Redirecting intracellular trafficking and the secretion pattern of FSH dramatically enhances ovarian function in mice

Huizhen Wang[a], Melissa Larson[a], Albina Jablonka-Shariff[b], Christopher A. Pearl[b], William L. Miller[c], P. Michael Conn[d], Irving Boime[b], and T. Rajendra Kumar[a,e,1]

Departments of [a]Molecular and Integrative Physiology and [e]Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City, KS 66160; [b]Department of Developmental Biology, Washington University School of Medicine, St. Louis, MO 63110; [c]Department of Molecular and Structural Biochemistry, North Carolina State University, Raleigh, NC 27695; and [d]Departments of Internal Medicine and Cell Biology and Biochemistry, Texas Tech University Health Sciences Center, Lubbock, TX 79430

Edited by R. Michael Roberts, University of Missouri, Columbia, MO, and approved February 28, 2014 (received for review November 14, 2013)

FSH and luteinizing hormone (LH) are secreted constitutively or in pulses, respectively, from pituitary gonadotropes in many vertebrates, and regulate ovarian function. The molecular basis for this evolutionarily conserved gonadotropin-specific secretion pattern is not understood. Here, we show that the carboxyterminal heptapeptide in LH is a gonadotropin-sorting determinant in vivo that directs pulsatile secretion. FSH containing this heptapeptide enters the regulated pathway in gonadotropes of transgenic mice, and is released in response to gonadotropin-releasing hormone, similar to LH. FSH released from the LH secretory pathway rescued ovarian defects in *Fshb*-null mice as efficiently as constitutively secreted FSH. Interestingly, the rerouted FSH enhanced ovarian follicle survival, caused a dramatic increase in number of ovulations, and prolonged female reproductive lifespan. Furthermore, the rerouted FSH vastly improved the in vivo fertilization competency of eggs, their subsequent development in vitro and when transplanted, the ability to produce offspring. Our study demonstrates the feasibility to fine-tune the target tissue responses by modifying the intracellular trafficking and secretory fate of a pituitary trophic hormone. The approach to interconvert the secretory fate of proteins in vivo has pathophysiological significance, and could explain the etiology of several hormone hyperstimulation and resistance syndromes.

protein sorting | regulated secretion | dense core granules | folliculogenesis

During vertebrate evolution, the female reproductive pattern underwent a remarkable transition from spawning of large number of eggs in primitive species under favorable conditions to more tightly controlled ovarian cycles in higher vertebrates, such that only a limited number (rodents) or a single (human and nonhuman primates) egg is released per cycle (1, 2). Coincident with this event, the single pituitary gonadotropic hormone that exists in primitive vertebrates has given rise to two gonadotropins, FSH and luteinizing hormone (LH), which coordinate gametogenesis and steroidogenesis (3–7). FSH and LH are heterodimeric glycoproteins that contain a common α-subunit and a hormone-specific β-subunit (3). Although synthesized in the same cell, the gonadotrope, FSH is mostly constitutively released in many species, whereas LH is stored in dense core granules (DCGs) and secreted in pulses via the regulated pathway in response to gonadotropin-releasing hormone (GnRH) (8, 9). Although this pattern is evolutionarily conserved, how distinct modes of gonadotropin secretion affect ovarian development and target cell responses remain unclear. Although models in which variations in secretion of gonadotropins have been achieved in vivo (10) and in vitro, including basolateral and apically polarized secretion (11), the in vivo consequences of altered mode of gonadotropin trafficking and release (constitutive vs. regulated) from pituitary are untested. We sought to identify why the two gonadotropins, LH and FSH, expressed in the same pituitary cell have evolved to exit via different routes to regulate ovarian physiology.

Results

Strategy to Redirect FSH from the Constitutive to Regulated Pathway. Our in vitro screens indicated that a carboxyterminal (C′)-heptapeptide in the human LHβ (LSGLLFL) (12) or a modified FSHβ containing this peptide (13) favors secretion of corresponding dimers via the regulated pathway in heterologous somatotrope cells. Based on these initial data, we engineered human transgenes encoding WT or mutant FSHβ containing the C′-heptapeptide (Fig. 1*A*), generated multiple lines of transgenic mice, and introduced transgenes representing three independent lines in each case separately onto *Fshb*-null background (14). This genetic rescue strategy facilitated monitoring of the intracellular behavior of only the human transgene-encoded WT or mutant FSH in the absence of endogenous mouse FSH (Fig. 1 *B*–*H*). We found the transgenes are appropriately targeted to gonadotropes and expressed in a sexually dimorphic pattern (Fig. 1 *D* and *E*) similar to FSH in pituitaries of control mice (Fig. 1 *C* and *F*). Western blot analysis under nonreducing conditions further confirmed that the interspecies FSH heterodimers (mouse α plus human FSHβ WT or mutant) are assembled and readily detected in pituitaries of transgene-expressing mice (Fig. 1 *G* and *H*). Based on similar levels of RNA and protein expression as those in control mice, we performed most of the analyses on two lines each, line 17 and line 31 expressing human $FSHB^{WT}$ and line 100 and line 300 expressing $FSHB^{Mut}$ transgenes, on *Fshb*-null genetic background.

Significance

Although the two pituitary gonadotropins luteinizing hormone (LH) and FSH are synthesized in the same cell, gonadotrope, their intracellular trafficking and secretion pattern are distinct. LH is released as pulses via the regulated pathway, whereas FSH is constitutively secreted. Why this hormone-specific pattern has evolved and whether the target organ, the ovary, senses specific hormone release pattern is not understood. In this paper, a gonadotrope-specific sorting determinant on LH has been identified, and FSH was diverted into the LH secretory pathway in vivo. FSH released like LH from pituitary dramatically enhanced the ovarian responses. Our studies provide a molecular basis for the evolution of distinct patterns of gonadotropin secretion and explain the origin of estrus cycles in mammals.

Author contributions: T.R.K. designed research; H.W., M.L., and T.R.K. performed research; A.J.-S., C.A.P., W.L.M., P.M.C., and I.B. contributed new reagents/analytic tools; H.W. and T.R.K. analyzed data; and T.R.K. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

[1]To whom correspondence should be addressed. E-mail: tkumar@kumc.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1321404111/-/DCSupplemental.

