***Pcsk6* mutant mice exhibit progressive loss of ovarian function, altered gene expression, and formation of ovarian pathology**

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Abstract

Bioactivation of precursor proteins by members of the proprotein convertase (PC) family is essential for normal reproduction. The *Pcsk6* gene is a member of the PC family that is expressed in numerous ovarian cell types including granulosa cells and oocytes. We hypothesized that loss of PCSK6 would produce adverse effects in the mouse ovary. Mice incapable of expressing PCSK6 (*Pcsk6*^{tm1Rob}) were obtained, and reproductive parameters (serum hormones, whelping interval, estrus cyclicity, and fertility) were compared to *Pcsk6*^{+/+} mice. While *Pcsk6*^{tm1Rob} female mice are fertile, they manifest reduced reproductive capacity at an accelerated rate relative to *Pcsk6*^{+/+} mice. Reproductive senescence is typically reached by 9 months of age and is correlated with loss of estrus cyclicity, elevated serum FSH levels, and gross alterations in ovarian morphology. A wide range of ovarian morphologies were identified encompassing mild, such as an apparent reduction in follicle number, to moderate – ovarian atrophy with a complete absence of follicles – to severe, manifesting as normal ovarian structures replaced by benign ovarian tumors, including tubulostromal adenomas. Targeted gene expression profiling highlighted changes in RNA expression of molecules involved in processes such as steroidogenesis, gonadotropin signaling, transcriptional regulation, autocrine/paracrine signaling, cholesterol handling, and proprotein bioactivation. These results show that PCSK6 activity plays a role in maintaining normal cellular and tissue homeostasis in the ovary.

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Introduction

The subtilisin-like proprotein convertase (PC) family of serine endoproteases is one class of enzymes that facilitate proprotein cleavage into their bioactive mature forms (reviewed in Seidah & Chretien (1999)). There is growing evidence that alterations in PC activity contribute to human pathologies including cancer, viral infections, and metabolic disorders (Taylor *et al.* 2003, Bassi *et al.* 2005, Maxwell & Breslow 2005, Seidah *et al.* 2006, Iatan *et al.* 2009). Seven mammalian PCs cleave precursor proteins at basic amino acid residues with the motif (K/R)-(X)_n-(K/R) (K=lysine, R=arginine, X=any amino acid): PCSK1–7. The PCs SKI-1/S1P and PCSK9 cleave substrates at non-basic residues. PC substrates encompass numerous protein precursors such as peptide hormones, growth and differentiation factors, receptors, proteases, and adhesion molecules (Seidah & Chretien 1999, Seidah *et al.* 2006). Known substrates that participate in reproductive physiology include pro-GNRH, pro-IGF1, IGF1 pro-receptor, pro-fertilins, pro-pituitary adenylate cyclase-activating polypeptide,

and members of the transforming growth factor β (TGFB) superfamily (Seidah & Chretien 1999).

PCSK6 exhibits a broad pattern of expression including heart, brain, placenta, lung, liver, uterine endometrium, and ovary (Kiefer *et al.* 1991, Fu *et al.* 2003, Freyer *et al.* 2007, Diaz *et al.* 2008). PCSK6 is a secreted, heparin-binding protein that is tethered to the plasma membrane through binding with members of the tissue inhibitors of metalloproteinase family (Tsuji *et al.* 2003, Jin *et al.* 2005, Nour *et al.* 2005). While PCSK6 is capable of cleaving a variety of substrates *in vitro*, it has been difficult to assign specific substrate partners to individual proteases due to redundant proteolytic activities shared among related family members, e.g. PCSK3 (furin). The best evidence for *bona fide* targets of PCSK6 comes from a combination of *in vivo* and *in vitro* experiments. These substrates include nodal (Constam & Robertson 2000, Beck *et al.* 2002), which is critical for body axis formation, and endothelial lipase (Gauster *et al.* 2005, Jin *et al.* 2005, Nour *et al.* 2005), a member of the triglyceride lipase family that regulates plasma lipoprotein concentrations (Ishida *et al.* 2003, Ma *et al.* 2003).

In mice, PCSK6 protein is encoded by the *Pcsk6* gene (Mbikay *et al.* 1995). Recent work from Diaz *et al.* (2008) has shown that expression of *Pcsk6* RNA in the mouse ovary corresponds primarily to granulosa cells of small to medium preantral follicles. St Germain *et al.* (2005) have shown that *Pcsk6* was expressed by oocytes. Diaz *et al.* have further shown that *Pcsk6* mRNA expression was suppressed during the preantral to antral transition through oocyte-derived factors. These investigators speculated that PCSK6 might have an important function in the proteolytic processing of ovarian substrates critical to maintain normal follicular biology. Although the dynamic change in PCSK6 expression suggests that an alteration in substrate processing or activity occurs during the preantral to antral transition, it is not definitively known whether PCSK6 activity is required for follicle maturation or any other aspects of ovarian function.

