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GCNF-dependent repression of *BMP-15* and *GDF-9* mediates gamete regulation of female fertility

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To determine the function of *germ cell nuclear factor* (*GCNF*) in female reproduction, we generated an oocyte-specific *GCNF* knockout mouse model (*GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$). These mice displayed hypofertility due to prolonged diestrus phase of the estrous cycle and aberrant steroidogenesis. These reproductive defects were secondary to a primary defect in the oocytes, in which expression of the paracrine transforming growth factor-β signaling molecules, *bone morphogenetic protein 15* (*BMP-15*) and *growth differentiation factor 9* (*GDF-9*), were up-regulated in *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ females at diestrus. This was a direct effect of GCNF, as molecular studies showed that GCNF bound to DR0 elements within the *BMP-15* and *GDF-9* gene promoters and repressed their reporter activities. Consistent with these findings, abnormal double-oocyte follicles, indicative of aberrant *BMP-15*/*GDF-9* expression, were observed in *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ females. The Cre/loxP knockout of GCNF in the oocyte has uncovered a new regulatory pathway in ovarian function. Our results show that GCNF directly regulates paracrine communication between the oocyte and somatic cells by regulating the expression of *BMP-15* and *GDF-9*, to affect female fertility.

Keywords: Cre/loxP/gene regulation/nuclear receptor/oocyte/ovary

Introduction

Germ cell nuclear factor (GCNF/RTR/NCNF, NR6A1) is an orphan member of the nuclear receptor superfamily (Chen *et al*., 1994; Nuclear Receptor Nomenclature Committee, 1999). GCNF functions as a transcription factor that binds to a direct repeat of the sequence AGGTCA with 0 base pair (bp) spacing between the half sites (DR0) element to repress gene transcription *in vitro* and *in vivo* (Chen *et al*., 1994; Yan *et al*., 1997; Cooney *et al*., 1998; Fuhrmann *et al*., 2001). It is expressed in early mouse embryos after the onset of gastrulation (Susens *et al*., 1997; Chung *et al*., 2001) and is essential for normal embryonic development and repression of *Oct4* expression in somatic cells of early mouse embryos (Chung *et al*., 2001; Fuhrmann *et al*., 2001). The DNA binding domain (DBD) of GCNF is essential for the function of GCNF during embryonic development and for *Oct4* repression as *GCNF*$^{lox/lox}$ embryos, in which the DBD of GCNF is deleted but the ligand binding domain (LBD) of GCNF is expressed in the mouse embryo, phenocopy *GCNF* null mutant (*GCNF*$^{-/-}$) mouse embryos (Lan *et al*., 2002). In adult vertebrates, *GCNF* is predominantly expressed in the gonads of several species, including mouse, rat and human (Chen *et al*., 1994; Katz *et al*., 1997; Agoulnik *et al*., 1998; Zhang *et al*., 1998). In the murine testis, *GCNF* is expressed in postmeiotic round spermatids at both the mRNA and protein levels (Chen *et al*., 1994; Katz *et al*., 1997; Lan *et al*., 2003), while in human testis, it is expressed in pachytene spermatocytes (Agoulnik *et al*., 1998). In the ovary, *GCNF* is expressed in oocytes in mouse, *Xenopus* and zebrafish (Chen *et al*., 1994; Joos *et al*., 1996; Katz *et al*., 1997; Braat *et al*., 1999). In the mouse ovary, *GCNF* is exclusively expressed in the oocytes of primary, secondary and pre-ovulatory follicles, but not primordial follicles, at both the mRNA and protein levels (Chen *et al*., 1994; Katz *et al*., 1997; Lan *et al*., 2003). GCNF is also present in ovulated oocytes and pre-implantation embryos, indicating that GCNF may play a maternal role in zygotic development prior to implantation (Lan *et al*., 2003). The oocyte-specific expression pattern indicates that GCNF may be a transcription factor that plays a role in regulating some aspect of oocyte function.

To determine the role of GCNF during female reproduction and by-pass the embryonic lethality, we employed a Cre/loxP strategy (Figure 1A), similar to a previous report (Gu *et al*., 1994), to inactivate the *GCNF* gene by deleting the DBD encoding exon of the *GCNF* gene specifically in the oocytes of adult mice. By breeding the floxed *GCNF* mice with male *zona pellucida protein 3* (*Zp3*) gene promoter-driven *Cre* transgenic mice (de Vries *et al*., 2000), we generated oocyte-specific *GCNF* knockout mice. The phenotype of these oocyte-specific *GCNF* knockout mice was determined. More importantly, the molecular mechanism of the phenotype was characterized. This study provides direct evidence that there exists a paracrine communication between oocytes and adjacent somatic steroidogenic cells during folliculogenesis that modulates the estrous cycle during female reproduction, and this function is mediated by GCNF-dependent regulation of *bone morphogenetic protein 15* (*BMP-15*) and *growth differentiation factor 9* (*GDF-9*) expression.

Results

Generation of GCNF$^{fl/fl}$ *mice*

To generate the floxed *GCNF* mice, we constructed a targeting vector that introduced a gene selection cassette, consisting of the *neomycin* (*Neo*) and herpes simplex virus *thymidine kinase* (*TK*) genes flanked by loxP sites, downstream of exon 4 of the *GCNF* gene and a single loxP site 500 bp upstream of exon 4 in the *GCNF* gene

