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

Huizhen Wang†, Ian Graham‡, Richard Hastings§, Sumedha Gunewardena¶, Michelle L. Brinkmeier⁎, P. Michael Conn†, Sally A. Camper†, and T. Rajendra Kumar∗‡§

From the Departments of †Molecular and Integrative Physiology, **Center for Reproductive Sciences, Institute for Reproductive Health and Regenerative Medicine, and §Flow Cytometry Core Laboratory, University of Kansas Medical Center, Kansas City, Kansas 66160, ‡Department of Molecular and Human Genetics, University of Michigan, Ann Arbor, Michigan 48109, and §Departments of Internal Medicine, Cell Biology, and Biochemistry, Texas Tech University, Lubbock, Texas 79430

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) 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.).
† To whom correspondence should be addressed: University of Kansas Medical Center, 3901 Rainbow Blvd., HLSIC Bldg. 3073, Kansas City, KS 66160. Tel.: 913-588-0414; E-mail: tkumar@kumc.edu.

2 The abbreviations used are: LH, luteinizing hormone; cKO, conditional knockout; CL, corpora lutea; CV, coefficient of variation; GnRH, gonadotrope-pinning hormone; miRNA, microRNA; qPCR, quantitative PCR; RT, Rosa tomato; TSH, thyroid-stimulating hormone; SF-1, steroidogenic factor-1.
Mice with a Deletion of Dicer in Gonadotropes

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 gonadotroph 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 ovaulation 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. 1A). Dicerflox/flox males, a generous gift from Dr. Brian Harfe (66) were first crossed with bLhb-Cre+ female mice (67) to obtain DicerΔ/Δ Cre+ mice. These mice were subsequently intercrossed to finally generate DicerΔ/Δ Cre++ mice, hereafter referred to as Dicercko mice. In the subsequent rounds of breeding, we also set up DicerΔ/Δ Cre−/++ mice with DicerΔ/Δ Cre++ mice to increase the frequency of generating the desired Dicercko mice. To genetically label gonadotropes in vivo, we set up DicerΔ/Δ Cre++ males with ROSATOMATO (Gt(Rosa)26Sor<tm4-(ACTB-tdTomato,-EGFP) Luo>) (The Jackson Laboratory), referred to as RT mice, and eventually generated DicerΔ/Δ Cre++ RTKI/KI or DicerΔ/Δ Cre++ RTKI/KI mice. Mice lacking either Fshb (68) or Lhb (69) or both were used as controls. ErsfΔ/+ mice were purchased from The Jackson Laboratory, Bar Harbor, ME and intercrossed to generate Ersf−/− 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 AriaII flow sorter (BD Biosciences). A UV laser at 488 nm was used to excite GFP protein. The GFP+ fraction representing the gonadotrophs 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 miRNA, mRNA, 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
Mice with a Deletion of Dicer in Gonadotropes

Sensitivity of LH was measured by a sandwich immunoradiometric assay (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.

Epipodymal 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).
Mice with a Deletion of Dicer in Gonadotropes

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. 1A). 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. 1B). 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. 1C). 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. 1D). 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 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. 3A). As expected, the expression of gonadotropin subunit mRNAs was nearly undetectable in the GFP− negative non-gonadotrope population of cells (Fig. 3A) 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. 3B) revealed that expression of only gonadotropin subunits, i.e. FSHβ and LHβ, but not other non-gonadotrope markers, i.e. TSHβ (thyrotropes), adrenocorticotropic hor-
Collectively, these results indicate that loss of thesis and secretion in the absence of DICER-dependent removes gonadal negative feedback, affects gonadotropin synthesis and secretion in the male (1, 10, 11). We next tested whether castration, which serves as key trophic hormones for male fertility. FSH regulates Sertoli and germ cell number, and sperm (68). LH is required for post-castration rise in gonadotropin synthesis and secretion in male mice.

**Mice with a Deletion of Dicer in Gonadotropes**

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. 3D). 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. 3D). 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. 3E). 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. 3E). 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. 3F).