Based on the pattern of expression in the mouse ovary and the putative PCSK6 substrates that participate in reproductive physiology, we hypothesized that loss of PCSK6 expression would generate an ovarian phenotype. In particular, we would predict a defect in follicle biology that may be complemented by alterations in ovarian morphology. To investigate the relative contribution of *Pcsk6* deletion to ovarian biology in an experimental model system, we obtained mice that were incapable of expressing PCSK6 (*Pcsk6*^{tm1Rob}) from the laboratory of Dr Daniel Constam (Swiss Institute for Experimental Cancer Research; Constam & Robertson 2000). Approximately 25% of *Pcsk6*^{tm1Rob} mice exhibit left-right axis defects, severe craniofacial abnormalities, and embryonic lethality by embryonic day (e) 15.5 (Constam & Robertson 2000). Approximately, for the 75% of mice that are viable, it is reported that these animals are capable of reproducing (Constam & Robertson 2000), but no in-depth analyses of their reproductive biology have been published. With the availability of viable *Pcsk6*^{tm1Rob} mice, we can determine whether the loss of PCSK6 has an impact on ovarian biology. This study examined ovarian morphology, ovulation, fertility, hormone production, and gene expression in *Pcsk6*^{tm1Rob} mice. We determined that loss of *Pcsk6* expression results in premature reproductive senescence at ~9 months (mo) of age in addition to the development of ovarian morphological pathologies ranging from an apparent reduction in follicle number to tumor formation.

Results

Pcsk6^{tm1Rob} female mice have a reduced reproductive capacity

To determine the effect that PCSK6 expression may have on female reproductive capacity, *Pcsk6*^{+/+} and *Pcsk6*^{tm1Rob} female mice were mated with *Pcsk6*^{+/+} male mice. Pairs capable of producing a minimum of

three litters over the study period were included in these analyses (one *Pcsk6*^{+/+} pair and three *Pcsk6*^{+/+} male × *Pcsk6*^{tm1Rob} female pairs were excluded from these analyses). The comparison of the mean number of pups born per litter was not statistically significant; 6.6 for *Pcsk6*^{+/+} pairs (8 pairs, 44 litters) and 7.5 for *Pcsk6*^{+/+} male × *Pcsk6*^{tm1Rob} female pairs (9 pairs, 49 litters; Table 1). Also, while the total number of litters born was not significantly different between the groups, the *Pcsk6*^{tm1Rob} mice produced the majority of their litters up to 6 mo of age, with fewer litters born between 6 and 9 mo. By contrast, the number of litters born to *Pcsk6*^{+/+} animals was evenly distributed throughout the breeding period. We observed that for mice used to maintain our breeding colonies, ~25% of *Pcsk6*^{+/+} male × *Pcsk6*^{tm1Rob} female matings (5 of 18 pairings) never produced a single litter, whereas only 1 of 21 matings of *Pcsk6*^{+/+} pairs did not produce offspring. Female *Pcsk6*^{tm1Rob} mice showed a significantly increased whelping interval (32.4 ± 3.1 days) compared with *Pcsk6*^{+/+} mice (23.5 ± 0.6 days; Table 1, *P* < 0.05). Furthermore, the median age of last litter for *Pcsk6*^{tm1Rob} female mice was 2 mo earlier than that of *Pcsk6*^{+/+} mice (8 vs 10 mo). While not statistically different, a loss of 2 months of breeding capacity is biologically important and likely reflective of aberrant ovarian biology in these mice. The percentage of mice exhibiting regular estrous cyclicity was also compared (Table 2). As early as 7 mo, the proportion of *Pcsk6*^{tm1Rob} mice cycling regularly had decreased to 58%, and by 9–10 mo, this proportion had dropped to 16% and remained

Table 1 Number of litters, average litter size, and whelping interval for wild-type (+/+) and *Pcsk6*^{tm1Rob} (–/–) mice.

Genotype female × male	Number of litters ^a	Average litter size ^a	Average whelping interval (days) ^b
+/+ × +/+			
259 × 244	7	4.8	24
264 × 275	5	8.8	22
265 × 276	9	3.6	21.5
287 × 288	7	8.3	26
289 × 290	9	6.4	22.9
322 × 324	2	7.5	26
1367 × 1368	3	7.0	23.5
1369 × 1370	2	6.5	22
Total = 44		Mean = 6.6	Mean = 23.5
–/– × +/+			
123 × 75	7	9.0	37.8
190 × 243	7	11.0	32.5
268 × 242	7	8.4	29.8
308 × 247	7	8.6	28.8
317 × 245	6	7.0	25.4
318 × 246	2	2.5	46
402 × 378	3	6.7	21
403 × 320	8	7.7	23.6
419 × 415	2	7.0	47
Total = 49		Mean = 7.5	Mean = 32.4

^aNo statistically significant differences between +/+ × +/+ and –/– × +/+ pairings. ^bWhelping interval is significantly different between +/+ × +/+ and –/– × +/+ pairings (*P* < 0.05).

Table 2 Examination of estrous cyclicity in virgin female mice stratified by age and genotype.

Age (months)	Incidence of regular estrus cyclicity (%)	
	<i>Pcsk6</i> ^{+/+}	<i>Pcsk6</i> ^{tm1Rob}
4–6	100 (28)	95 (20)
7–8	82 (17)	58 (29)
9–10	95 (21)	16 (24)
11–13	63 (19)	25 (31)

The number in parentheses indicates the number of animals examined for each condition.

low thereafter. Abnormal estrous cycles typically manifested as complete acyclicity. By contrast, the majority of *Pcsk6*^{+/+} mice cycled regularly until 13 mo.