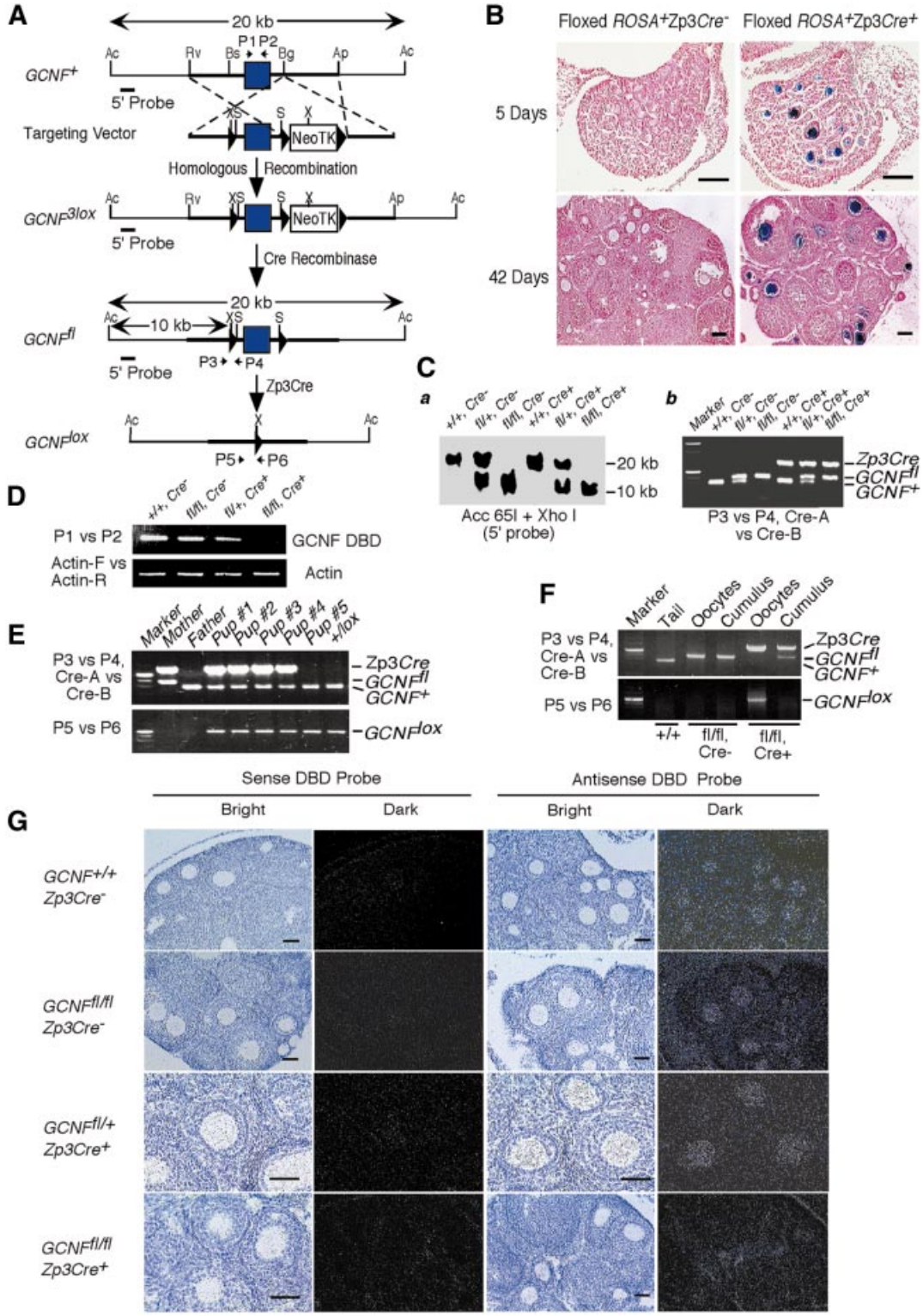

Fig. 1. Generation of an oocyte-specific *GCNF* knockout mouse model. (**A**) Structures of the $GCNF^{3lox}$, $GCNF^{fl}$ and $GCNF^{lox}$ alleles and the targeting vector. LoxP sites, filled triangles; the DBD-encoding exon, filled box. Restriction enzyme sites of *Acc*65I (Ac), *Apa*I (Ap), *Bgl*I (Bg), *Bsi*EI (Bs), *Eco*RV (Rv), *Xho*I (X) and *Sal*I (S) in each allele are shown. Positions of PCR primers (P1 to P6), and the 5′ probe for Southern blot analysis are indicated. (**B**) Cre recombinase activity on the floxed ROSA transgene in the ovaries determined by β-galactosidase staining. Bar scale, 200 μm. (**C**) Genotype analysis of tail biopsies. (a) Southern blot analysis showing a 20 kb band for the $GCNF^{+}$ allele and a 10 kb band for the $GCNF^{fl}$ allele. (b) PCR analysis using primers (P3 and P4) to distinguish the $GCNF^{fl}$ and $GCNF^{+}$ alleles, and primers (Cre-A and Cre-B) to determine the presence of Zp3*Cre* transgene. (**D**) RT–PCR analysis showing the loss of the DBD encoding region of the GCNF mRNA in the $GCNF^{fl/fl}$Zp3Cre^{+} ovary. (**E**) Genotyping of tail DNA showing the complete deletion of the GCNF DBD encoding exon in the progenies from the cross between $GCNF^{fl/fl}$Zp3Cre^{+} females and wild-type males. (**F**) PCR analysis showing complete deletion of the GCNF DBD encoding exon in ovulated oocytes, but not in cumulus cells. (**G**) *In situ* hybridization showing the loss of the DBD region of the GCNF mRNA in the oocytes of $GCNF^{fl/fl}$Zp3Cre^{+} mice. Bar scale, 50 μm.

(Lan *et al*., 2002). Subsequent electroporation of embryonic stem (ES) cells (129 AB1.2 cells) with the targeting vector and selection in Geneticin® (G418) led to ES cell clones containing a $GCNF^{3lox}$ allele (Figure 1A). Two correctly targeted ES cell clones carrying the $GCNF^{3lox}$ allele were amplified and then transiently transfected with a Cre expression plasmid (pOG231) (O'Gorman *et al*., 1997) to delete the *NeoTK* selection cassette and leave loxP sites upstream and downstream of exon 4. Southern blot and PCR analyses demonstrated the absence of the *NeoTK* cassette, and the presence of two loxP sites flanking the DBD encoding exon in eight of 80 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil (FIAU)-resistant ES cell clones (our unpublished data). Five $GCNF^{fl/+}$ ES clones were injected into C57BL/6 blastocysts to generate chimeric mice. Chimeric mice from each clone transmitted the $GCNF^{fl}$ allele to their offspring. Intercrossing of heterozygous floxed *GCNF* mice ($GCNF^{fl/+}$) from each line produced $GCNF^{+/+}$, $GCNF^{fl/+}$ and $GCNF^{fl/fl}$ progenies at the expected 1:2:1 Mendelian ratio, for both males and females. $GCNF^{fl/fl}$ mice were healthy and fertile, and had normal *GCNF* expression in the testis (our unpublished data) and ovary (Figure 1G). These results indicate that insertion of loxP sites upstream and downstream of the DBD encoding exon of the *GCNF* gene does not cause any hypomorphic effects due to disrupted expression.

Oocyte-specific Cre recombinase activity

Previously generated Zp3*Cre* transgenic mice have been shown to express Cre recombinase specifically in the ovary (de Vries *et al*., 2000). To confirm the cell specificity of Cre recombinase in Zp3*Cre* transgenic mice, we used the floxed *ROSA* reporter mice (Soriano, 1999). X-gal staining of tissues from progeny of matings between male Zp3*Cre* transgenic mice with female floxed *ROSA* mice at different ages was performed. Specific β-galactosidase activity was observed only in the ovary (Figure 1B), and not in the oviduct, uterus, heart, kidney, liver, pituitary and brain in the adult floxed $ROSA^{+}$Zp3Cre^{+} females (our unpublished data). Within the ovary, the Cre recombinase activity was observed only in oocytes from the primary to pre-ovulatory stages; no expression was noted in oocytes of primordial follicles or in somatic cells (Figure 1B). Therefore, the Cre recombinase in the Zp3*Cre* transgenic mice is specifically expressed in the oocytes of primary, secondary and pre-ovulatory follicles, which is similar to the *GCNF* ovarian expression pattern (Katz *et al*., 1997; Lan *et al*., 2003), and is efficiently able to delete the floxed DNA fragment in the mouse genome, as 100% of those oocytes at the primary and later follicular stages stained positively for β-galactosidase activity.

Creation of oocyte-specific GCNF knockout mice

Breeding of male Zp3*Cre* transgenic mice with female $GCNF^{fl/fl}$ mice produced bigenic heterozygous animals ($GCNF^{fl/+}$Zp3Cre^{+}). Male $GCNF^{fl/+}$Zp3Cre^{+} mice were then bred with $GCNF^{fl/+}$ females to generate $GCNF^{fl/fl}$Zp3Cre^{+} offspring with an expected Mendelian rate of 12.5%. The genotypes of these animals were determined by Southern blot and PCR analyses (Figure 1C). About half of $GCNF^{fl/fl}$Zp3Cre^{+} mice (310/625) were females, that were oocyte-specific *GCNF* knockout mice. These oocyte-specific *GCNF* knockout mice grew normally and were indistinguishable from their $GCNF^{+/+}$, $GCNF^{fl/fl}$ and $GCNF^{fl/+}$Zp3Cre^{+} littermates (our unpublished data).

To determine whether the DBD encoding region of *GCNF* mRNA was completely deleted by the Cre recombinase in the $GCNF^{fl/fl}$Zp3Cre^{+} ovaries, total ovarian RNA was isolated from 1-month-old littermates of $GCNF^{fl/fl}$Zp3Cre^{+} mice and three control groups including $GCNF^{+/+}$, $GCNF^{fl/fl}$ and $GCNF^{fl/+}$Zp3Cre^{+} mice, and then subjected to RT–PCR with specific primers for exon 4 of the *GCNF* gene encoding the DBD. As shown in Figure 1D, the DBD region of *GCNF* mRNA was absent in the $GCNF^{fl/fl}$Zp3Cre^{+} ovary, but was present in the ovaries of all three control littermates. *In situ* hybridization also showed that the DBD-encoding region of the *GCNF* transcript was completely deleted in the $GCNF^{fl/fl}$Zp3Cre^{+} ovaries (Figure 1G). To confirm the above results, female $GCNF^{fl/fl}$Zp3Cre^{+} mice were bred with $GCNF^{+/+}$ males, and the genotype of the offspring was determined by PCR analysis. As shown in Figure 1E, all the progeny lacked a $GCNF^{fl}$ allele; rather they had a recombined $GCNF^{lox}$ allele generated by the Cre recombinase in the oocytes. PCR analysis of genomic DNA isolated from ovulated oocytes and cumulus granulosa cells showed the absence of $GCNF^{fl}$ allele and presence of $GCNF^{lox}$ allele in ovulated oocytes but not cumulus granulosa cells, confirming complete deletion of $GCNF^{fl}$ allele by Zp3Cre recombinase specifically in oocytes and not the adjacent granulosa cells in $GCNF^{fl/fl}$Zp3Cre^{+} females (Figure 1F). No recombined $GCNF^{lox}$ allele was present in genomic DNA isolated from pituitary and brain of adult $GCNF^{fl/fl}$Zp3Cre^{+} females (our unpublished data). Taken together, these results show that the GCNF DBD-encoding exon was completely deleted by the Zp3Cre recombinase only in oocytes, not in granulosa cells, nor in other organs such as the uterus, pituitary or brain.