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. 3G). 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 miRNAs, 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 miRNAs encoding transcriptional repressors. In the absence or severe suppression of a set of these miRNAs in purified gonadotropes (Fig. 4A), 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. 4C) in the pituitaries of Dicer cKO mice compared with those in control mice. Other known repressor-encoding mRNAs such as Mss1, 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 Acrv2 but not Foxl2 mRNAs (Fig. 4D and data not shown) and SF-1 (Fig. 4E) 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...
Mice with a Deletion of Dicer in Gonadotropes

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 (GFPe) and non-gonadotrope (GFPe) cells were obtained from the pituitaries of adult male control and Dicer cKO mice. Loss of Dicer in gonadotropes led to suppression of all three gonadotropin subunit mRNAs compared with that in corresponding controls (p < 0.01). Note that GFPe non-gonadotrope cells do not express gonadotropin subunits, confirming the validity of the flow sorting to enrich GFPe 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 (TSHβ), 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+/−), 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 and ACTH, prolactin, GH, and TSH were measured by RIA in intact and castrated Ctrl and Dicer cKO male mice were measured by RIA. 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 RIA 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 Ppiil 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. 5F). 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. 5C) and histology of cauda epididymis showed a significantly lower number of sperm in mutants (Fig. 5F). 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. 5D). 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. 5E).
Mice with a Deletion of Dicer in Gonadotropes

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. 5G). 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. 5G). 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. 5G), 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. 5G). 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. 5H). Consistent with the Leydig cell marker expression, serum testosterone levels were not significantly different in Dicer cKO mutants compared with those in controls (Fig. 5I). However, 50% of mutant males in which testes size was moderately affected showed elevated serum testosterone (Fig. 5I). 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. 5I). Interestingly, immunofluorescence of gross testis from mutants on an RT genetic background did confirm that Cre was active locally within the testis (Fig. 6A), and expression of Dicer was significantly reduced in the testes of Dicer cKO male mutants (Fig. 6B).

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. 7A, middle and right panels), Fshb mRNA was unaffected in pituitary in the absence of Dicer (Fig. 7A, left panel) compared with controls. As expected, gonadotropin subunit-encoding mRNAs were undetectable in GFP− non-gonadotropes (Fig. 7A). 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. 7B). In contrast to LH expression, although Fshb RNA levels remain unchanged, the corresponding protein was undetectable in pituitary sections obtained from Dicer cKO male mice compared with that in control sections (Fig. 7B). 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 non-gonadotropes (Fig. 7C) 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.

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 Gnhr and Acrv2 mRNAs that encode two major signaling pathway receptors required for activation of gonadotropon 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.
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. 7D), an increase in the corresponding gonadotropin β-subunit proteins in pituitaries determined by Western blot analysis (Fig. 7E), and resulted in elevated serum gonadotropin levels controls (Fig. 7F). 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.
Mice with a Deletion of Dicer in Gonadotropes

Altered Expression of Transcriptional Activators and Repressors in Pituitaries of Dicer cKO Female Mice—Loss of DICER-dependent miRNAs in the pituitaries of female Dicer cKO 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 assessed 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. 8A). 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. 8B). Moreover, in pituitaries of female Esr1−/− mice, Cga and Lhb, but not Fshb mRNAs, were significantly up-regulated by real time qPCR (Fig. 8C) 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 Gnhr and Nr5a1 (encodes SF-1) mRNAs were both significantly suppressed (Fig. 8D). 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. 8E). 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. 9A). 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. 9B) and litter size, i.e. number of pups per litter (Fig. 9C). 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+/-) 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+/- mice. Abnormal morphology with mostly Sertoli cell-only like phenotype (black star) was also apparent in some tubules within the same testis sections. Consistent with the tests phenotype, very few sperm were present in the epididymis of mutant males compared with control groups of mice (F, lower panels). G. expression of tests markers was shown. Immunolocalization was done on formalin-fixed testis sections using antibodies against Sertoli (SOX9), PAN germ cell (GCNA1), undifferentiated spermatogonia (PLZF; white arrow), mitotic germ cell (pHistone H3), and round spermatid (SP10)-specific markers. Note that histologically normal tubules in tests of Dicer cKO mouse express all the testis cell markers similar to those in control sections. However, abnormal tubules in Dicer cKO mouse tests 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 tests (H). Taqman real time qPCR assays (I) indicate that Sertoli and Leydig cell mRNAs were aberrantly regulated in the tests 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 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.