The ovulatory capacity of *Pcsk6*^{tm1Rob} mice is decreased in response to exogenous hormonal stimulation

Superovulation in response to exogenous gonadotropins was tested in mice at 30 d and 3 mo. At 30 d, there was no statistically significant difference in the number of oocytes released by *Pcsk6*^{+/+} (40.8 ± 5.1) or *Pcsk6*^{tm1Rob} (51.8 ± 5.4) mice. By 3 mo, *Pcsk6*^{tm1Rob} mice showed a significantly reduced ovulatory response (24.0 ± 3.1) compared with *Pcsk6*^{+/+} (35.5 ± 3.8) animals ($P < 0.05$).

Basal and eCG-stimulated serum steroids are equivalent between young female *Pcsk6*^{+/+} and *Pcsk6*^{tm1Rob} mice

Saline- and eCG-stimulated serum steroid levels (progesterone (P₄), testosterone, and estradiol (E₂)) from cycling female mice ranging from 30 to 48 days were measured at 48 and 60 h post injection (Table 3). It should be noted that our initial studies examined serum steroid levels at 48 h in *Pcsk6*^{+/+} and *Pcsk6*^{tm1Rob} mice, but we did not observe the expected change in E₂ production frequently reported in the literature. This is likely a factor inherent to our colony. As such, we extended our analyses to examine responses at 60 h post eCG injection. Increases in serum steroids were measured in both genotypes in response to eCG.

***Pcsk6*^{tm1Rob} female mice exhibit altered gene expression in response to exogenous eCG**

Ovarian expression of selected target genes from female mice ranging from 30 to 48 days was examined by quantitative RT-PCR (QPCR) 60 h after saline or eCG injection (Fig. 1; Supplementary Figure 1, see section on supplementary data given at the end of this article, 48 h). Similarities in the expected responses to eCG were observed for several known target genes; the relative basal level or magnitude of the response was

significantly lower for *Fshr* and *Cyp19a1* in *Pcsk6*^{tm1Rob} mice respectively.

RNA levels for the PCSK6 substrate endothelial lipase (*Lipg*) were significantly increased by eCG treatment, and the magnitude of response was significantly higher in *Pcsk6*^{tm1Rob} mice. Transcript levels for nodal were also increased by eCG in *Pcsk6*^{+/+} mice, but there was no increase observed in *Pcsk6*^{tm1Rob} mice. Analysis of PC transcript levels showed that the expression of *Pcsk4* was significantly decreased in response to eCG, whereas the expression of *Pcsk9* was increased. There was little difference in the basal or eCG-induced expression profile for *Pcsk3*, *Pcsk5*, or *Pcsk7* (not shown). Moreover, in *Pcsk6*^{+/+} mice, the expression of *Pcsk6* RNA was significantly lower at 48 h in response to eCG, most likely reflecting the suppressive effects produced by fully grown oocytes as the number of mature antral follicles increases (Diaz *et al.* 2008). However, *Pcsk6* RNA expression returned to basal levels by 60 h post eCG treatment. The dynamic change in expression suggests a role for select PCs and their substrates during follicle maturation, and demonstrates altered regulation of several hormonally responsive genes in *Pcsk6*^{tm1Rob} mice.

***Pcsk6*^{tm1Rob} female mice exhibit a premature rise in serum FSH levels**

Serum FSH and LH were measured in mice ranging from 3 to 15 mo (Table 4). FSH levels remained normal in *Pcsk6*^{+/+} mice until they were 13–15 mo, the age at which these mice show signs of reproductive senescence (Nelson *et al.* 1982). By contrast, FSH levels were prematurely elevated at 9 mo in *Pcsk6*^{tm1Rob} mice. No differences in serum LH were identified at any age (Table 4).

Ovaries from *Pcsk6*^{tm1Rob} mice display abnormal morphologies

The morphology of ovaries from female mice at 1–20 mo was compared. We observed that there did not appear to be gross abnormalities with any other organ systems,

Table 3 Mean serum steroid levels from saline- or eCG-treated female mice at 48 or 60 h post injection.

Genotype	Treat-ment	Progesterone (ng/ml)	Testosterone (ng/ml)	Estradiol (pg/ml)
48 h				
<i>Pcsk6</i> ^{+/+}	Saline	3.3 ± 1.3	42.8 ± 2.8	–
	eCG	15.1 ± 3.2 ^a	460.2 ± 145.4 ^a	–
<i>Pcsk6</i> ^{tm1Rob}	Saline	3.4 ± 0.4	56.34 ± 8.5	–
	eCG	21.3 ± 3.8 ^a	392.0 ± 160.3	–
60 h				
<i>Pcsk6</i> ^{+/+}	Saline	11.20 ± 3.9	0.1 ± 0.04	44.4 ± 8.6
	eCG	88.81 ± 9.7 ^a	0.9 ± 0.14 ^a	137.2 ± 16.7 ^a
<i>Pcsk6</i> ^{tm1Rob}	Saline	14.08 ± 9.1	0.09 ± 0.007	48.53 ± 17.3
	eCG	63.25 ± 13.0 ^a	1.2 ± 0.2 ^a	177.9 ± 14.7 ^a

Estradiol was undetectable at 48 h.

^aSignificant difference between saline- and eCG-treated $P < 0.01$.

