per litter. These observations reinforce that female reproductive function, unlike that in the male, is more sensitive to suppression or the absence of gonadotropins. Consistent with suppression of both FSH and LH levels, ovarian cycles were markedly impaired, and ovarian histology showed the presence of a rare corpus luteum in *Dicer* cKO female mice. These phenotypes were secondary to suppressed gonadotropins because exogenous gonadotropins pharmacologically rescued *Dicer* cKO female mice. It is interesting to note that *Dicer* cKO female mice responded to exogenous gonadotropins somewhat intermediately compared with similarly treated control and double mutants lacking both FSH and LH. Whether there are any differences in egg/embryo quality among these genotypes of mice remains to be examined further.

In summary, our studies indicate that gonadotrope-specific deletion of *Dicer* results in altered gonadotropin homeostasis and as a secondary consequence reproductive dysfunction leading to reduced fertility. Our studies have also unraveled a previously unrecognized role for DICER-dependent miRNA pathway in post-gonadectomy rise in gonadotropin synthesis and secretion. Identification of miRNAs that selectively target individual gonadotropin subunits would provide novel *in vivo* therapeutic reagents and provide us with a feasible approach to manipulate pituitary gonadotropin secretion as desired.

Acknowledgments—We thank Warren B. Nothnick for a critical reading of the manuscript, Brian Harfe for generously providing Dicer$^{f/f}$ mice, Irving Boime for providing recombinant FSH, antisera against FSH, and human chorionic gonadotropin, Albert Parlow for pituitary hormone standards and antisera, and George Enders, Ken Morohashi, Buck Hales, and Prabhakara Reddi for antibodies. The Ligand Assay and Analysis Core at the Center for Research in Reproduction at the University of Virginia received support from Eunice Kennedy Shriver NICHD, National Institutes of Health U54-(Specialized Cooperative Centers Program in Reproduction) Grant HD28934.

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