Reduced fertility and prolonged estrous cycle in the $GCNF^{fl/fl}Zp3Cre^{+}$ females

As indicated in Figure 1E, $GCNF^{fl/fl}$Zp3Cre^{+} female mice are fertile. To further determine whether there is any fertility defect in these mice, the females and their control littermates of three different genotypes ($GCNF^{+/+}$, $GCNF^{fl/fl}$ and $GCNF^{fl/+}$Zp3Cre^{+}) were bred with stud males for up to 1 year. As shown in Figure 2A, the number of pups per litter at day 5 after birth in the knockout group ($GCNF^{fl/fl}$Zp3Cre^{+}) was significantly reduced ($P < 0.001$) compared with the three control groups ($GCNF^{+/+}$, $GCNF^{fl/fl}$ and $GCNF^{fl/+}$Zp3Cre^{+}). Significantly, the number of litters per month in the knockout group was reduced ~50% compared with those in the three control groups (Figure 2B). To determine whether the reduced number of litters per month was due to defects in the estrous cycle, animals (2–4 months old) were subjected to a daily Pap smear test for two consecutive months to determine the length of the estrous cycle and the four stages of the estrous cycle (diestrus, proestrus, estrus and metestrus). As shown in Figure 2C, the average length of the estrous cycle in $GCNF^{fl/fl}$Zp3Cre^{+} mice were much longer than those in the three control groups ($P < 0.001$). This prolonged estrous cycle was due to prolonged diestrus. No differences were observed at the other stages

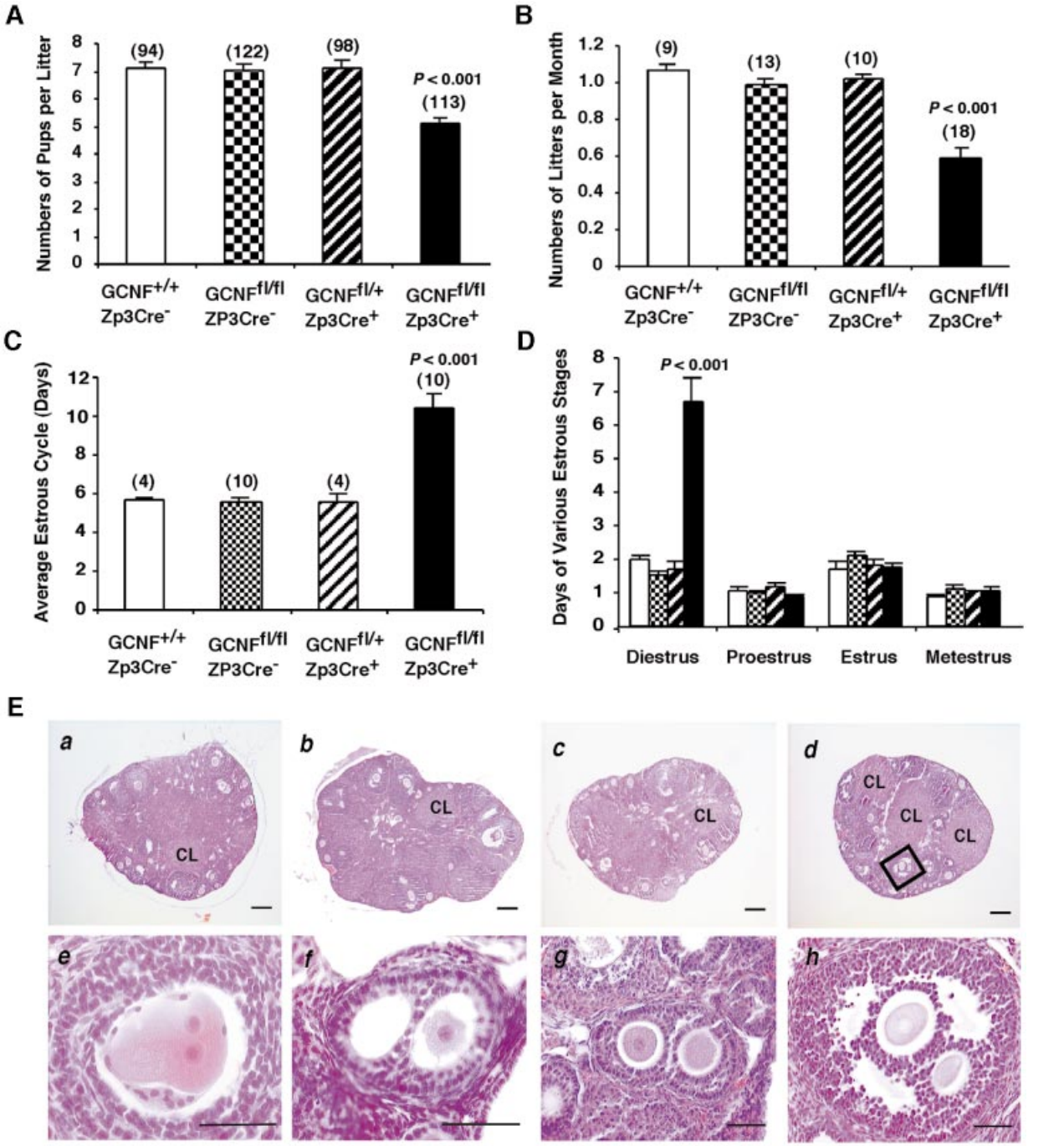

Fig. 2. Oocyte-specific *GCNF* knockout mice display hypofertility, abnormal estrous cycle and double-oocyte follicles. (**A**) Reduced numbers of pups per litter and (**B**) reduced numbers of litters per month from the breeding of $GCNF^{fl/fl}$Zp3Cre^{+} females with wild-type males for 1 year. (**C**) Prolonged length of the estrous cycle in the $GCNF^{fl/fl}$Zp3Cre^{+} females. (**D**) Prolonged diestrus of the estrous cycle in the $GCNF^{fl/fl}$Zp3Cre^{+} females. (**E**) Normal ovarian histology except the presence of double oocyte follicles in the $GCNF^{fl/fl}$Zp3Cre^{+} females. (a) $GCNF^{+/+}$, (b) $GCNF^{fl/fl}$, (c) $GCNF^{fl/+}$Zp3Cre^{+} and (d) $GCNF^{fl/fl}$Zp3Cre^{+} ovaries were 3-month-old littermates. CL, corpus lutea. (e–h) The presence of double-oocyte follicles at the primary (e and f), secondary (g) and antral (h) stages in $GCNF^{fl/fl}$Zp3Cre^{+} ovaries. High magnification of the box in (d) is shown in (h). The bar scale in (a)–(d) is 200 μm. In (e)–(h) the bar scale is 50 μm.

of the estrous cycle (Figure 2D). This prolonged diestrus in the knockout mice probably accounts for the reduced number of litters per month.

Presence of double oocyte follicles in the $GCNF^{fl/fl}Zp3Cre^{+}$ females

There was no difference in the size and weight of ovaries among all four animal groups [$GCNF^{+/+}$, 0.487 ± 0.031 ovary weight (mg)/body weight (g), n = 3; $GCNF^{fl/fl}$, 0.424 ± 0.046, n = 3; $GCNF^{fl/+}$Zp3Cre^{+}, 0.447 ± 0.027, n = 3; and $GCNF^{fl/fl}$Zp3Cre^{+}, 0.432 ± 0.038, n = 3]. Histological studies showed that follicles at different stages and corpora lutea were present in the $GCNF^{fl/fl}$Zp3Cre^{+} mice, similar to the three control groups ($GCNF^{+/+}$, $GCNF^{fl/fl}$ and $GCNF^{fl/+}$Zp3Cre^{+}) (Figure 2E, a–d). Careful analysis of the ovarian histology revealed the