Figure 1 Relative expression 60 h post eCG injection. Gene expression was analyzed by QPCR in ovaries isolated from 30- to 48-day-old *Pcsk6^{+/+}* (WT) and *Pcsk6^{tm1Rob}* mice 60 h post eCG injection. *Pcsk6^{+/+}* + PBS ($N=10$), *Pcsk6^{+/+}* + eCG ($N=12$), *Pcsk6^{tm1Rob}* + PBS ($N=4$), *Pcsk6^{tm1Rob}* + eCG ($N=5$). #Significant difference between saline- and eCG-treated groups, *significant difference between *Pcsk6^{+/+}* and *Pcsk6^{tm1Rob}* groups; $P<0.05$.

with the exception that *Pcsk6^{tm1Rob}* mice are often born with only one eye, the other eye socket being empty. This is consistent with the cyclopia phenotype as part of the complex craniofacial abnormalities observed in e13.5–15.5 embryos as described originally (Constam & Robertson 2000).

A morphological diagnosis of normal was defined as ovaries containing follicular structures representing the different stages of maturation, corpora lutea, and ovarian surface epithelial and interstitial cells that did not exhibit any unusual features (e.g. hyperplasia). As a normal aspect of the follicle cycle, the presence of degenerating follicles was observed in sexually mature animals from both groups. Prior to 6 mo, the majority of ovaries were identified as normal (Fig. 2A and B). Occasionally, interstitial cell hyperplasia with hypertrophy, represented by clusters of large, multinucleated cells with granular cytoplasm, was observed to be present in ovaries of *Pcsk6^{+/+}* and *Pcsk6^{tm1Rob}* mice starting at 4.5 mo (Fig. 2C and D, arrow). While corpus luteal hyperplasia (Fig. 2E, arrows) and luteomas (Fig. 2F, arrows) were

observed in *Pcsk6^{+/+}* and *Pcsk6^{tm1Rob}* mice, luteomas were first observed in *Pcsk6^{tm1Rob}* mice starting as early as 3 mo; whereas luteomas were only observed in *Pcsk6^{+/+}* mice starting at 9 mo. From 9 mo onward, there was a similar incidence of luteoma formation in both genotypes (*Pcsk6^{+/+}* = 20.6% (7/34) and *Pcsk6^{tm1Rob}* = 23.9% (11/46)); however, the *Pcsk6^{tm1Rob}* ovaries typically displayed additional pathologies, such as cysts or a lack of follicles at any stage.

Approximately, 20% of *Pcsk6^{tm1Rob}* mice between 6 and 14 mo had ovaries with normal histological features similar to those from age-matched *Pcsk6^{+/+}* mice (Fig. 3A). The majority of the ovaries from *Pcsk6^{tm1Rob}* animals displayed complex morphologies including an apparent reduction or absence of follicles (Fig. 3B–E), ovarian atrophy (Fig. 3C), proteinaceous (Fig. 3E) or hemorrhagic (Fig. 3F) cysts in the presence or absence of follicles, and epithelial inclusion cysts (Fig. 3G and H). The lumen of cysts was typically lined with one to five cell layers of columnar or cuboidal epithelial cells (Fig. 3H). Small proteinaceous cysts were occasionally observed in *Pcsk6^{+/+}* mice prior to 14 mo ($n=4/46$), but healthy follicles representing the different stages of maturation were always present in these ovaries.

The most severely affected ovaries from *Pcsk6^{tm1Rob}* mice exhibited complete ovarian atrophy with a lack of follicular structures (Fig. 3C). Furthermore, ~11% (11/97) of the *Pcsk6^{tm1Rob}* animals developed tubulostromal adenomas, composed of a mass of dilated tubules lined by non-ciliated cuboidal epithelium (Fig. 4A and B). These structures were typically separated by various sized large round to polygonal cells resembling stromal interstitial cells. Other benign tumors, such as cystadenoma ($n=5$; Fig. 4C and D) or granulosa cell tumor ($n=2$; Fig. 4E and F), were also identified. Normal ovarian architecture was typically obliterated in *Pcsk6^{tm1Rob}* mice presenting with tubulostromal adenoma, cystadenoma, or granulosa cell tumors. As was the case with the original females used to establish the colony (Supplementary Figure 2 and Supplementary Table 1, see section on supplementary data given at the

Table 4 Mean serum FSH and LH levels.

Age (months)	<i>n</i>	Genotype	Mean FSH ± S.E.M. (ng/ml)	Mean LH ± S.E.M. (ng/ml)
3–6	15	<i>Pcsk6^{+/+}</i>	9.1 ± 1.8	1.4 ± 0.4
	12	<i>Pcsk6^{tm1Rob}</i>	17.9 ± 7.4	0.9 ± 0.3
7–8	7	<i>Pcsk6^{+/+}</i>	6.9 ± 0.7	0.4 ± 0.08
	14	<i>Pcsk6^{tm1Rob}</i>	13.4 ± 5.4	0.7 ± 0.1
9	9	<i>Pcsk6^{+/+}</i>	10.8 ± 5.4	0.7 ± 0.1
	11	<i>Pcsk6^{tm1Rob}</i>	33.8 ± 8.9 ^a	0.8 ± 0.2
10–12	10	<i>Pcsk6^{+/+}</i>	7.3 ± 0.9	0.8 ± 0.1
	17	<i>Pcsk6^{tm1Rob}</i>	36.3 ± 8.3 ^a	1.3 ± 0.6
13–15	13	<i>Pcsk6^{+/+}</i>	33.8 ± 5.7	0.9 ± 0.3
	15	<i>Pcsk6^{tm1Rob}</i>	43.7 ± 4.5	0.9 ± 0.2

^asignificant difference between *Pcsk6^{+/+}* and *Pcsk6^{tm1Rob}* serum levels, $P<0.05$.