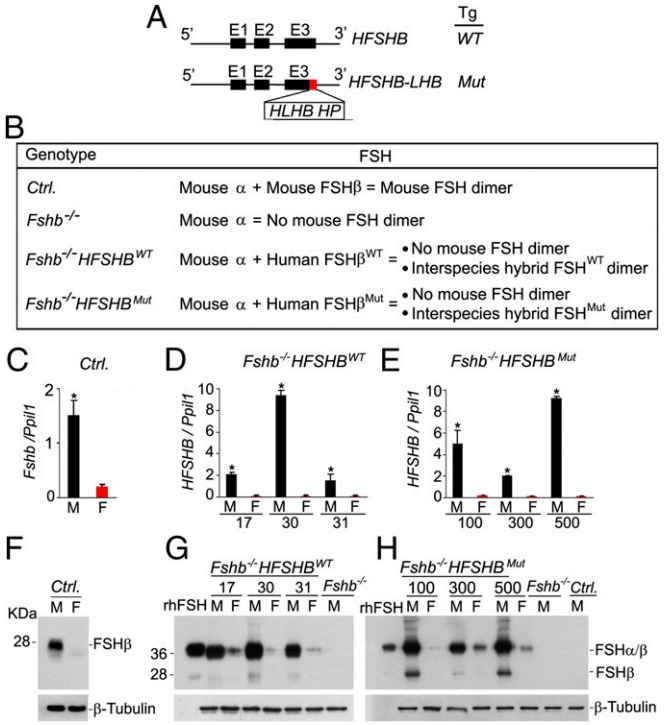

Fig. 1. Human *FSHB* transgenes are expressed in pituitaries. (*A*) *HFSHB* WT and mutant (Mut) transgene (*HFSHB-LHB*) are shown. The 3′ end of Mut gene is fused with the 21 nt of human *LHB* gene sequences (*HLHB HP*) that encode the C′-heptapeptide shown as a red box. (*B*) The genotypes and predicted FSH heterodimer subunit combinations are shown. (*C–E*) TaqMan real-time PCR assay by using mouse *Fshb* or human *FSHB* primers/probes on RNA from adult control WT FSH or mutant FSH transgene-expressing males (black bars) and females (red bars). *Ppil1* expression was used as an internal control for all real-time PCR assays. In each case, RNA from at least three adult mice was used in triplicate, and mean ± SEM values are presented (*$P < 0.01$, male vs. female). (*F–H*) Western blot analysis of pituitary proteins under nondenaturing conditions detects human FSH hybrid dimers by using a human FSH-specific mouse monoclonal 4B antibody that also detects uncombined FSHβ subunit. A goat polyclonal antiserum detects mouse FSHβ in *Ctrl* (*F*) mouse pituitary extracts under denaturing conditions. Recombinant human FSH (rhFSH) and *Fshb*$^{-/-}$ mouse pituitary extracts served as positive and negative controls, respectively, to confirm the specificity of the FSH antibodies. Expression of β-tubulin was used as loading control. F, female; M, male.

Secreted proteins that enter regulated pathway exit the trans-Golgi network into DCGs (15). First, we assessed the colocalization of FSHβ with chromogranin-A (Chr-A) (Fig. 2 *A–D*), a known chaperone protein in gonadotropes (16). Four to six times more gonadotropes ($P < 0.01$; $n = 250$ cells) showed colocalization of Chr-A with FSHβ in pituitary sections from *HSFHB*^{Mut} compared with that observed in control and *HSFHB*^{WT} mice (Fig. 2 *A* and *B*). This number was similar to that obtained with LHβ and Chr-A double labeling (Fig. 2 *C* and *D*).

Finally, we tested colocalization of FSHβ (Fig. 2 *E* and *F*) or LHβ (Fig. 2 *G* and *H*) with Rab27, a resident DCG-specific protein in gonadotropes (17). FSHβ and Rab27 were colocalized as discrete punctae in six to eight fold more gonadotropes of *HSFHB*^{Mut} mice compared with control or *HSFHB*^{WT} mouse pituitary sections ($P < 0.01$; $n = 250$ cells; Fig. 2 *E* and *F*). This pattern of Rab27 colocalization was similar to LHβ in gonadotropes of control mice (Fig. 2 *G* and *H*) and identical in all three independent lines of *HSFHB*^{Mut} mice. Collectively, these data indicate that mutant FSH is packaged into Rab27-positive DCGs and rerouted like LH to the regulated secretory pathway in gonadotropes.

Response of LH and FSH to acute GnRH is distinct; LH is released as a bolus, whereas FSH shows no response and exits constitutively from gonadotropes (8, 13). If the mutant FSH enters the regulated pathway, it should be released as a bolus in response to GnRH. We injected adult mice with buserelin, a stable GnRH agonist, and assayed serum FSH levels after 2 h by using an RIA that detects FSH in controls and the interspecies hybrid FSH heterodimer produced in *HFSHB* transgene expressing *Fshb*-null mice (18). FSH levels were elevated 2.5–4 times more in response to buserelin vs. baseline PBS