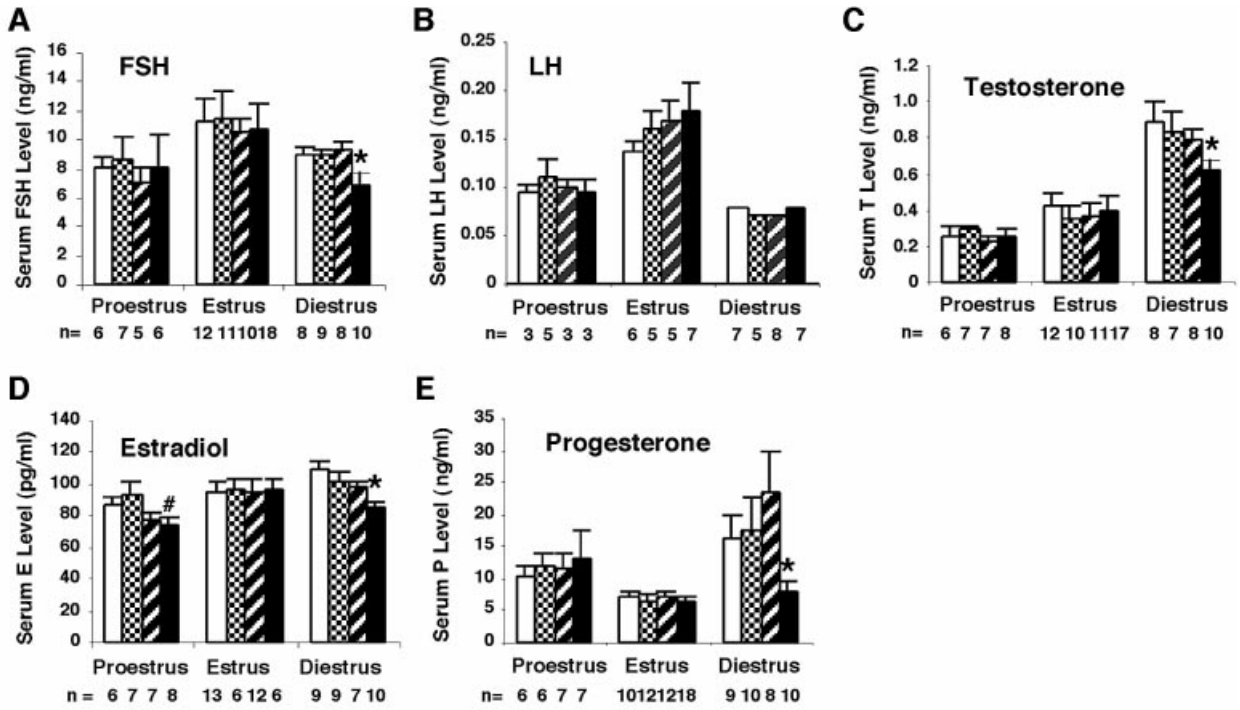

Fig. 3. Serum levels of gonadotropins and steroid hormones in the $GCNF^{fl/fl}$Zp3Cre^{+} females during the estrous cycle. (**A**) FSH, (**B**) LH, (**C**) testosterone, (**D**) estradiol and (**E**) progesterone levels in the $GCNF^{fl/fl}$Zp3Cre^{+} (filled bar), and three control groups, $GCNF^{+/+}$ (open bar), $GCNF^{fl/fl}$ (grid bar) and $GCNF^{fl/+}$Zp3Cre^{+} (striped bar), are presented as means ± SEs among the indicated numbers of samples in each group in the bar graph. $^{*}P < 0.05$, when compared with three control groups at diestrus. $^{\#}P < 0.05$, when compared with $GCNF^{+/+}$ or $GCNF^{fl/fl}$ at estrus.

presence of double oocytes within some follicles in the oocyte-specific *GCNF* knockout mice (Figure 2E, d–h). Histological studies on serial ovarian sections showed that no animals in the control groups, $GCNF^{+/+}$ (seven mice), $GCNF^{fl/fl}$ (six mice) and $GCNF^{fl/+}$Zp3Cre^{+} (five mice) had any follicles containing double oocytes. However, in the $GCNF^{fl/fl}$Zp3Cre^{+} mice, six of seven mice at diestrus had one to three follicles containing double oocytes. Among these six mice, four mice at the age ranging from 1 to 12 months had only one double-oocyte follicle. A 3-month-old $GCNF^{fl/fl}$Zp3Cre^{+} mouse had two double-oocyte follicles, while another $GCNF^{fl/fl}$Zp3Cre^{+} mouse at the same age had three double-oocyte follicles. These double-oocyte follicles may be derived from a single oocyte, because two nucleus-like structures were observed in one oocyte (Figure 2E, e). The double-oocyte follicles continued to develop from the primary stage to the antral stage, and no obvious defects in the histology of somatic cells of these double-oocyte follicles were observed (Figure 2E, f–h).

Reduced steroid hormone levels in the $GCNF^{fl/fl}Zp3Cre^{+}$ mice at diestrus

The prolonged estrus cycle indicated that there could be alterations in the levels of circulating gonadotropins and steroid hormones in the $GCNF^{fl/fl}$Zp3Cre^{+} mice. Using standard radioimmunoassays, hormone levels of 2- to 3-month-old mice at various stages of the estrous cycle were determined in a double-blind test. As shown in Figure 3 and Supplementary table, no significant changes in follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone and progesterone levels were observed in the $GCNF^{fl/fl}$Zp3Cre^{+} mice at proestrus and estrus. No significant changes in estradiol levels were observed in the $GCNF^{fl/fl}$Zp3Cre^{+} females at estrus (Figure 3D). Although estradiol levels in the $GCNF^{fl/fl}$Zp3Cre^{+} females at proestrus were lower than those of $GCNF^{+/+}$ and $GCNF^{fl/fl}$ controls, there was no significant change in the estradiol levels between $GCNF^{fl/fl}$Zp3Cre^{+} and $GCNF^{fl/+}$Zp3Cre^{+} controls (Figure 3D). However, at diestrus, gonadotropin FSH, but not LH levels, and steroid hormones including testosterone, estradiol and progesterone levels were significantly reduced in the $GCNF^{fl/fl}$Zp3Cre^{+} mice, when compared with the three control groups ($GCNF^{+/+}$, $GCNF^{fl/fl}$ and $GCNF^{fl/+}$Zp3Cre^{+}) ($P < 0.05$). The reduced steroid hormone levels at diestrus could account for the abnormal estrous cycle observed in the $GCNF^{fl/fl}$Zp3Cre^{+} mice.

Mis-expression of key steroidogenic genes in the $GCNF^{fl/fl}Zp3Cre^{+}$ mice at diestrus

Since steroid hormone levels in the $GCNF^{fl/fl}$Zp3Cre^{+} females at diestrus were reduced (Figure 3), the expression of key enzymatic and regulatory genes involved in steroidogenesis in the somatic cells of the ovary was analyzed. As determined by RT–PCR or northern blot analyses (Figure 4A–D), the expression levels of *steroidogenic acute regulatory protein* (*StAR*) and *3β-hydroxysteroid dehydrogenase I* (*3βHSD I*) were reduced in the $GCNF^{fl/fl}$Zp3Cre^{+}ovaries at diestrus compared with control animals. In contrast, the expression of *17-α hydroxylase* (*17αOH*) was increased compared with control animals (Figure 4E and F). Quantitative RT–PCR analyses confirmed the above results (Supplementary figure 1). Other ovarian steroidogenic enzymatic genes including *cholesterol side chain cleavage cytochrome*