7F). Thus, DICER-dependent miRNAs in gonadotropes are essential for ovariectomy-induced up-regulation of gonadotropin in female mice.
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.
Mice with a Deletion of Dicer in Gonadotropes

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

(Fig. 9D). However, we rarely found CL in ovarian sections obtained from 9 of 10 (90%) age-matched Dicer cKO females (Fig. 9D). 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. 9D). 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. 9E). Serum estradiol levels were not significantly different (Fig. 9F), but progesterone levels were suppressed in Dicer cKO mutants compared with those in control mice (Fig. 9G). 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. 10F). 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
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 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-
Mice with a Deletion of Dicer in Gonadotropes

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 gonadotropes-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 gonadotrope 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. Notchick for a critical reading of the manuscript, Brian Harfe for generously providing Dicer Thy1 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 Mora-hashi, 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 US4-(Specialized Cooperative Centers Program in Reproduction) Grant HD28934.

REFERENCES

1. Bousfield, G. R., Jia, L., and Ward, D. N. (2006) Gonadotropins: chemistry and biosynthesis. In Kobil and Neil's Physiology of Reproduction (Neill, J. D., ed.) pp 1581–1624, 3rd Ed., Elsevier Press, New York.
2. Pierce, J. G., and Parsons, T. F. (1981) Glycoprotein hormones: structure and function. Annu. Rev. Biochem. 50, 465–495.
3. Davis, S. W., Castinetti, F., Carvalho, L. R., Ellsworth, B. S., Potok, M. A., Lyons, R. H., Brinkmeier, M. L., Raetzman, L. T., Carninci, P., Mortensen, A. H., Hayashizaki, Y., Arnhold, I. J., Mendonça, B. B., Brue, T., and Camper, S. A. (2010) Molecular mechanisms of pituitary organogenesis: in search of novel regulatory genes. Mol. Cell. Endocrinol. 323, 4–19.
4. Drouin, J. (2006) Molecular mechanisms of pituitary differentiation and regulation: implications for hormone deficiencies and hormone resistance syndromes. Front. Horm. Res. 35, 74–87.
5. Mollard, P., Hodson, D. J., Lafont, C., Rizzoti, K., and Drouin, J. (2012) A tridimensional view of pituitary development and function. Trends Endocrinol. Metab. 23, 261–269.
6. Watkins-Chow, D. E., and Camper, S. A. (1998) How many homeobox genes does it take to make a pituitary gland? Trends Genet. 14, 284–290.
7. Zhu, X., Gleiberman, A. S., and Rosenfeld, M. G. (2007) Molecular physiology of pituitary development: signaling and transcriptional networks. Physiol. Rev. 87, 933–963.
8. Zhu, X., Lin, C. R., Prefontaine, G. G., Tollkuhn, J., and Rosenfeld, M. G. (2005) Genetic control of pituitary development and hypopituitarism. Curr. Opin. Genet. Dev. 15, 332–340.
9. Japón, M. A., Rubinstein, M., and Low, M. J. (1994) In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. J. Histochem. Cytochem. 42, 1117–1125.
10. Ciccone, N. A., and Kaiser, U. B. (2009) The biology of gonadotropin regulation. Curr. Opin. Endocrinol. Diabetes Obes. 16, 321–327.
11. Gregory, S. J., and Kaiser, U. B. (2004) Regulation of gonadotropins by inhibin and activin. Semin. Reprod. Med. 22, 253–267.
12. Thompson, I. R., and Kaiser, U. B. (2014) GnRH pulse frequency-dependent differential regulation of LH and FSH gene expression. Mol. Cell. Endocrinol. 385, 28–35.
13. Fortin, J., Boehm, U., Deng, C. X., Treier, M., and Bernard, D. J. (2014) Follicle-stimulating hormone synthesis and fertility depend on SMAD4 and FOXL2. FASEB J. 28, 3396–3410.
14. Bilezikjian, L. M., Justice, N. J., Blackler, A. N., Wiater, E., and Vale, W. W. (2012) Cell-type specific modulation of pituitary cells by activin, inhibin, and follistatin. Mol. Cell. Endocrinol. 359, 43–52.
15. Melamed, P., Kadir, M. N., Wijeweera, A., and Seah, S. (2006) Transcription of gonadotropin β subunit genes involves cross-talk between the transcription factors and co-regulators that mediate actions of the regulatory hormones. Mol. Cell. Endocrinol. 252, 167–183.
16. Bakke, M., Zhao, L., and Parker, K. L. (2001) Approaches to define the role of SF-1 at different levels of the hypothalamic-pituitary-steroidogenic organ axis. Mol. Cell. Endocrinol. 179, 33–37.
17. Xie, H., Cherrington, B. D., Meadows, J. D., Witham, E. A., and Mellon, P. L. (2013) Msx1 homeodomain protein represses the egsu and GnRH receptor genes during gonadotrope development. Mol. Endocrinol. 27, 422–436.
18. Arriola, D. J., Mayo, S. L., Skarra, D. V., Benson, C. A., and Thackray, V. G. (2012) FOXO1 transcription factor inhibits luteinizing hormone β gene expression in pituitary gonadotrope cells. J. Biol. Chem. 287, 33424–33435.
19. Bernard, D. J., Fortin, J., Wang, Y., and Lamba, P. (2010) Mechanisms of FSH synthesis: what we know, what we don’t, and why you should care. Fertil. Steril. 93, 2465–2485.
20. Coss, D., Mellon, P. L., and Thackray, V. G. (2010) A FoxF1 in the Smad house: activin regulation of FSH synthesis: what we know, what we don’t, and why you should care. Fertil. Steril. 93, 2465–2485.
21. Salisbury, T. B., Binder, A. K., and Nilson, J. H. (2008) Welcoming the TALE homeodomain proteins Pbx1 and Pbx2 to the FSH synthesis: what we know, what we don’t, and why you should care. Fertil. Steril. 93, 2465–2485.
22. Thackray, V. G., Mellon, P. L., and Coss, D. (2010) Hormones in synergy: regulation of the pituitary gonadotropin genes. Mol. Cell. Endocrinol. 314, 192–203.
23. Li, H., Xi, Q., Xiong, Y., Cheng, X., Qi, Q., Yang, L., Shu, G., Wang, S., Wang, L., Gao, P., Zhu, X., Jiang, Q., Zhang, Y., and Yuan, L. (2011) A comprehensive expression profile of microRNAs in porcine pituitary. PLoS ONE 6, e24883.
24. Zhang, L., Cai, Z., Wei, S., Zhou, H., Zhou, H., Jiang, X., and Xu, N. (2013) MicroRNA expression profiling of the porcine developing hypothalamus and pituitary tissue. Int. J. Mol. Sci. 14, 20326–20339.
25. Carroll, R. S., Corrigan, A. Z., Vale, W., and Chin, W. W. (1991) Activin stabilizes follicle-stimulating hormone-β messenger ribonucleic acid levels. Endocrinology 129, 1721–1726.
26. Bailey, J. S., Rave-Harel, N., McGillivray, S. M., Coss, D., and Mellon, P. L. (2004) Activin regulation of the follicle-stimulating hormone β-subunit gene involves Smads and the TALE homeodomain proteins Pbx1 and
Mice with a Deletion of Dicer in Gonadotropes