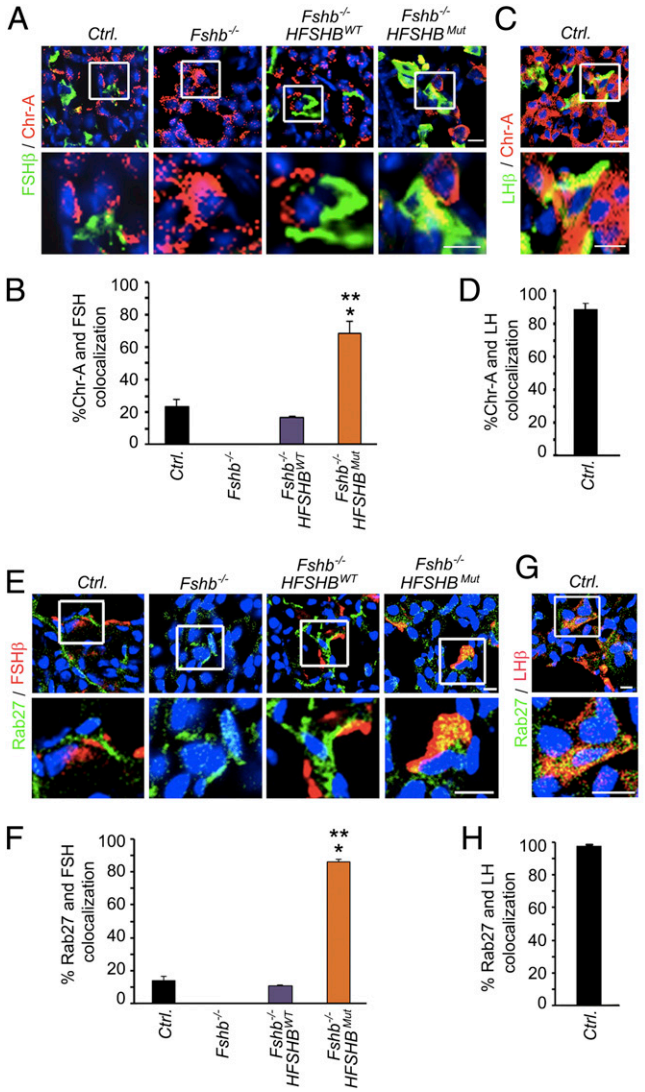

Fig. 2. Rerouted FSH enters the regulated pathway and is stored in DCGs in gonadotropes. (*A* and *C*) Confocal microscopy on pituitary sections from control (*Ctrl.*), *Fshb*$^{-/-}$, *Fshb*$^{-/-}$ *HFSHB*^{WT}, and *Fshb*$^{-/-}$ *HFSHB*^{Mut} mice dually labeled with Chr-A and an FSHβ (*A*) or LHβ (*C*) antibody. (*E* and *G*) Colocalization of FSH and Rab27, a DCG marker, was determined by confocal microscopy on pituitary sections from the same genotypes with a Rab27 antiserum and FSHβ 4B monoclonal antibody (*E*) or hCGβ (*G*) that detects LH. The nuclei are stained by TO-PRO-3 dye. The area represented by the white box in merged images has been enlarged and shown in *A*, *C*, *E*, and *F*. Approximately 250 cells from multiple pituitary sections of three mice per genotype were counted. (*B*, *D*, *F*, and *H*) The percentage of colabeled gonadotropes is represented as mean ± SEM (*$P < 0.001$ for *Fshb*$^{-/-}$ *HFSHB*^{Mut} vs. control or *Fshb*$^{-/-}$ and **$P < 0.001$ for *Fshb*$^{-/-}$ *HFSHB*^{WT} mice). (Scale bar: 25 μm.) Note the colocalization in mice expressing the rerouted FSH is nearly identical to that of LH in control sections (*C* and *I*).

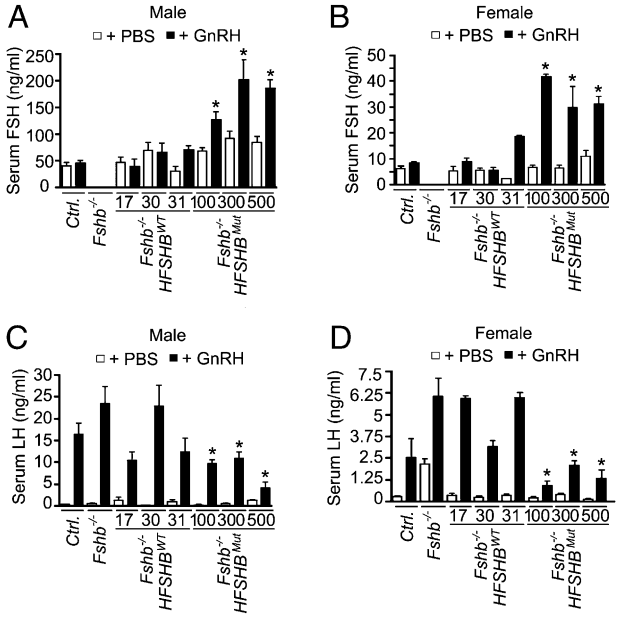

Fig. 3. Mutant FSH is released in response to acute GnRH treatment. Adult male (*A* and *C*) and female (*B* and *D*) mice were i.p. injected with PBS solution (150 μL per mouse) or the GnRH analog buserelin (2 μg/150 μL per mouse). GnRH response (dark bars) was measured after 3 h and compared with that in PBS solution controls (open bars) by serum RIAs for FSH (*A* and *B*) and LH (*C* and *D*). Note that mutant but not WT FSH is released in response to buserelin. Mice expressing mutant FSH have reduced basal LH levels and show a blunted response to acute GnRH analog treatment (*C* and *D*). Three independent lines expressing WT or mutant HFSHB transgenes on an *Fshb*-null background are used. Data are means ± SEM; $n = 7$–10 mice (*$P < 0.01$, GnRH response vs. PBS solution).

solution ($P < 0.01$; $n = 10$) in all three lines of $HSFHB^{Mut}$ male (Fig. 3*A*) and female (Fig. 3*B*) mice. Although basal FSH levels were higher in males than females ($P < 0.01$; $n = 10$), the ratio of FSH release in response to buserelin vs. PBS solution was significantly higher in females ($P < 0.01$; $n = 10$). In contrast, buserelin treatment did not cause any significant FSH release in control and $Fshb^{-/-}$ $HSFHB^{WT}$ male and female mice ($P > 0.05$; $n = 10$). Immunogold EM analysis with FSH-specific 4B monoclonal antibody further confirmed that the mutant FSH is stored in DCGs and is released upon acute GnRH treatment (*SI Appendix*, Fig. S1 *A* and *B*, arrows). Thus, mutant FSH enters the regulated pathway of sorting, is stored in DCGs in gonadotropes, and is released in response to acute GnRH treatment. This response mimics a characteristic pattern of LH storage and release seen in control mice.