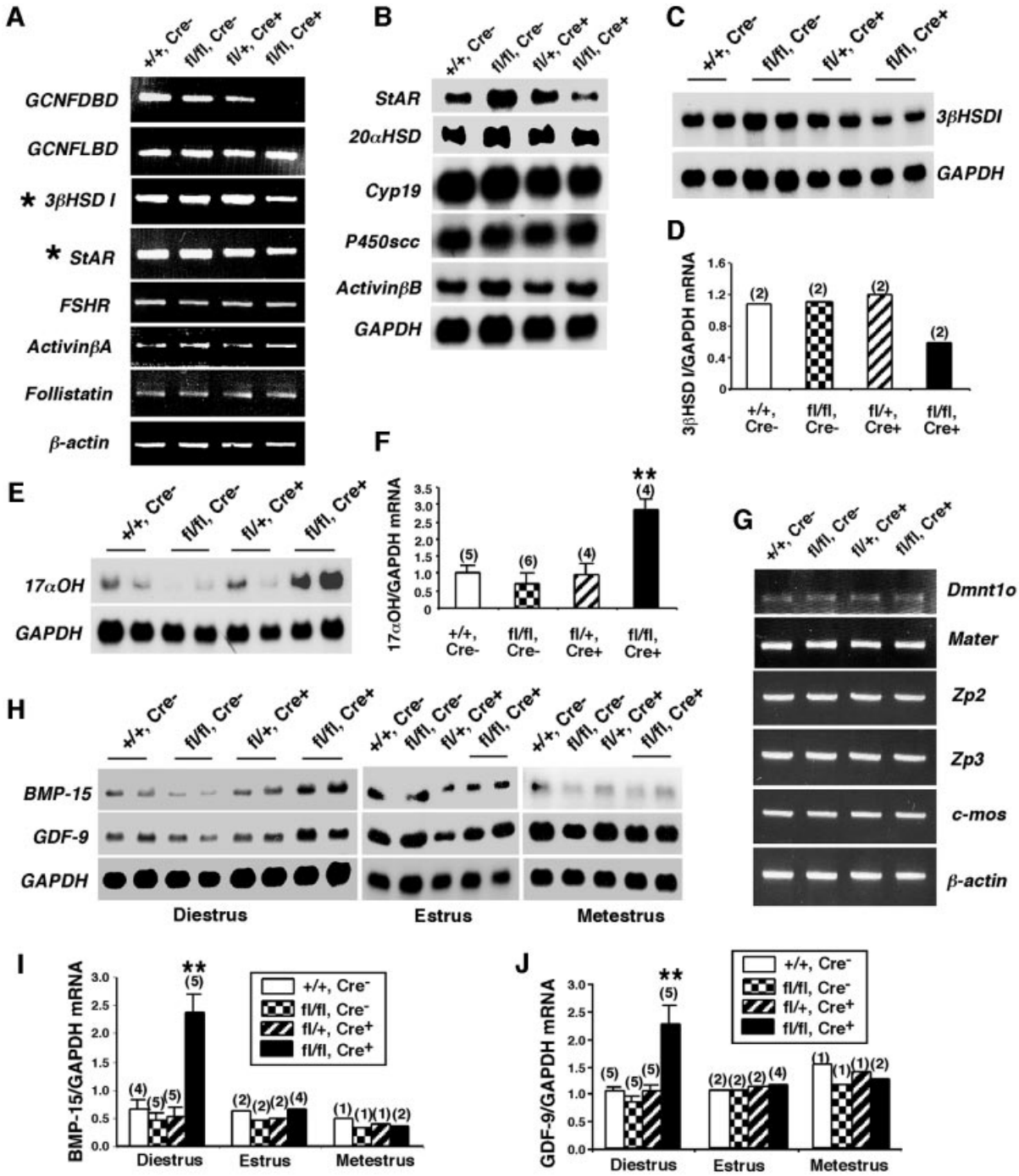

Fig. 4. Expression of ovarian marker genes in the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ ovary. (A–F) Expression of key ovarian genes expressed in somatic cells of *GCNF*$^{+/+}$ (+/+, Cre–), *GCNF*$^{fl/fl}$ (fl/fl, Cre–), *GCNF*$^{fl/+}$Zp3*Cre*$^{+}$ (fl/+, Cre+) and *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ (fl/fl, Cre+) ovaries at diestrus (day 1–2). (**A**) RT–PCR showing the reduced *StAR* and *3βHSD I* expression in the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ mice. (B–F) Nothern blot analyses showing the mis-expression of (**B**) *StAR*, (**C** and **D**) *3βHSD I* and (**E** and **F**) *17αOH* in *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ *mice. Quantitative 3βHSD I* and *17αOH* mRNA levels in the northern blots are shown in the bar graph in (D) and (F), respectively. (**G**–**J**) Expression of oocyte marker genes. (G) RT–PCR showing expression of oocyte genes, *Dmnt1o*, *Mater*, *Zp2*, *Zp3* and c-*mos*, in ovaries. Experiments were repeated twice using two individual animals. (H) Representative radiographs of northern blot analyses showing the *BMP-15* and *GDF-9* expression. (I and J) Quantitative ovarian (I) BMP-15 and (J) GDF-9 mRNA levels in northern blots. For (F), (I) and (J), relative mRNA levels (normalized to GAPDH mRNA levels) are presented as means ± SEs from various numbers of animal samples (indicated by alphabetic numbers) of each genotype. $^{**}P < 0.01$ compared with three other groups at diestrus.

P450 protein (*P450scc*), *Cyp19* (aromatase) and *20α-hydroxysteroid dehydrogenase* (*20αHSD*) were not affected in the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ ovaries, neither were *FSHR*, *activin βA*, *activin βB*, *follistatin*, *LHR* and *inhibin α*, which are expressed in the somatic cells of the ovary (Figure 4A and B, and Supplementary figure 1). Therefore, inactivation of the *GCNF* gene in the oocytes indirectly caused mis-expression of key components of the steroidogenic pathway in somatic cells, such as *StAR*, *3βHSD I* and *17αOH*, which leads to reduced steroid hormone levels in the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ females at diestrus. Indeed, we have searched the *StAR*, *3βHSD I* and *17αOH* gene promoters

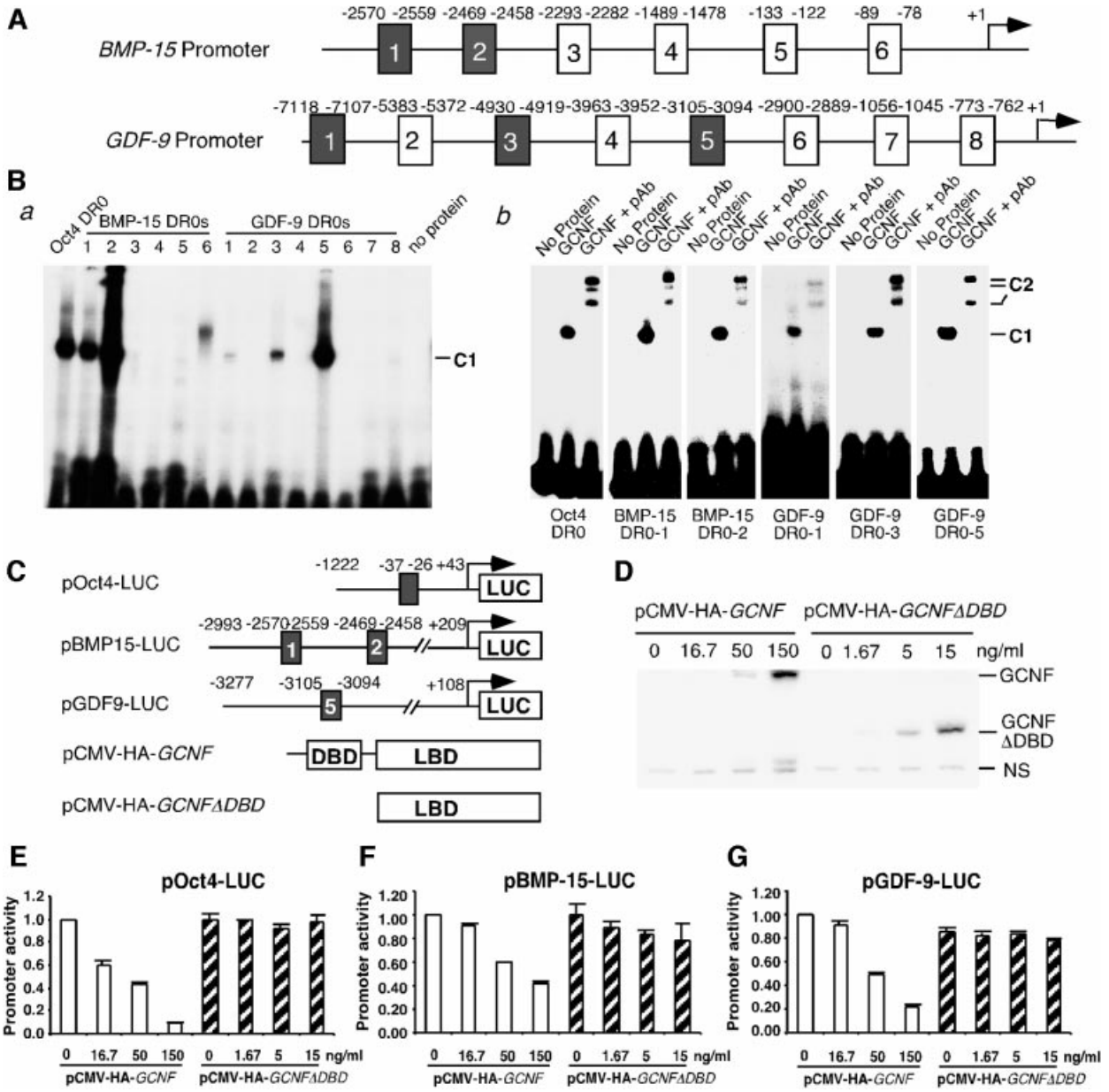

Fig. 5. Direct regulation of the *BMP-15* and *GDF-9* expression by GCNF. (**A**) Schematic representation of putative DR0s in the mouse *BMP-15* and *GDF-9* promoters. (**B**) EMSAs showing the binding of *in vitro* translated GCNF to multiple DR0 elements in the *BMP-15* and *GDF-9* promoters. (a) GCNF was incubated with different ^{32}P-labeled oligonucleotide probes containing the putative DR0 elements. (b) Retarded DNA-protein complexes in EMSAs by anti-GCNF antibodies (pAb). C1, GCNF–DR0 DNA complexes; C2, antibody retarded GCNF–DR0 DNA complexes. (**C**) Schematic representation of GCNF expression plasmids and luciferase reporter plasmids containing *Oct4*, *BMP-15* or *GDF-9* promoters. (**D**) Western blot analysis showing the expression levels of full-length GCNF protein and GCNF mutant protein (GCNFΔDBD) in transfected CHO cells using anti-HA antibodies. NS, non-specific protein. (E, G) Dose-dependent repression of the (**E**) *Oct4*, (**F**) *BMP-15* and (**G**) *GDF-9* expression by GCNF, not GCNFΔDBD, in CHO cells. Promoter activities are presented as means ± SDs of the percentages of the total promoter activities (without pCMV-*HA-GCNF* plasmid) and represent three independent measurements.