Prep1. Mol. Endocrinol. 18, 1158–1170
27. Bernard, D. J. (2004) Both SMAD2 and SMAD3 mediate activin-stimulated expression of the follicle-stimulating hormone β subunit in mouse gonadotrope cells. Mol. Endocrinol. 18, 606–623
28. Graham, K. E., Nusser, K. D., and Low, M. J. (1999) LβT2 gonadotrophs secrete follicle stimulating hormone (FSH) in response to activin A. J. Endocrinol. 162, R1–R5
29. Hanson, H. J., Sebastian, J., Strahl, B. D., Wu, J. C., and Miller, W. L. (2001) Transcriptional regulation of the ovine follicle-stimulating hormone-β gene by activin and gonadotropin-releasing hormone (GnRH): involvement of two proximal activator protein-1 sites for GnRH stimulation. Endocrinology 142, 2267–2274
30. Jacobs, S. B., Coss, D., McGillivray, S. M., and Mellon, P. L. (2003) Nuclear factor Y and steroidogenic factor 1 physically and functionally interact to contribute to cell-specific expression of the mouse Follicle-stimulating hormone-β gene. Mol. Endocrinol. 17, 1470–1483
31. Pernasetti, F., Vasilyev, V. V., Rosenberg, S. B., Bailey, J. S., Huang, H. J., Pernasetti, F., Vasilyev, V. V., Rosenberg, S. B., Bailey, J. S., Huang, H. J., and Baum, D. J. (2014) MicroRNAs as biomarkers for pituitary adenoma pathogenesis. J. Endocrinol. Invest. 162, R1–R5
32. Pernasetti, F., Vasilyev, V. V., Rosenberg, S. B., Bailey, J. S., Huang, H. J., Pernasetti, F., Vasilyev, V. V., Rosenberg, S. B., Bailey, J. S., Huang, H. J., and Baum, D. J. (2014) MicroRNAs as biomarkers for pituitary adenoma pathogenesis. J. Endocrinol. Invest. 162, R1–R5
33. Suszko, M. I., Lo, D. J., Suh, H., Camper, S. A., and Woodruff, T. K. (2003) Identification of differentially expressed microRNAs by microarray: a possible role for microRNA genes in pituitary adenomas. Mol. Endocrinol. 162, R1–R5
34. Weiss, J., Guendner, M. J., Halvorson, L. M., and Jameson, J. L. (1995) Regulation of the rat follicle-stimulating hormone subunit promoter by activin. Mol. Endocrinol. 17, 318–332
35. West, B. E., Parker, G. E., Savage, J. J., Kiratipranon, P., Toomey, K. S., Beach, L. R., Colvin, S. C., Sloop, K. W., and Rhodes, S. J. (2004) Regulation of the follicle-stimulating hormone-β gene by the LH3XH LHR-homodimer transcription factor. Endocrinology 145, 4666–4679
36. Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297
37. Bartel, D. P. (2009) MicroRNAs: target recognition and regulatory functions. Cell 136, 215–233
38. Hannon, G. J., Rivas, F. V., Murchison, E. P., and Stentz, J. A. (2006) The expanding universe of noncoding RNAs. Cold Spring Harb. Symp. Quant. Biol. 71, 551–564
39. He, L., and Hannon, G. J. (2004) MicroRNAs: small RNAs with a big role in gene regulation. Nat. Rev. Genet. 5, 522–531
40. Murchison, E. P., and Hannon, G. J. (2004) miRNAs on the move: miRNA and microRNA processing enzyme Dicer in POMC-expressing cells leads to pituitary dysfunction, neurodegeneration and development of obesity. Mol. Metab. 2, 74–85
41. Bottoni, A., Zatelli, M. C., Ferracin, M., Tagliati, F., Piccin, D., Vignali, C., Calin, G. A., Negrini, M., Croce, C. M., and Dei Uberti, E. C. (2007) Identification of differentially expressed microRNAs by microarray: a possible role for microRNA genes in pituitary adenomas. J. Cell. Physiol. 210, 370–377
42. Di Ieva, A., Butz, H., Niamah, M., Rotondo, F., De Rosa, S., Sav, A., Yousef, G. M., Kovacs, K., and Cusimano, M. D. (2014) MicroRNAs as biomarkers in pituitary tumors. Neurosurgery 75, 181–189; discussion 188–189
43. Gadela, M. R., Kasuki, L., Dénès, J., Trivelin, G., and Korbonits, M. (2013) MicroRNAs: suggested role in pituitary adenoma pathogenesis. J. Endocrinol. Invest. 36, 889–895
44. Godoy, J., Nishimura, M., and Webster, N. J. (2011) Gonadotropin-releasing hormone induces miR-132 and miR-212 to regulate cellular morphology and migration in immortalized LβT2 pituitary gonadotrope cells. Mol.
Mice with a Deletion of Dicer in Gonadotropes