Rerouting FSH into the Regulated Pathway Alters LH Packaging and Storage. If the rerouted FSH exits via regulated pathway, it may affect LH intracellular trafficking and homeostasis. To address this, we measured LH levels in the same serum samples analyzed for FSH. Significantly lower levels of LH were present in serum of mice expressing the mutant FSH, and less LH was released in response to acute GnRH treatment (Fig. 3 *C* and *D*). Expression analyses of pituitaries from mice expressing mutant FSH indicated comparable *Lhb* mRNA levels (*SI Appendix*, Fig. S2 *A* and *B*) but a reduced amount of LHβ protein (*SI Appendix*, Fig. S2 *D* and *E*). These LH levels are not significantly different from those in *Lhb* heterozygous mutant mice ($P > 0.05$ in *SI Appendix*, Fig. S2 *C* and *J*) and are not known to result in overt phenotypes (19), and thus explain the normal fertility of rerouted FSH-expressing mice (as detailed later). Moreover, the temporal secretion pattern of LH is not altered in mice expressing mutant FSH because all aspects of the estrus cycle stage-dependent LH regulation, mRNA (*SI Appendix*, Fig. S2*G*), protein (*SI Appendix*, Fig. S2*H*), and secretion (*SI Appendix*, Fig. S2*I*) are similar to those in mice expressing WT FSH. However, it is possible that rerouted FSH and LH compete for DCGs, and this could result in reduction in LH levels observed in mutant FSH-expressing mice. To test this, we reduced mutant FSH expression by deleting one allele of *activin receptor-2*, a key signaling pathway in gonadotropes required for FSH expression (20) (*SI Appendix*, Fig. S3 *C* and *E*; serum FSH levels are two to four times less when expressed on an $Acvr2^{+/-}$ genetic background vs. on an $Acvr2^{+/+}$ background), and this reduction in mutant FSH restored LH levels to control values (*SI Appendix*, Fig. S3 *B*, *D*, and *F*; serum LH levels are four times greater when expressed on an $Acvr2^{+/-}$ genetic background vs. on an $Acvr2^{+/+}$ background). These data provide direct genetic evidence that the intracellular trafficking of mutant FSH is identical to that of LH in the regulated pathway. The rerouted FSH alters the LH secretion dynamics, likely by competing for a limited number of secretory granules in gonadotropes.

Rerouted FSH Rescues *Fshb*-Null Mice. Loss of FSH results in female infertility as a consequence of folliculogenesis arrest at the preantral stage (18). We next examined if rerouted FSH released from the LH pathway can rescue female *Fshb*-null mice. At 9 wk of age, $Fshb^{-/-}$ females displayed hypoplastic ovaries and uteri (Fig. 4*A*) indicative of anovulatory and noncyclic phenotype compared with age-matched controls (18). In contrast, ovarian and uterine morphology was indistinguishable in controls, or $Fshb^{-/-}$ mice that express WT or mutant FSH (Fig. 4*A*). The presence of antral follicles and abundant corpora lutea (CL) in ovarian sections indicated normal estrus cycles were present in control and $Fshb^{-/-}$ mice expressing WT or mutant FSH (Fig. 4*B*). FSH regulates aromatase (*Cyp19a1*) that is required for estrogen production in granulosa cells (21). TaqMan quantitative PCR (qPCR) assays confirmed that *Cyp19a1* levels were suppressed in *Fshb*-null mice ($P < 0.01$; $n = 3$ mice) and restored to levels similar to those in control and $Fshb^{-/-}$ mice expressing WT or mutant FSH at 9 wk of age (Fig. 4*C*). Although serum estradiol levels were similar to control groups, progesterone levels were high in $Fshb^{-/-}$ mice that express mutant FSH (*SI Appendix*, Fig. S4) and breeding characteristics were similar between control, $Fshb^{-/-}$ $HSFHB^{WT}$, and $Fshb^{-/-}$ $HSFHB^{Mut}$ females (*SI Appendix*, Tables S1 and S2). Thus, rerouted FSH dimer functionally rescues *Fshb*-null females as efficiently as the constitutively secreted WT FSH. Mutant FSH also functionally rescued *Fshb*-null males similar to WT FSH (*SI Appendix*, Fig. S5 and Tables S1 and S2).

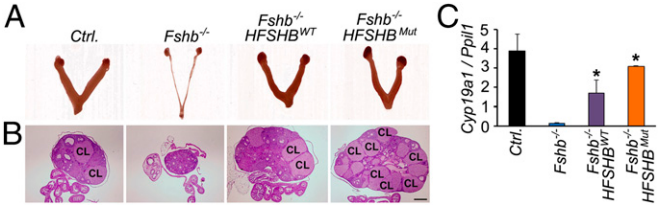

Fig. 4. Mutant FSH rescues *Fshb*-null female mice. (*A*) Morphology of ovaries and uteri from female mice at 9 wk of age shows rescue of *Fshb*-null females by WT and mutant FSH is indistinguishable. (*B*) Ovarian histology shows multiple CLs, indicating estrus cycles resumed normally. (Scale bar: 100 μm.) (*C*) Increased expression of aromatase (*Cyp19a1*) by mRNA TaqMan real-time qPCR assay further confirms the rescue. *Ppil1* mRNA expression was used as an internal control. Triplicate RNA samples from four to six mice per genotype were used for qPCR assays. Data are mean ± SEM. *Ppil1* encodes peptidyl-prolyl *cis-trans* isomerase-like 1.