for DR0 elements and found none, which, in conjunction with no expression of *GCNF* or Cre activity in the somatic cells of the ovary in the oocyte-specific *GCNF* knockouts (Figure 1), supports the notion that the effects of loss of GCNF in the oocyte on somatic gene expression is indirect.

Overexpression of oocyte-specific GDF-9 and BMP-15 genes in the GCNF$^{fl/fl}$Zp3Cre^{+} mice at diestrus

Since GCNF is exclusively expressed in oocytes (Chen *et al.*, 1994; Katz *et al.*, 1997) and functions as a transcription factor to regulate gene expression (Fuhrmann *et al.*, 2001), the target genes for GCNF in the ovary must be expressed in the oocytes. These potential GCNF target gene products can be secreted from the oocyte, and directly or indirectly regulate steroidogenesis in the somatic cells of the ovary. Oocyte-secreted transforming growth factor-β (TGF-β) family members, GDF-9 and BMP-15, are two candidate GCNF target genes, as GDF-9 and BMP-15 have been shown to be involved in paracrine signaling to adjacent somatic cells during steroidogenesis (Dong *et al.*, 1996;

Elvin *et al*., 1999a,b, 2000; Otsuka *et al*., 2000, 2001; Solovyeva *et al*., 2000; Vitt *et al*., 2000a,b). To gain insight into the molecular mechanisms of the ovarian defects in *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ females, the expression levels of several known oocyte-specific genes, including *BMP-15* and *GDF-9* in *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ females, were determined. As shown by RT–PCR (Figure 4G), the expression of *zona pellucida protein 2* (*Zp2*), *Zp3*, c-*mos*, *maternal antigen that embryos require* (*Mater*) and *oocyte-specific DNA methyltransferase-1* (*Dnmt1o*) were not altered in the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ ovaries at diestrus. However, the expression levels of *BMP-15* and *GDF-9* were increased >2-fold in the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ ovaries at diestrus but not at estrus or metestrus, compared with the levels in *GCNF*$^{+/+}$, *GCNF*$^{fl/fl}$ and *GCNF*$^{fl/+}$Zp3*Cre*$^{+}$ ovaries, as determined by northern blot analyses (Figure 4H–J). These results indicate that *BMP-15* and *GDF-9* are two potential target genes whose expression can be negatively regulated by GCNF within the oocyte at diestrus.

Expression of BMP-15 and GDF-9 is directly regulated by GCNF

The increased expression of *BMP-15* and *GDF-9* genes in *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ ovaries at diestrus (Figure 4H–J) prompted us to test whether these two genes can be directly regulated by GCNF. We searched the 5′ DNA sequences of mouse *BMP-15* and *GDF-9* genes in the Celera mouse genome database, and found that there are multiple putative DR0 elements in these two promoters with 1–4 bp mismatches from the consensus DR0 sequence (Figure 5A). Electrophoretic mobility shift assays (EMSAs) were then performed to determine whether GCNF can bind to these putative DR0 elements (Figure 5B). Radiolabeled DR0 oligonucleotides from the *Oct4* promoter, as previously reported (Fuhrmann *et al*., 2001), were used as a positive control for EMSAs. We found that two BMP-15 DR0s (BMP-15 DR0-1 and BMP15 DR0-2) and three GDF-9 DR0s (GDF-9 DR0-1, GDF-9 DR0-3 and GDF-9 DR0-5) can bind *in vitro* translated GCNF, forming a GCNF–DR0 DNA complex (Figure 5B, a). Addition of specific anti-GCNF antibodies (Lan *et al*., 2003) reduced the mobility of these GCNF–DR0 DNA complexes (Figure 5B, b). Although a DNA–protein complex was observed in EMSA using BMP15 DR0-6 as a probe (Figure 5B, a), addition of anti-GCNF antibodies did not super-shift this complex (our unpublished data), indicating that this complex does not contain GCNF. Our results suggest that GCNF can bind to multiple DR0 elements in the *BMP-15* and *GDF-9* promoters, forming GCNF–DR0 DNA complexes.

Having established that GCNF can bind to DR0 elements in the *BMP-15* and *GDF-9* promoters, we next performed promoter reporter analysis to determine whether GCNF can directly regulate the transcription of the *BMP-15* and *GDF-9* genes using transient transfection techniques in Chinese hamster ovary (CHO) cells. Promoter reporter plasmids pBMP-15-LUC, pGDF-9-LUC or pOct4-LUC were transfected into cultured CHO cells along with pCMV-HA-*GCNF* or pCMV-HA-*GCNFΔDBD* (Figure 5C). The expression levels of GCNF and GCNFΔDBD were determined by western blot analysis using anti-HA antibodies. As shown in Figure 5D, the amounts of GCNF protein in CHO cells transfected with 16.7, 50 and 150 ng/ml of pCMV-HA-*GCNF* were comparable to the levels of GCNFΔDBD in CHO cells transfected with 1.67, 5 and 15 ng/ml of pCMV-HA-*GCNFΔDBD*, respectively. In CHO cells transfected with pOct4-LUC plasmid, GCNF repressed *Oct4* reporter activity in a dose-dependent manner, whereas the deletion mutant of GCNF (GCNFΔDBD) did not have any effect on the *Oct4* promoter activity (Figure 5E). These results confirm our previous observation that the DBD of GCNF is essential for GCNF to repress *Oct4* expression in somatic cells of early mouse embryos and that the LBD of GCNF does not have DNA binding-independent activities during early mouse development (Lan *et al*., 2002). In cells transfected with pBMP-15-LUC, the *BMP-15* promoter activity was also repressed by GCNF, but not by GCNFΔDBD, in a dose-dependent manner (Figure 5F). Similarly, the *GDF-9* reporter activity in transfected CHO cells was repressed by GCNF, but not by GCNFΔDBD, in a dose-dependent manner (Figure 5G). As GCNF bound to BMP-15 DR0-1 and BMP-15 DR0-2 in the *BMP-15* promoter and GDF-9 DR0-5 in the *GDF-9* promoter (Figure 5B), and GCNFΔDBD did not have DNA binding activity to the consensus DR0 elements (our unpublished data), the repression of *BMP-15* and *GDF-9* reporter activity by GCNF might be due to the binding of GCNF to the DR0 elements via the DBD of GCNF. These results support our *in vivo* observation that the expression levels of *GDF-9* and *BMP-15* were increased in *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ ovaries at diestrus (Figure 4H–J), indicating that *GDF-9* and *BMP-15* are two direct target genes of GCNF in the oocytes at diestrus.