Pitx2 deletion in pituitary gonadotropes is compatible with gonadal development, puberty, and fertility. *Genesis* **46**, 507–514

Kumar, T. R., Wang, Y., Lu, N., and Matzuk, M. M. (1997) Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat. Genet.* **15**, 201–204

Ma, X., Dong, Y., Matzuk, M. M., and Kumar, T. R. (2004) Targeted disruption of luteinizing hormone beta subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility. *Proc. Natl. Acad. Sci. U.S.A.* **101**, 17294–17299

Kumar, T. R., and Low, M. J. (1995) Hormonal regulation of human follicle-stimulating hormone beta subunit gene expression. *Mol. Endocrinol.* **9**, 628–637

Wang, H., Larson, M., Jablonka-Shariff, A., Pearl, C. A., Miller, W. L., Conn, P. M., Boime, I., and Kumar, T. R. (2014) Redirecting intracellular trafficking and the secretion pattern of FSH dramatically enhances ovarian function in mice. *Proc. Natl. Acad. Sci. U.S.A.* **111**, 5735–5740

Panneerdoss, S., Chang, Y. F., Buddavarapu, K. C., Chen, H. I., Shetty, G., Wang, H., Chen, Y., Kumar, T. R., and Rao, M. K. (2012) Androgen-responsive microRNAs in mouse Sertoli cells. *PloS ONE* **7**, e41146