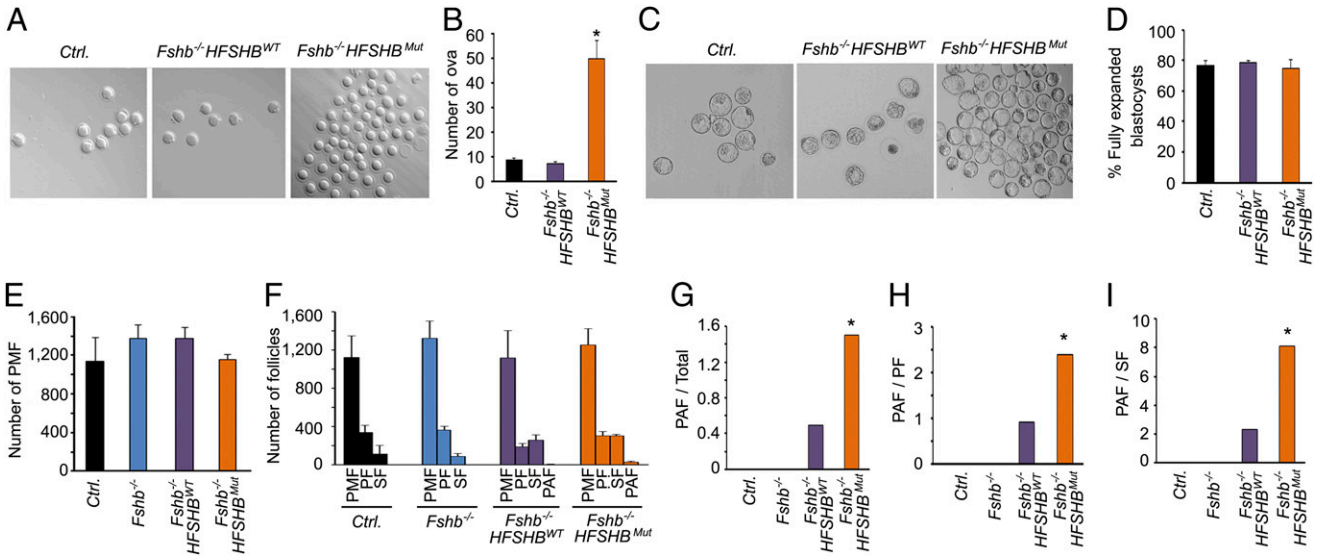

Fig. 5. Rerouted FSH increases ovulation efficiency as a result of enhanced follicle survival. (*A*) Morphology of fertilized one-cell embryos retrieved from oviducts of naturally mated mice with vaginal plugs. (*B*) Six times more one-cell embryos are present in oviducts of mutant FSH-expressing mice. (*C*) In vitro culture of fertilized eggs results in viable and apparently healthy blastocysts in all genotypes. (*D*) Quantification of fully expanded blastocysts reveals comparable efficiencies across all genotypes. All data are mean ± SEM; $n = 10$ mice per group (*$P < 0.01$ in *B* and *$P > 0.05$ in *D*). (*E*) PMF counts obtained from mice at 3 d of age are not different across genotypes, indicating pulsatile FSH does not increase the PMF pool. (*F*) Follicle counts in ovaries of mice at 3 wk of age show that the number of PMF, primary (PF) and secondary (SF) follicles are nearly identical. Preantral follicles (i.e., PAFs) begin to appear in WT and mutant FSH-expressing mice. (*G–I*) Ratio of PAF to total (*G*), PF (*H*), or SF (*I*) is significantly higher in serial sections of the entire ovary from mutant FSH-expressing mice compared with those in WT FSH-expressing mice. Follicle counts were estimated as described previously (44).

Rerouted FSH Enhances Ovulation Efficiency and Prolongs Female Reproductive Lifespan. Because the mouse uterus can have only limited implantations, and counting the pups will not give an accurate estimate of ovulations (22), we set up natural matings with proven fertile males and counted the number of fertilized eggs retrieved from oviducts. We found that the number of ovulations is dramatically increased (by six times) in *Fshb*-null mice expressing mutant FSH compared with WT FSH or normal control mice (Fig. 5 *A* and *B*). These fertilized one-cell embryos, when cultured in vitro, fully expanded into blastocysts as efficiently as controls (Fig. 5 *C* and *D*). To test the in vivo developmental competency of eggs, in a separate set of experiments, we first derived two-cell embryos from fertilized eggs collected from oviducts of mutant FSH-expressing mice. We then transplanted these embryos into oviducts of pseudopregnant female mice and obtained viable pups carrying mutant FSH transgene at an efficiency better than that achieved in typical superovulation and transgenic mouse production protocols (*SI Appendix*, Table S3). Thus, pulsatile FSH released like LH dramatically enhances the number of ovulations.

We tested three possibilities to explain the superior ovulation performance in mice expressing FSH from the LH pathway. First, ovarian follicle counts revealed that the number of primordial follicles (PMF) and the total number of follicles are nearly identical across all genotypes, ruling out the possibility that increased PMF or total follicle number is the reason for enhanced ovulation performance (Fig. 5 *E* and *F*).

We found that more preantral follicles are present in ovaries of mutant FSH-expressing mice (Fig. 5 *G–I*). Second, we tested the ovulation performance in aged mice at 6 mo and up to 1 y. We found that mutant FSH-expressing mice continue to produce more eggs than the age-matched controls, as evident by the increased number of CLs in ovarian histological sections (*SI Appendix*, Fig. S5). These data suggest ovarian folliculogenesis is not rapidly accelerated in mutant FSH-expressing mice, because it would have led to premature ovarian failure. Consistent with this, we found expression levels of phospho-PTEN, FoxO1, and FoxO3a, the key factors normally regulated during the initial phases of follicle development (23, 24), are not significantly different than those in controls (*SI Appendix*, Figs. S6 and S7). Third, in vivo BrdU labeling experiments confirmed an increase in follicle size and proliferation of granulosa cells (*SI Appendix*, Fig. S8 *A–C*) in ovaries of mice expressing rerouted FSH. Most importantly, the percentage of atretic follicles assessed by expression of the proapoptotic factor, cleaved caspase 3 is significantly reduced ($P < 0.05$) in ovaries of rerouted FSH-expressing mice at 21d of age, at which time point atresia is normally more pronounced in control mice (*SI Appendix*, Fig. S8 *D* and *E*). Furthermore, qPCR assays indicated that rerouting FSH into the LH pathway itself did not significantly affect expression of the known pituitary markers (*SI Appendix*, Fig. S9) that could also influence ovarian phenotypes. Together, these data confirm that diverting FSH into the LH secretion pathway results in enhanced follicle survival by blocking atresia and prolongs female reproductive lifespan.