Figure 2 Ovarian morphology common in *Pcsk6*^{+/+} and *Pcsk6*^{tm1Rob} mice. Representative images of normal ovarian morphology in *Pcsk6*^{+/+} (A) and *Pcsk6*^{tm1Rob} (B) mice at 3 mo. Interstitial cell hyperplasia and hypertrophy were observed in some 4.5-month-old *Pcsk6*^{+/+} (C) and *Pcsk6*^{tm1Rob} (D) mice (arrows). Corpora luteal hyperplasia (E) and multiple luteomas (F) were also observed in both *Pcsk6*^{+/+} and *Pcsk6*^{tm1Rob} mice (arrows). (E) *Pcsk6*^{+/+} mouse at 6 mo. (F) *Pcsk6*^{tm1Rob} at 4 mo mouse. Scale bars are indicated with a line below the image. For A and B, the scale bar is equivalent to 800 μm; C and D, 50 μm; E and F, 600 μm.

end of this article), many *Pcsk6*^{tm1Rob} animals that were fertile were found to possess abnormal ovarian morphologies after they were harvested (Figs 3B, C, E, 4A and C). However, *Pcsk6*^{tm1Rob} mice often reached reproductive senescence at an earlier age than *Pcsk6*^{+/+} littermates (~8 mo). After 14 mo, ovarian atrophy was commonly observed in all animals. No adenomas or granulosa cell tumors were identified in any *Pcsk6*^{+/+} animals up to 20 mo.

Aging *Pcsk6*^{-/-} female mice exhibit differential expression of genes that contribute to reproductive function

To obtain further insight into biochemical pathways or effector molecules that may be altered in aging mice, total RNA isolated from one ovary of estrus stage-matched 6-mo-old *Pcsk6*^{+/+} and *Pcsk6*^{tm1Rob} female mice was used for QPCR analysis (four animals in each

group; Table 5). This age was selected because gross morphological differences were typically not detected prior to 6 mo, and we hypothesized that this time point might provide an expression profile reflective of molecular alterations preceding gross morphological changes. Mild interstitial cell hyperplasia was observed in the contralateral ovary of one *Pcsk6*^{+/+} mouse (Fig. 3C) and two *Pcsk6*^{tm1Rob} mice, but the others were morphologically indistinguishable. These analyses identified changes in expression of genes associated with gonadotropin signaling, steroid hormone production, or reproductive growth factors. For example, *Fshr* expression was increased 2.4-fold, which correlated with increased expression of downstream targets including LH receptor (*Lhcgr*) and *Cyp19a1*, 5.0- and 6.2-fold respectively. At 6 mo, *Nr5a1* RNA expression was elevated ~2.5-fold in the *Pcsk6*^{tm1Rob} ovaries relative to the *Pcsk6*^{+/+} mice, whereas no detectable increase was observed in *Nr5a2*.

Figure 3 Variations of ovarian morphology exhibited by *Pcsk6*^{tm1Rob} mice. Representative H&E-stained ovarian sections from *Pcsk6*^{+/+} and *Pcsk6*^{tm1Rob} mice are shown. (A) *Pcsk6*^{+/+} mouse at 9 mo (#27). (B–E) *Pcsk6*^{tm1Rob} mice at 9 mo exhibit mild (B; mouse #33) to severe (C; mouse #1260) ovarian atrophy with a reduced number of follicles relative to age-matched mice, a disorganized ovarian organ structure with corporal luteal hyperplasia and minimal interstitial tissue (D; mouse #134), ovarian atrophy with proteinaceous cystic follicles (E; mouse #9), hemorrhagic cysts (F; mouse #1152, 12 mo), and epithelial inclusion cysts (F and G). (G) Tissues representing the right and left ovaries of mouse #1254 (9 mo). There is a complete absence of stroma and mesenchymal structures for the right ovary replaced by a single large cyst-like structure lined by one (bottom panel) to multiple (top panel) cell layers of columnar epithelium (H). The left ovary exhibits interstitial cell hyperplasia, follicular degeneration, and dilated follicular cysts lined by one cell layer thick epithelium. Scale bars are indicated with a line below the image. For A, B, C, D, and F, the scale bar is equivalent to 400 μ m; E, 125 μ m; G, 500 μ m; H, 50 μ m.

Embryologically, proteolytic processing of nodal by PCSK6 and PCSK3 are required to promote autoregulation of *Nodal* transcription (Beck *et al.* 2002). Total RNA levels of *Nodal* remained the same in *Pcsk6*^{+/+} and *Pcsk6*^{tm1Rob} ovaries at baseline, suggesting that inactivation of PCSK6 alone is insufficient to alter *Nodal* autoregulation. By contrast, expression of *Lipg* was reduced by 92% in *Pcsk6*^{tm1Rob} ovaries. Analysis of PC expression indicated that *Pcsk3* and *Pcsk4* were reduced, *Pcsk9* was lower, but not significantly, and *Pcsk5* and *Pcsk7* were unaffected. The expression of several signaling molecules (*Wnt4*, epiregulin, and *Bmp4*) and signaling regulators (*Sfrp4* and *Grem1*) was also significantly altered in ovaries from *Pcsk6*^{tm1Rob} mice. Collectively, these expression data indicate that

changes occur at the molecular level in *Pcsk6*^{tm1Rob} ovaries that affect proteins involved in a number of cellular processes in multiple ovarian cell types prior to the formation of gross pathologies.