Discussion

The oocyte-specific *GCNF* expression pattern suggests that GCNF may play a role in female reproduction (Chen *et al*., 1994; Katz *et al*., 1997). The embryonic lethality of the *GCNF* knockout mice prevented us from analyzing its functional role during female reproduction (Chung *et al*., 2001; Lan *et al*., 2002). In this study, we successfully generated floxed GCNF mice that can by-pass early embryonic lethality using a Cre/loxP targeting strategy (Figure 1A). Using Zp3*Cre* transgenic mice (de Vries *et al*., 2000), we were able to generate oocyte-specific *GCNF* knockout mice, in which the *GCNF* gene was inactivated specifically in oocytes, not in granulosa cells, or in other organs such as pituitary, hypothalamus or uterus (Figure 1). Oocyte-specific *GCNF* knockout mice display reduced fertility (Figure 2), which is likely to be due to the prolonged estrous cycle, as the reduced numbers of estrous cycles per month (54% of the means of the three control groups) were almost the same as the numbers of litters per month (57% of the means of the three control groups) in the oocyte-specific *GCNF* knockout mice (Figure 2B and C). The prolonged estrous cycle observed in the oocyte-specific *GCNF* knockout mice appears to result from defects in ovarian somatic cells such as granulosa, thecal and luteal cells. In fact, circulating steroid hormones including estradiol, progesterone and testosterone levels, and gonadotropins FSH, but not LH, levels were reduced significantly at the diestrus stage of the estrous cycle in the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ mice (Figure 3). The reduced steroid hormone and FSH levels (Figure 3)

indicate that there is a defect in steroidogenesis in the somatic cells within the ovaries of the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ females at diestrus. To support this notion, ovarian somatic cell marker genes, *StAR*, *3βHSD I* and *17αOH*, which are involved in steroidogenesis, were found to be mis-expressed in the ovary at diestrus (Figure 4A–F, and Supplementary figure 1). The reduced expression of *StAR* (Figure 4A and B, and Supplementary figure 1), which mediates the rate-limiting step in steroidogenesis (Stocco, 2001), alone may well cause the reduced estradiol, progesterone and testosterone levels in the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ females at diestrus. The reduced expression of *3βHSD I* (Figure 4A, C and D, and Supplementary figure 1), an enzyme involved in the conversion of pregnenolone to progesterone (Bain *et al*., 1991), in combination with the increased expression of *17αOH* (Figure 4E and F, and Supplementary figure 1), an enzyme involved in the conversion of progesterone to 17α-hydroxypregnenolone (Lieberman and Warne, 2001), could well explain why progesterone levels are reduced more markedly than either estradiol or testosterone levels in the oocyte-specific *GCNF* knockout mice at diestrus (Figure 3). The reduced steroidogenesis could also be due to the reduced FSH levels in the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ mice at diestrus (Figure 3), as FSH/FSHR signaling is required for female fertility, normal folliculogenesis and steroidogenesis (Kumar *et al*., 1997; Dierich *et al*., 1998; Danilovich *et al*., 2002; Eimerl and Orly, 2002). However, the mechanism by which inactivation of GCNF in oocytes causes reduction in serum FSH levels in the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ mice at diestrus remains to be elucidated. Taken together, the oocyte-specific *GCNF* knockout mice have defects in steroidogenesis of the ovary at the diestrus stage of the estrous cycle, and these defects in steroidogenesis are likely the cause of the abnormal estrous cycle, leading to female subfertility.

Importantly, because *GCNF* is expressed in germ cells (Chen *et al*., 1994; Katz *et al*., 1997) and functions to repress gene transcription (Fuhrmann *et al*., 2001), the aberrant steroidogenesis observed in the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ females is likely due to mis-regulated oocyte paracrine signaling molecules. There is a large body of evidence indicating that the oocyte plays an active role in orchestrating and coordinating the development of follicles by secreting a factor that affects granulosa cell differentiation, steroidogenesis and proliferation (Vanderhyden and Tonary, 1995; Eppig, 2001; Eppig *et al*., 2002; Matzuk *et al*., 2002). Two TGF-β family members, GDF-9 and BMP-15, are oocyte-secreted factors involved in the regulation of adjacent somatic cell function in the ovary (Eppig, 2001; Matzuk *et al*., 2002). *GDF-9*$^{-/-}$ mice are infertile with defects in follicular development beyond the primary stage and thecal cell development (Dong *et al*., 1996; Elvin *et al*., 1999b). Elevated progesterone and FSH levels and mis-expression of ovarian steroidogenic genes in somatic cells such as *17αOH* in thecal cells have been reported in *GDF-9*$^{-/-}$ mice (Dong *et al*., 1996; Elvin *et al*., 1999b). Regulation of steroidogenesis by recombinant GDF-9 has also been documented, not only in granulosa cells (Elvin *et al*., 1999a, 2000; Vitt *et al*., 2000a), but also in thecal cells (Solovyeva *et al*., 2000; Vitt *et al*., 2000b). BMP-15, another oocyte-secreted TGF-β family member, also modulates follicular development and steroidogenesis in ovarian somatic cells (Otsuka *et al*., 2000, 2001; Yan *et al*., 2001). Therefore, reduced steroid hormone levels and mis-expression of *StAR*, *3βHSD I* and *17αOH* in the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ ovary at diestrus (Figures 3 and 4A–F, and Supplementary figure 1) are likely due to aberrant expression of *BMP-15* and *GDF-9* in the oocyte. In fact, we found that *BMP-15* and *GDF-9* expression levels were up-regulated in the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ ovary at diestrus (Figure 4H–J). As ovarian size and weight of *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ females and ovarian morphology were similar to those of control littermates (*GCNF*$^{+/+}$, *GCNF*$^{fl/fl}$ and *GCNF*$^{fl/+}$Zp3*Cre*$^{+}$ mice) (Figure 2E), and there were no changes in expression levels of oocyte-specific genes, *Dnmt1o*, *Mater*, *Zp2*, *Zp3* and c-*mos* (Figure 4G), the increased expression levels of *BMP-15* and *GDF-9* at diestrus (Figure 4H–J) are not due to increased oocyte numbers within the ovary nor oocyte size. Rather, our results indicate that the expression of the *BMP-15* and *GDF-9* genes can be negatively regulated by GCNF within the oocyte at diestrus. Using EMSAs, we were able to show that GCNF can bind to multiple DR0 elements in the *BMP-15* and *GDF-9* promoters (Figure 5B). In cultured CHO cells, both the *BMP-15* and *GDF-9* promoter activities were repressed by GCNF (Figure 5F and G). These results demonstrate that *BMP-15* and *GDF-9* are direct target genes of GCNF in oocytes. The diestrus stage-specific overexpression of *BMP-15* and *GDF-9* (Figure 4H–J) correlates well with the reduced steroid hormone levels (Figure 3) and mis-expression of *StAR*, *3βHSD I* and *17αOH* in the *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ mice at diestrus (Figure 4A–F, and Supplementary figure 1). Therefore, overexpression of *GDF-9* and *BMP-15* in oocytes is a primary defect in the oocyte-specific *GCNF* knockout mice, and might be the cause of aberrant expression of ovarian steroidogenic genes, *StAR*, *3βHSD I* and *17αOH* in the somatic cells, which leads to reduced circulating steroid hormone levels at the diestrus stage of the estrous cycle.

BMP-15$^{-/-}$ mice are subfertile with reduced litter sizes and reduced numbers of litters per month and have multiple oocyte follicles, and introducing a mutant *GDF-9* allele into *BMP-15*$^{-/-}$ mice further reduces female fertility (Yan *et al*., 2001), whereas the *GDF-9* knockout mice are infertile (Dong *et al*., 1996). Ablation of BMPRIB/ALK6, a putative BMP-15/GDF-9 receptor that is expressed in the granulosa cells of the ovary, also causes prolonged diestrus of the estrous cycle with reduced estradiol biosynthesis in the mouse ovary (Yi *et al*., 2001). These loss-of-function studies show that BMP-15/GDF-9 signaling in the ovary is important for proper follicular development and efficient female fertility. In this study, we found that the inactivation of the *GCNF* gene in oocytes, which directly caused overexpression of *BMP-15* and *GDF-9* (gain-of-function) at the diestrus stage of the estrous cycle (Figure 4H–J), also led to female hypofertility, with reduced litter sizes, reduced numbers of litters per month, a prolonged estrous cycle and the formation of double oocyte follicles (Figure 2). Therefore, it appears that the maintenance of precise expression levels of *BMP-15* and *GDF-9* in oocytes is essential for efficient female fertility and proper follicular development. The presence of regulatory feedback systems between the oocyte *BMP-15*/*GDF-9* and granulosa cell *kit ligand* characterized

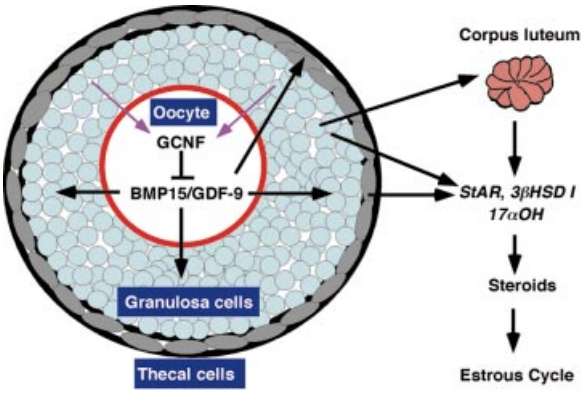

Fig. 6. A model for GCNF regulation of BMP-15/GDF-9 signaling during female reproduction. GCNF represses BMP-15/GDF-9 expression, which in turn affects steroidogenesis and female fertility.

recently (Elvin *et al*., 1999b; Joyce *et al*., 2000; Otsuka and Shimasaki, 2002) could be the mechanism of maintaining the appropriate levels of BMP-15 and GDF-9 in oocytes required to exert their physiological functions.