Rerouted FSH Alters the Ovarian Gene Responses. We next tested if ovarian granulosa cells interpret and decode the rerouted FSH signaling inputs any differently than those elicited by constitutively secreted FSH. We found marked differences in gene expression patterns of major groups of ovarian genes (*SI Appendix*, Fig. S10*A*). The first group includes granulosa cell prosurvival genes (*Igf1* and *Bcl2*) that are preferentially up-regulated by rerouted FSH. This pattern of FSH release also significantly up-regulated the second group, consisting of the previously known FSH-responsive genes *Esr1*, *Kcnj8*, *S100a6*, *S100g*, and *Tagln* (21, 25). Surprisingly, the third group consisted of typical (pulsatile) LH-responsive genes including *Cap1*, *Fas*, *Ptgs2*, *Star*, and *Tsg6* (26). It is likely that this third set of genes "senses" the pulsatile nature of the gonadotropin signal input, irrespective of whether the signal is FSH or LH. We further confirmed that this set of gene responses is not a result of rerouted FSH cross-talking via activation of LH receptors, because total phospho-CREB and phospho-PKA substrate levels in ovaries are not elevated and similar to those seen in mice expressing FSH constitutively (*SI Appendix*, Fig. S10 *B*

and *C*). Additionally, mutant FSH did not rescue *Lhb*-null mice (*SI Appendix*, Fig. S10*D*), and these data genetically confirm that it does not bind and activate LH receptors on ovarian cells. The final group consists of genes (*Apaf1*, *Fasl*, *Inhbb*, *Ereg*, *Casp3*, *Lhr*, *Dok1*, *Cebpb*) that did not show any preferential response to constitutively secreted or rerouted FSH. Thus, rerouted FSH up-regulates prosurvival factors and evokes some LH-like responses in granulosa cells, causes a reduction in follicular atresia, and enhances ovulations (Fig. 6).

Discussion

Protein secretion occurs by two distinct modalities. In the first mode, proteins are secreted basolaterally or apically from polarized epithelial cells and genetic modification of this pathway has been studied in fly models in vivo (27). The second secretion route involves intracellular vesicular trafficking of proteins via the constitutive and regulated pathways (28, 29). The consequences of modifying these secretion pathways have not been tested in vivo. In the pituitary, GnRH pulse characteristics are critical for differential regulation of LH and FSH (30–33). Emerging evidence suggests GnRH pulses are interpreted at the level of gonadotropin β-subunit promoters, downstream signaling cascades involving MAPKs and ultimately reflected in corresponding subunit biosynthesis (31, 33). Here, we have not changed the GnRH pulsatile pattern or transcriptional effects of pulsatile GnRH on promoters driving *WT* and *Mut HFSHB* transgenes. Instead, we genetically altered the intracellular trafficking of FSH by fusing the FSHβ subunit with a carboxyl-terminal heptapeptide that is normally present in the LHβ subunit. This genetic strategy allowed us to divert FSH from a constitutive to the regulated pathway by which LH normally exits gonadotropes.

The reproductive strategy in primitive vertebrates involves presence of a single gonadotropin and production of a large number of eggs at one time. Although this mode of reproduction is highly efficient, it is limited by the availability of appropriate environmental conditions, large metabolic demands, and a shortened reproductive lifespan. To circumvent these limitations, we suggest that higher vertebrates have evolved a different strategy involving two gonadotropins and a cyclic process termed estrus or menstrual cycle, in which only a limited number of eggs or single egg per cycle is released. This process is more or less precisely timed, metabolically less demanding compared with release of a large number of eggs at one time, and occurs over a longer period. This reproductive strategy perhaps necessitated that one gonadotrophic hormone (i.e., FSH) is released continuously in low levels, acts as a mitogen, and favors follicle recruitment and selection during the initial phase of the cycle. FSH itself primes the receptor for the second hormone (i.e., LH), which is stored and packaged in DCGs, released as a pulse each time, and of which the highest amplitude pulse (i.e., LH surge) causes the release of egg(s). Because continuous exposure to LH during initial phases of ovarian folliculogenesis would cause premature luteinization (34) or ovarian hyperstimulation (35, 36), both of which are detrimental to egg production, we predict that the carboxyl-terminal heptapeptide of LHβ has evolved to direct LH into DCGs. This intracellular compartmentalization of LH, distinct from FSH, thus ensures LH to be released in response to hypothalamic GnRH only in quanta, and preventing continuous release. Our data are consistent with the hypothesis that continuous (i.e., FSH) and pulsatile (i.e., LH) gonadotropin secretion patterns have evolved to regulate production of only a limited number of eggs per cycle, as genetically diverting FSH into the LH secretion pathway resulted in release of more number of eggs. Our genetic and physiologic studies thus provide a molecular basis for the origin of estrus cycles.

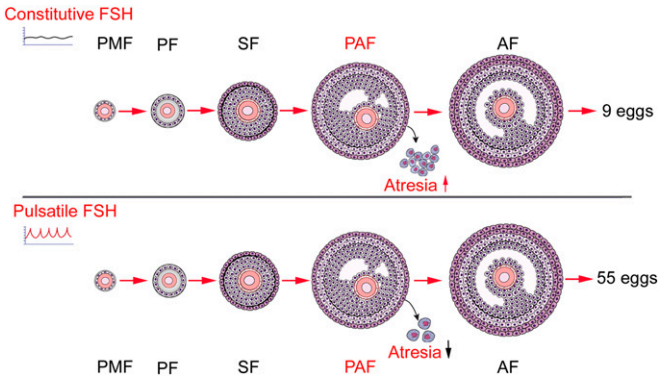

Fig. 6. A model for enhanced follicle survival and ovulation efficiency by pulsatile FSH. Reduced follicle atresia occurs in mice expressing pulsatile (*Lower*) compared with constitutive (*Upper*) FSH, thereby leading to enhanced follicle survival and increased ovulations.

Our studies indicate that the C′-terminal heptapeptide is a key determinant for gonadotropin sorting in vivo. It is not known if the heptapeptide would change FSHβ glycosylation and consequently serum $t_{1/2}$ of mutant FSH. In vitro metabolic labeling studies (13) and our Western blot data (Fig. 1) show that mutant FSH migrates identical to WT FSH. Any gross changes in glycosylation would affect the electrophoretic mobility, a typical glycosylation-dependent characteristic of gonadotropins (3). However, more detailed structure–function studies are required to confirm changes in glycosylation and $t_{1/2}$ of the mutant FSH.