Kumar, T. R., Fairchild-Huntress, V., and Low, M. J. (1992) Gonadotrope-specific expression of the human follicle-stimulating hormone beta subunit gene in pituitaries of transgenic mice. *Mol. Endocrinol.* **6**, 81–90

Kumar, T. R., and Low, M. J. (1993) Gonadal steroid hormone regulation of human and mouse follicle stimulating hormone beta subunit gene expression *in vivo*. *Mol. Endocrinol.* **7**, 898–906

Foulkes, W. D., Priest, J. R., and Duchaine, T. F. (2014) DICER1: mutations, microRNAs and mechanisms. *Nat. Rev. Cancer* **14**, 662–672

Ha, M., and Kim, V. N. (2014) Regulation of microRNA biogenesis. *Nat. Rev. Mol. Cell Biol.* **15**, 509–524

Lau, E. (2014) Non-coding RNA: interrogating miRNA-target interactions *in vivo*. *Nat. Rev. Genet.* **15**, 642

Shenoy, A., and Blblloch, R. H. (2014) Regulation of microRNA function in somatic stem cell proliferation and differentiation. *Nat. Rev. Mol. Cell Biol.* **15**, 565–576

Kumar, T. R., Varani, S., Wreford, N. G., Telfer, N. M., de Kretser, D. M., and Matzuk, M. M. (2001) Male reproductive phenotypes in double mutant mice lacking both FSHβ and activin receptor IIA. *Endocrinology* **142**, 3512–3518

Wreford, N. G., Rajendra Kumar, T., Matzuk, M. M., and de Kretser, D. M. (2001) Analysis of the testicular phenotype of the follicle-stimulating hormone beta subunit knockout and the activin type II receptor knockout mice by stereological analysis. *Endocrinology* **142**, 2916–2920

Koopman, P. (2001) Sry, Sox9, and mammalian sex determination. *EXS* **91**, 25–56

McClelland, K., Bowles, J., and Koopman, P. (2012) Male sex determination: insights into molecular mechanisms. *Asian J. Androl.* **14**, 164–171

Svingen, T., and Koopman, P. (2013) Building the mammalian testis: origins, differentiation, and assembly of the component cell populations. *Genes Dev.* **27**, 2409–2426

Hewitt, S. C., and Korach, K. S. (2003) Oestrogen receptor knockout mice: roles for oestrogen receptors α and β in reproductive tissues. *Reproduction* **125**, 143–149

Kumar, T. R. (2005) What have we learned about gonadotropin function from gonadotropin subunit and receptor knockout mice? *Reproduction* **130**, 293–302

Kumar, T. R. (2005) Gonadotropin gene targeting and biological implications. *Endocrine* **26**, 227–233

Lei, Z. M., Mishra, S., Zou, W., Xu, B., Foltz, M., Li, X., and Rao, C. V. (2001) Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. *Mol. Endocrinol.* **15**, 184–200

Zhang, F. P., Poutanen, M., Wilbertz, J., and Huhtaniemi, I. (2001) Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. *Mol. Endocrinol.* **15**, 172–183

Pérez-Millán, M. I., Zeidler, M. G., Saunders, T. L., Camper, S. A., and Davis, S. W. (2013) Efficient, specific, developmentally appropriate cre-mediated recombination in anterior pituitary gonadotropes and thyrotropes. *Genesis* **51**, 785–792

Wen, S., Schwarz, J. R., Niculescu, D., Dinu, C., Bauer, C. K., Hirdes, W., and Boehm, U. (2008) Functional characterization of genetically labeled gonadotropes. *Endocrinology* **149**, 2701–2711

Kumar, T. R., Schuff, K. G., Nusser, K. D., and Low, M. J. (2006) Gonadotropin gene targeting and biological implications. *Endocrinology* **147**, 103–115

O’Shaughnessy, P. J., Bennett, M. K., Scott, I. S., and Charlton, H. M. (1992) Effects of FSH on Leydig cell morphology and function in the hypogonadal mouse. *J. Endocrinol.* **135**, 517–525

O’Shaughnessy, P. J., Monteiro, A., Verhoeven, G., De Gendt, K., and Abel, M. H. (2010) Effect of FSH on testicular morphology and spermatogenesis in gonadotrophin-deficient hypogonadal mice lacking androgen receptors. *Reproduction* **139**, 177–184