Similar to *BMP-15*$^{-/-}$, *GDF-9*$^{+/-}$*BMP-15*$^{-/-}$ (Yan *et al*., 2001), *FSHR*$^{+/-}$ (Danilovich and Sairam, 2002), genistein- or diethylstibestrol-treated (Iguchi *et al*., 1990; Jefferson *et al*., 2002), and rat inhibin α transgenic (McMullen *et al*., 2001) mice, some double-oocyte follicles were observed in the oocyte-specific *GCNF* knockout (Figure 2E). This phenotype is unlikely to be due to the aberrant ovarian expression of *FSHR* and *inhibin* α expression in the somatic cells, as expression of these two genes were not affected in *GCNF*$^{fl/fl}$Zp3*Cre*$^{+}$ mice at diestrus (Figure 4, and Supplementary figure 1). Rather, it could be due to defects in the granulosa cells (e.g. steroidogenesis and estradiol-induced estrogen receptor expression) at diestrus caused by the abnormal GDF-9 and BMP-15 signaling from the oocyte, which was resulted from the inactivation of GCNF in the oocyte.

Based on our results, we propose a model for the function of GCNF in the ovary during female reproduction (Figure 6). In the oocyte, GCNF binds to DR0 elements in the *GDF-9* and *BMP-15* promoters and represses their transcription to maintain appropriate levels of GDF-9 and BMP-15 at diestrus. The loss of repression activity of GCNF causes abnormal *BMP-15/GDF-9* expression in oocytes, leading to aberrant BMP-15 and GDF-9 signaling to the somatic cells of the ovary. Mis-expression of *StAR*, *3βHSD I* and *17αOH* in the somatic cells of the ovary, caused by abnormal BMP-15/GDF-9 signaling, results in reduced steroid hormone levels, leading to an aberrant estrous cycle that affects female fertility. Somatic cells may play a feedback role in regulating oocyte GCNF activity (pink arrows in Figure 6) in a ligand-dependent or -independent manner.

In summary, we have successfully generated an oocyte-specific *GCNF* knockout mouse model that displays hypofertility, abnormal estrous cycle, aberrant steroidogenesis and double-oocyte follicles in the ovaries. These reproductive defects are secondary to the primary defect, which is up-regulation of the TGF-β family members BMP-15 and GDF-9 in oocytes at diestrus. We show that GCNF can directly repress expression of the *BMP-15* and *GDF-9* genes, an effect mediated via direct interactions of GCNF with multiple DR0 elements in the promoters of both genes. The oocyte-specific *GCNF* knockout mouse model has uncovered a new regulatory pathway in ovarian function. We conclude that a paracrine regulatory pathway exists among oocytes and adjacent somatic cells to modulate the estrous cycle during female reproduction, and this function is mediated by BMP-15 and GDF-9, whose expression is regulated by GCNF in oocytes.

Materials and methods

Generation of GCNF^{fl} mice and mouse genotyping

GCNF^{fl} mice were generated by homologous recombination in the AB1.2 ES cell line using the Cre/loxP system (Figure 1A). *GCNF*$^{fl/+}$ ES clones were obtained after *GCNF*^{3lox} ES cells (Lan *et al*., 2002) were transiently transfected with a Cre expression vector, pOG231 (O'Gorman *et al*., 1997). Microinjection of *GCNF*$^{fl/+}$ ES cells into mouse C57BL/6 blastocysts produced chimeric animals. Breeding of male chimeras (>90% agouti coat color) with C57BL/6 females produced *GCNF*$^{fl/+}$ mice. Mouse tail genomic DNA was extracted and genotyped either by Southern blot analysis using a 5′ *GCNF* probe (Chung *et al*., 2001) or by PCR using different sets of primers listed in the Supplementary data.

RNA analysis

Total ovarian RNA was isolated using Trizol reagent (Invitrogen). RT–PCR and quantitative RT–PCR analyses are described in the Supplementary data. *In situ* hybridization and northern blot analyses were performed as described previously (Katz *et al*., 1997). Mouse cDNA probes used for northern blot analyses were as follows (nucleotides and DDBJ/EMBL/GenBank accession Nos): *StAR* (821–1436 of L36062), *17αOH* (813–1433 of M64863), *P450scc* (19–696 of NM_019779), *Cyp19* (64–544 of NM_007810), *20αHSD* (376–1060 of AB059565), *activin* β*B* (14–548 of X83376), *3βHSD I* (466–1214 of M58567), *GDF-9* (17–1360 of L06444) and *BMP-15* (1–2000 of AF082348). The same blots were then reprobed with glyceraldedyde-3-phosphate dehydrogenase (*GAPDH*) as a loading and quality control. Quantitative mRNA levels of *3βHSD I*, *17αOH*, *BMP-15* and *GDF-9* were determined by ImageQuant program, and normalized to the GAPDH mRNA levels in the northern blots.

Collection of oocytes and cumulus cells, classification of the estrous cycle and hormone assays

Oocytes and cumulus cells were collect from 1- to 2-month-old animals after PMSG/hCG treatment as described previously (Elvin *et al*., 2000). The estrous cycle of adult mice were determined as reported previously (Jablonka-Shariff *et al*., 1999; Nothnick, 2000). Each cycle length was determined as the length of time between two consecutive occurrences of estrus in the animals that had progressed through at least three consecutive estrous cycles. Serum estradiol, progesterone, testosterone, FSH and LH levels were determined by the Core Ligand and Assay Laboratory (University of Virginia) using radioimmunoassay kits.

Histological analysis

Ovaries were fixed in 4% paraformaldehyde, dehydrated and then embedded in paraffin. The paraffin-embedded tissues were serially sectioned at 7 μm thickness and stained with hematoxylin and eosin. β-galactosidase staining of tissue samples was performed according to the manufacturers protocol (Specialty Media, NJ). After staining, tissues were post-fixed, dehydrated, embedded and then sectioned followed by counter-staining with 0.1% Nuclear Fast Red.

EMSAs

EMSAs were performed as described previously (Cooney *et al*., 1998). Production of *in vitro* translated GCNF and GCNF antibodies were described previously (Lan *et al*., 2003). The *Oct4* DR0 sequences of the double-stranded oligonucleotide probe for EMSAs were as reported previously (Fuhrmann *et al*., 2001). The sequences of the double-stranded oligonucleotide probes, containing putative DR0 elements in the *BMP-15* and *GDF-9* promoter, for EMSAs are listed in the Supplementary data.

Cell culture, western blot analysis, plasmid construction and transient transfection assays

CHO cells were cultured in Ham's F12 media (Invitrogen) with 10% fetal calf serum. A *BMP-15* promoter DNA fragment from –2993 to +209 nt was obtained from a BAC clone (RP24-294F7, BAC-PAC; CHORI, CA) and inserted into the *Kpn*I and *Hin*dIII sites of the pGL-3-basic luciferase reporter vector (Promega) to generate the pBMP-15-LUC plasmid. The *GDF-9* promoter reporter plasmid, pGDF-9-LUC, was generously provided by Dr M.Matzuk, in which a *GDF-9* promoter DNA fragment (–3277 to +108 nt) containing GDF-9 DR0-5 was cloned into the pGL3-basic reporter vector. The *Oct4* promoter reporter plasmid, pOct4-LUC (Fuhrmann *et al*., 2001), was used as a positive control for the transfection experiments. Expression vectors, pCMV-*GCNF* and pCMV-*GCNFΔDBD*, were constructed by inserting the full-length mouse *GCNF* cDNA (Chen *et al*., 1994) and the LBD encoding mouse *GCNF* cDNA sequences into the pCMV-HA vector (Promega), respectively. Cells were transiently transfected with plasmids for 48 h, using Fugene 6 (Roche) as described previously (Fuhrmann *et al*., 2001). Western blot analysis with anti-HA antibodies was performed as reported previously (Lan *et al*., 2003). Luciferase activities in the samples were determined using a firefly luciferase assay system (Promega), and normalized to the co-reporter activity of the pRL-TK Renilla luciferase control vector (Promega).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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