Although rerouting FSH into the LH pathway vastly improved the number of eggs released per cycle and extended the female reproductive lifespan in a rodent model (mouse), this perhaps is not the evolutionarily preferred route for FSH secretion in humans and nonhuman primates, in which only one egg per cycle is released. Various other factors, including anatomical organization of the uterus, implantation efficiency, allocation of energy resources, and keeping with metabolic demands, would explain the constraints for multiple ovulations and necessitated that FSH release is more constitutive in these species. However, our genetic manipulation of the pattern of FSH secretion could have clinical implications particularly in artificial reproductive technology protocols wherein the number and quality of eggs retrieved are often the limiting factors in aged women.

Because mutations in constitutive/regulated protein sorting machinery impair protein secretion and are often associated with diseases (37), we propose that defects in gonadotropin routing and secretion pattern could cause some forms of infertility. It will be feasible for us in the future to genetically divert LH from the regulated (pulsatile) into the constitutive secretory pathway, mimicking FSH release, and analyze the consequences on an *Lhb*-null background (19). Our studies should also be more broadly applicable for genetically manipulating other pulsatile signaling systems, such as insulin (28, 38), parathyroid hormone (39), thyroid-stimulating hormone (40), and neuropeptides (41), to explain the pathophysiological basis of various human diseases associated with these systems.

Materials and Methods

Transgenic Mice. The transgenes encoding WT and mutant human FSHβ were cloned (13, 42) and transgenic mice produced as described previously (43). Transgenes were introduced onto *Fshb*-null background in two steps (14). *Lhb* and *Acvr2* mice were previously generated and characterized (19, 20). All animal procedures were per National Institutes of Health guidelines and approved by the University of Kansas Medical Center institutional animal use and care committee.

Real-Time qPCR Assays. TaqMan real-time PCR assays were performed on triplicate cDNA samples from three to five independent mice by using preinventoried primer/probe mix combos (Applied Biosystems).

Western Blot Analysis. Western blot analysis on pituitary and ovary proteins was performed as described previously (19). Expression of β-tubulin was used as an internal control.

Hormone Assays. Serum hormones (LH, FSH, testosterone, estradiol, and progesterone) were assayed at the Ligand Assay Core at the University of Virginia.

Confocal Microscopy. Frozen pituitary sections were incubated with appropriate primary and dye-conjugated secondary antibodies and visualized by using a Leica confocal microscope.

Histological Analysis. Tissues were formalin- or Bouin reagent-fixed, processed, and stained with periodic acid–Schiff reagent/hematoxylin (19). Testis tubule and ovarian follicle counts were calculated as described previously (14, 44).

Immunoelectron Microscopy. Pituitaries were fixed in 4% (wt/vol) paraformaldehyde/0.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and processed at the EM core.

Fertility Assays and Egg/Embryo Culture. Fertility assays, superovulation, collection of eggs, embryo culture, and transplantation were all done as described previously (43).

Statistical Analysis. All statistical analyses (*t* test and ANOVA followed by post hoc test) were performed by using Prism software.

Detailed methods are provided in *SI Appendix*.

ACKNOWLEDGMENTS. We thank Phillip Stevenson, Steven Curry, Colin O'Neil for technical assistance and Shyam Sharan (NCI-Frederick, Frederick, MD) for critically reading of the manuscript and suggestions. This study was supported in part by National Institutes of Health (NIH) Grants HD056082, AG029531, RR024214, RR016475, HD069751, CA166557 (to T.R.K.), and OD011092 (to P.M.C.). The Ligand Assay and Analysis Core at the Center for Research in Reproduction at the University of Virginia received support from the Eunice Kennedy Shriver National Institute of Child Health and Human Development/NIH (Specialized Cooperative Centers Program in Reproduction) Grant U54-HD28934.