Discussion

This study establishes the expression of the *Pcsk6* gene as a contributor to the maintenance of ovarian physiology and tissue integrity in aging C57Bl/6 mice. To date, there has been no postnatal phenotype described for the ~75% of viable *Pcsk6*^{tm1Rob} animals. Prior to 6 mo, the ovaries of *Pcsk6*^{tm1Rob} mice are typically indistinguishable from *Pcsk6*^{+/+} littermates at the level of their gross anatomy. *Pcsk6*^{tm1Rob} mice are fertile and efficiently

Figure 4 Benign tumor phenotypes of *Pcsk6*^{tm1Rob} mice. Representative H&E-stained ovarian sections from *Pcsk6*^{tm1Rob} mice are shown. (A) Tubulostromal adenoma in a 7-mo mouse (#32). This tissue mass is comprised of acinar and tubular epithelial structures, with no detectable normal ovarian features. The boxed area is shown in (B) at higher magnification; this depicts an inclusion cyst lined by ciliated columnar epithelium. (C) The right ovary of mouse #118 (13 mo) exhibits a cystadenoma, with no detectable normal ovarian features. Papillary projections are lined by ciliated epithelial cells (higher magnification shown in (D)). The left ovary of mouse #118 exhibited severe interstitial cell hyperplasia, a hemorrhagic cyst, and a tubulostromal adenoma. (E) The left ovary of mouse #1327 (13.5 mo) exhibits some follicular structures, in addition to a large epithelial cyst, and a granulosa cell tumor (higher magnification in (F)). The tumor is composed of nests of epithelial cells ranging in morphology from large cells with a pale, foamy cytoplasm to small, elongated cells that form tubule-like structures or large nests of cuboidal or polygonal cells with large basal oval-shaped nuclei. The right ovary of mouse #1327 was severely atrophied and scattered throughout, and there are frequent individual nests of cells similar to those described for the left ovary. There was also one dilated and hemorrhagic follicle and several small degenerate follicles. For A, the scale bar is equivalent to 250 μ m; B, 25 μ m; C, 800 μ m; D and F, 50 μ m; E, 1000 μ m.

raise pups to weaning, thus they possess the adequate follicular, endocrine, and neuroendocrine parameters for reproductive competence. However, at early ages, molecular abnormalities are evident in their altered responses to exogenous hormone. By 6 mo, ovarian gene expression profiles are significantly different from *Pcsk6*^{+/+} animals indicating molecular alterations prior to formation of pathology. As the animals age beyond 6 mo, there is an increase in serum FSH levels corresponding with reduced estrous cyclicity. In the most extreme scenarios, normal ovarian structures were absent and often replaced by large cysts or benign tumors. We hypothesize that the loss of PCSK6 activity produces an imbalance in substrate levels that is not fully compensated by other PC activity, resulting in the myriad of cellular and molecular changes displayed by the *Pcsk6*^{tm1Rob} mice.

Pcsk6 is expressed in theca cells at all stages of follicle maturation and granulosa cells prior to the antral stage

(Diaz *et al.* 2008). FSH increases *Pcsk6* RNA in preantral granulosa cell–oocyte complexes, and RNA expression is subsequently suppressed by oocyte-derived factors in antral follicles (Diaz *et al.* 2008). The phenotypic effects that we observed are likely due to altered substrate activity in preantral follicle granulosa cells and potentially any stage of follicle maturation in theca cells. Between 30 and 48 days of age, *Pcsk6*^{tm1Rob} mice are capable of responding to exogenous eCG showing the expected alterations in gene expression and enhanced steroid production. However, the basal level or degree of response of some genes is less in the *Pcsk6*^{tm1Rob} ovaries than that in the *Pcsk6*^{+/+} mice, e.g. *Fshr* and *Cyp19a1*. Of course, variation in gene expression levels may be due to changes in the number of cells expressing the transcript, transcriptional regulation, or RNA stability. Our data raise the possibility that there are inherent alterations in granulosa and theca cells of *Pcsk6*^{tm1Rob} mice that contribute to the reproductive abnormalities

Table 5 Relative fold gene expression in whole ovary of estrus stage-matched *Pcsk6*^{tm1Rob} compared with *Pcsk6*^{+/+} mice at 6 months of age.