1. Bronson HF (1989) *Mammalian Reproductive Biology* (Univ Chicago Press, Chicago).
2. Norris DO, Lopez KH eds (2011) *Hormones and Reproduction of Vertebrates* (Elsevier, New York), 1st Ed, Vol 5, pp 59–72.
3. Bousfield GR, Jia L, Ward DN (2006) Gonadotropins: Chemistry and biosynthesis. *Knobil and Neill's Physiology of Reproduction*, ed Neill JD (Elsevier, New York), 3rd Ed, Vol 1, pp 1581–1634.
4. Li MD, Ford JJ (1998) A comprehensive evolutionary analysis based on nucleotide and amino acid sequences of the alpha- and beta-subunits of glycoprotein hormone gene family. *J Endocrinol* 156(3):529–542.
5. Nagirnaja L, et al. (2010) Genomics and genetics of gonadotropin beta-subunit genes: Unique FSHB and duplicated LHB/CGB loci. *Mol Cell Endocrinol* 329(1-2):4–16.
6. Pierce JG, Parsons TF (1981) Glycoprotein hormones: Structure and function. *Annu Rev Biochem* 50:465–495.
7. Uchida K, et al. (2010) Evolutionary origin of a functional gonadotropin in the pituitary of the most primitive vertebrate, hagfish. *Proc Natl Acad Sci USA* 107(36): 15832–15837.
8. McNeilly AS, Crawford JL, Taragnat C, Nicol L, McNeilly JR (2003) The differential secretion of FSH and LH: Regulation through genes, feedback and packaging. *Reprod Suppl* 61:463–476.
9. Padmanabhan V, McFadden K, Mauger DT, Karsch FJ, Midgley AR, Jr. (1997) Neuroendocrine control of follicle-stimulating hormone (FSH) secretion. I. Direct evidence for separate episodic and basal components of FSH secretion. *Endocrinology* 138(1): 424–432.
10. Kumar TR (2007) Mouse models for gonadotropins: A 15-year saga. *Mol Cell Endocrinol* 260-262:249–254.
11. Jablonka-Shariff A, Garcia-Campayo V, Boime I (2002) Evolution of lutropin to chorionic gonadotropin generates a specific routing signal for apical release in vivo. *J Biol Chem* 277(2):879–882.
12. Jablonka-Shariff A, Pearl CA, Comstock A, Boime I (2008) A carboxyl-terminal sequence in the lutropin beta subunit contributes to the sorting of lutropin to the regulated pathway. *J Biol Chem* 283(17):11485–11492.
13. Pearl CA, Jablonka-Shariff A, Boime I (2010) Rerouting of a follicle-stimulating hormone analog to the regulated secretory pathway. *Endocrinology* 151(1):388–393.
14. Kumar TR, Low MJ, Matzuk MM (1998) Genetic rescue of follicle-stimulating hormone beta-deficient mice. *Endocrinology* 139(7):3289–3295.
15. Kim T, Gondré-Lewis MC, Arnaoutova I, Loh YP (2006) Dense-core secretory granule biogenesis. *Physiology (Bethesda)* 21:124–133.
16. Anouar Y, Duval J (1991) Differential expression of secretogranin II and chromogranin A genes in the female rat pituitary through sexual maturation and estrous cycle. *Endocrinology* 128(3):1374–1380.
17. Zhao S, Torii S, Yokota-Hashimoto H, Takeuchi T, Izumi T (2002) Involvement of Rab27b in the regulated secretion of pituitary hormones. *Endocrinology* 143(5): 1817–1824.
18. Kumar TR, Wang Y, Lu N, Matzuk MM (1997) Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. *Nat Genet* 15(2):201–204.
19. Ma X, Dong Y, Matzuk MM, Kumar TR (2004) Targeted disruption of luteinizing hormone beta-subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility. *Proc Natl Acad Sci USA* 101(49):17294–17299.
20. Matzuk MM, Kumar TR, Bradley A (1995) Different phenotypes for mice deficient in either activins or activin receptor type II. *Nature* 374(6520):356–360.
21. Burns KH, Yan C, Kumar TR, Matzuk MM (2001) Analysis of ovarian gene expression in follicle-stimulating hormone beta knockout mice. *Endocrinology* 142(7):2742–2751.
22. Wang H, Dey SK (2006) Roadmap to embryo implantation: Clues from mouse models. *Nat Rev Genet* 7(3):185–199.
23. Reddy P, Zheng W, Liu K (2010) Mechanisms maintaining the dormancy and survival of mammalian primordial follicles. *Trends Endocrinol Metab* 21(2):96–103.
24. Sullivan SD, Castrillon DH (2011) Insights into primary ovarian insufficiency through genetically engineered mouse models. *Semin Reprod Med* 29(4):283–298.
25. Burns KH, Owens GE, Ogbonna SC, Nilson JH, Matzuk MM (2003) Expression profiling analyses of gonadotropin responses and tumor development in the absence of inhibins. *Endocrinology* 144(10):4492–4507.
26. Fan HY, et al. (2009) MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. *Science* 324(5929):938–941.
27. Tojo SJ, Germeraad S, King DS, Fristrom JW (1987) Polarized secretion of an ectopic protein in Drosophila salivary glands in vivo. *EMBO J* 6(8):2249–2254.
28. Arvan P (2004) Secretory protein trafficking: Genetic and biochemical analysis. *Cell Biochem Biophys* 40(3, suppl):169–178.
29. Burgess TL, Kelly RB (1987) Constitutive and regulated secretion of proteins. *Annu Rev Cell Biol* 3:243–293.
30. Burger LL, Haisenleder DJ, Dalkin AC, Marshall JC (2004) Regulation of gonadotropin subunit gene transcription. *J Mol Endocrinol* 33(3):559–584.
31. Ciccone NA, Kaiser UB (2009) The biology of gonadotroph regulation. *Curr Opin Endocrinol Diabetes Obes* 16(4):321–327.
32. Marshall JC, et al. (1991) Gonadotropin-releasing hormone pulses: Regulators of gonadotropin synthesis and ovulatory cycles. *Recent Prog Horm Res* 47:155–187, discussion 188–189.
33. Thompson IR, Kaiser UB (2013) GnRH pulse frequency-dependent differential regulation of LH and FSH gene expression. *Mol Cell Endocrinol*, 10.1016/j.mce.2013.09.012.
34. Filicori M (1997) Cause for premature luteinization? *Fertil Steril* 67(6):1179–1181.
35. Matzuk MM, DeMayo FJ, Hadsell LA, Kumar TR (2003) Overexpression of human chorionic gonadotropin causes multiple reproductive defects in transgenic mice. *Biol Reprod* 69(1):338–346.
36. Nilson JH, Abbud RA, Keri RA, Quirk CC (2000) Chronic hypersecretion of luteinizing hormone in transgenic mice disrupts both ovarian and pituitary function, with some effects modified by the genetic background. *Recent Prog Horm Res* 55:69–89.
37. Lin WJ, Salton SR (2013) The regulated secretory pathway and human disease: Insights from gene variants and single nucleotide polymorphisms. *Front Endocrinol (Lausanne)* 4:96.
38. Hellman B (2009) Pulsatility of insulin release—a clinically important phenomenon. *Ups J Med Sci* 114(4):193–205.
39. Schaefer F (2000) Pulsatile parathyroid hormone secretion in health and disease. *Novartis Found Symp* 227:225–239.
40. Yamada M, Mori M (2008) Mechanisms related to the pathophysiology and management of central hypothyroidism. *Nat Clin Pract Endocrinol Metab* 4(12):683–694.
41. Tsutsumi R, Webster NJ (2009) GnRH pulsatility, the pituitary response and reproductive dysfunction. *Endocr J* 56(6):729–737.
42. Kumar TR, Schuff KG, Nusser KD, Low MJ (2006) Gonadotroph-specific expression of the human follicle stimulating hormone beta gene in transgenic mice. *Mol Cell Endocrinol* 247(1-2):103–115.
43. Kumar TR, Larson M, Wang H, McDermott J, Bronshteyn I (2009) Transgenic mouse technology: Principles and methods. *Methods Mol Biol* 590:335–362.
44. Kerr JB, et al. (2006) Quantification of healthy follicles in the neonatal and adult mouse ovary: Evidence for maintenance of primordial follicle supply. *Reproduction* 132(1):95–109.