Gene	Synonym	Mean expression relative to <i>Pcsk6</i> ^{+/+} (±S.E.M.)
<i>Nr5a1</i>	Steroidogenic factor 1	2.5±0.8*
<i>Nr5a2</i>	Liver receptor homolog-1	1.1±0.17
<i>Fshr</i>	FSH receptor	2.4±0.8*
<i>Lhcgr</i>	LH/choriogonadotropin receptor	5.0±0.7*
<i>Cyp11a1</i>	P450 side chain cleavage	0.7±0.4
<i>Hsd3b</i>	P450 hydroxysteroid dehydrogenase 3 beta	1.2±0.6
<i>Cyp17a1</i>	Cyp17a1	6.8±2.6*
<i>Cyp19a1</i>	P450 aromatase	6.2±1.8*
<i>Star</i>	Steroidogenic acute regulatory protein	1.4±0.8
<i>Ptgs1</i>	Cyclooxygenase 1	0.76±0.2
<i>Ptgs2</i>	Cyclooxygenase 2	1.6±0.7 ^a
<i>Sfrp4</i>	Secreted frizzled-related protein 4	0.22±0.07*
<i>Wnt4</i>	Wingless-related MMTV integration site 4	0.45±0.05*
<i>Amh</i>	Anti-Müllerian hormone	1.01±0.08
<i>Ereg</i>	Epiregulin	0.6±0.1
<i>Areg</i>	Amphiregulin	1.01±0.45
<i>Bmp4</i>	Bone morphogenetic protein 4	0.53±0.04*
<i>Bmp6</i>	Bone morphogenetic protein 6	0.49±0.14
<i>Grem1</i>	Gremlin	2.65±0.08
<i>Nodal</i>	Nodal	1.06±0.21
<i>Lipg</i>	Endothelial lipase	0.08±0.008*
<i>Pcsk3</i>	Furin	0.52±0.04*
<i>Pcsk4</i>	Proprotein convertase 4	0.53±0.2
<i>Pcsk5</i>	Proprotein convertase 5	0.9±0.2
<i>Pcsk7</i>	Proprotein convertase 7	0.75±0.13
<i>Pcsk9</i>	Proprotein convertase 9	0.6±0.2
<i>rpll</i>	RNA polymerase II	Standard for loading

Ovaries from four age-matched and estrus stage-matched animals were used for each genotype. Data represents fold differences of pooled data for each genotype. * $P < 0.01$.

^aThree of four *Pcsk6*^{tm1Rob} mice had increased *Ptgs2* expression averaging 1.6-fold compared to wild-type mice, whereas one *Pcsk6*^{tm1Rob} mouse showed >20-fold overexpression.

observed, including increased whelping interval and reduced estrus cyclicity. The fact that we do observe alterations in the responses for some (e.g. *Fshr*, *Cyp19a1*, *Nodal*), but not all (e.g. *Lhcgr*, *Nr5a1*, *Cyp11a1*, *Pcsk4*, *Pcsk9*), genes suggests that at the early ages examined, a difference in follicle cell number is unlikely to account for these changes. Moreover, the direction of the alteration between the *Pcsk6*^{+/+} and *Pcsk6*^{tm1Rob} mice is not always in the same direction, i.e. we do not always observe a decrease in the *Pcsk6*^{tm1Rob} animals suggestive of decreased cell numbers, sometimes there is an increase in gene expression (e.g. *Lipg*). As the animals age, more defects are apparent with increased serum FSH and alterations in basal gene expression. As these reproductive parameters are dependent on the production of protein and steroid hormones from healthy ovarian follicles, the aberrant physiological responses in *Pcsk6*^{tm1Rob} mice are most likely due to loss of negative endocrine feedback or changes in the local autocrine/paracrine milieu that compromise follicle health.

We observed elevated FSH levels starting at 9 mo of age in the *Pcsk6*^{tm1Rob} mice, reflective of a loss of endocrine feedback. Mice engineered to overexpress FSH under the direction of the metallothionein-1 promoter develop hemorrhagic and fluid-filled cysts; however, these mice develop ovarian pathology by 6–7 weeks (Kumar *et al.* 1999). It thus remains possible that the higher levels of FSH observed contribute to the formation of cystic phenotype in *Pcsk6*^{tm1Rob} mice. Future studies to conduct morphometric analysis of follicle loss and experiments using granulosa cells isolated from different stages of follicle maturation will provide greater insight into disruptions produced by loss of PCSK6 activity.

The phenotypes observed in the *Pcsk6*^{tm1Rob} ovaries are unlike many other single gene knockout or transgenic models described (Matzuk & Lamb 2002, Vanderhyden *et al.* 2003). Reduced oocyte number or accelerated oocyte loss is a precursor to pathologies observed in different mouse models (Matzuk & Lamb 2002, Vanderhyden *et al.* 2003, Vanderhyden 2005). In general, ovarian follicle depletion is associated with increased epithelial invaginations and inclusion cysts, as well as with formation of tubular adenomas. Invasive epithelial tubules and tubulostromal adenomas develop in the vast majority of mice with germ cell deficiencies, such as those produced by mutations in the *W* (*Kitl*) or *Sl* (*Kitl*) loci (Murphy 1972, Murphy & Beamer 1973, Ishimura *et al.* 1986, Vanderhyden *et al.* 2003). These abnormalities typically form by 7 mo and are directly correlated with oocyte depletion. *Pcsk6*^{tm1Rob} ovaries are frequently observed with a reduced number or complete absence of oocytes strongly suggesting that loss of oocytes participates in the formation of the tubular adenomas. *Pcsk6*^{tm1Rob} ovaries exhibit other benign tumor types including granulosa cell tumors or cystadenomas. Development of these pathologies indicates that substrates cleaved by PCSK6 likely play a role in aspects of ovarian cell function and survival.

The histopathological phenotype of the *Pcsk6*^{tm1Rob} ovaries is variable, indicating that there is incomplete penetrance of the phenotype produced by inactivation of the *Pcsk6* gene. This is consistent with the observations made in the original description of the *Pcsk6*^{tm1Rob} mouse, where multiple phenotypes resulting in embryonic lethality were produced in only 25% of the offspring (Constam & Robertson 2000). Constam & Robertson (2000) originally proposed that functional overlap between PCSK3 (furin) and PCSK6 might account for incomplete penetrance of the *Pcsk6*^{tm1Rob} phenotype. Unfortunately, inactivation of *Pcsk3* (Roebroek *et al.* 1998) produces 100% embryonic lethality, thus precluding the opportunity to study the contribution of *Pcsk3* in ovarian biology using a standard knockout model. Quantification of other PC transcripts at 6 mo suggests that loss of PCSK6 activity does not induce a mechanism to produce a compensatory increase in PC

transcription. Indeed, basal levels of *Pcsk3*, *Pcsk4*, and *Pcsk7* were decreased in *Pcsk6^{tm1Rob}* ovaries at 6 mo. Reduced expression of these PCs, and thus altered bioactive substrate levels, may contribute further to an abnormal cellular environment leading to the observed ovarian phenotypes.

Many of the genes that encode proteins participating in ovarian reproductive activities are regulated through signaling pathways that often converge with the Sf-1 and Lrh-1 transcription factors. At 6 mo, *Nr5a1* (Sf-1) RNA expression was elevated ~2.5-fold in the *Pcsk6^{-/-}* ovaries relative to the *Pcsk6^{+/+}* mice, whereas no detectable increase was observed in *Nr5a2* (Lrh-1). Expression of *Fshr*, an Sf-1-regulated gene (Levallet *et al.* 2001), was increased 2.4-fold. As anticipated, the RNA for a downstream target of FSH signaling, *Cyp19a1*, was also increased. There was no detectable change in additional genes associated with steroidogenesis, *Star*, *Cyp11a1*, or *Hsd3b*. The RNA of a luteal marker, *Sfrp4*, an inhibitor of WNT signaling (Kawano & Kypta 2003), was dramatically decreased. Collectively, these data show that there are significant changes in the RNA expression of transcriptional regulators, signaling inhibitors, and rate-limiting enzymes that may contribute to the formation of an abnormal environment in *Pcsk6^{tm1Rob}* ovaries leading to the formation of pathologies contributing to reduced reproductive capacity.

We also evaluated the expression levels of the PCSK6 substrates nodal and endothelial lipase. Nodal is a TGFB/BMP superfamily member that induces granulosa cell apoptosis, thus likely playing a role in follicular atresia (Wang *et al.* 2006). Nodal controls its own mRNA expression through an autoregulatory activity, which requires efficient nodal maturation via PCSK3 and PCSK6 proteolysis (Beck *et al.* 2002). The presence of either PCSK3 or PCSK6 is sufficient to cleave immature nodal. No differences in *Nodal* mRNA expression were measured in *Pcsk6^{tm1Rob}* compared with *Pcsk6^{+/+}* animals at basal levels; however, nodal expression was not induced by eCG in *Pcsk6^{tm1Rob}* ovaries compared with *Pcsk6^{+/+}* ovaries. We hypothesize that PCSK6 activity is required for nodal induction but is dispensable to maintain basal RNA expression levels. Unfortunately, antibodies to detect endogenous nodal protein are poor, and so we are currently unable to examine the degree of nodal maturation in the *Pcsk6^{tm1Rob}* ovary. Nodal activity is required to sustain *Bmp4* expression embryologically (Beck *et al.* 2002). We theorize that *Bmp4* RNA levels are lower because of reduced nodal maturation occurring through absent PCSK6, and reduced PCSK3, activity. Further investigation will be required to determine if nodal maturation is efficiently accomplished in *Pcsk6^{tm1Rob}* ovaries, and the effects that this may have on BMP activity.

Endothelial lipase is a member of the triglyceride lipase family and is tethered to the plasma membrane through heparan sulfate proteoglycans, where it

metabolizes high-density lipoprotein (HDL) particles allowing cholesterol ester uptake (Broedl *et al.* 2003, Fuki *et al.* 2003). Limited analysis of endothelial lipase expression in reproductive tissues demonstrate low levels of expression in the non-pregnant ovary and high levels in the pregnant ovary localized to the corpus luteum (Lindegaard *et al.* 2005). In a model examining human endothelial lipase expression in a transgenic mouse, the ovary was one of the sites of highest expression (Broedl *et al.* 2005). There is no mention in this study as to whether endothelial lipase is expressed in specific ovarian cell types. Our experiments revealed that *Lipg* RNA is increased in response to exogenous eCG, indicating that this gene product is likely needed for enhanced cholesterol uptake during a time when steroid production is increased. Moreover, at 30–48 days of age, *Lipg* RNA levels increased significantly more in response to eCG in *Pcsk6^{tm1Rob}* ovaries compared with wild-type animals. Since PCSK6 proteolytically cleaves endothelial lipase to reduce lipase activity, the increased RNA levels in *Pcsk6^{tm1Rob}* ovaries are unlikely due to a compensatory transcriptional response because of reduced bioactive protein levels. This is in contrast to increased levels of pituitary adenylate cyclase-activating polypeptide transcripts that were observed in the ovaries and testes of *Pcsk4* null animals (Li *et al.* 2000). Additional research will be required to investigate the underlying cause of the enhanced eCG response in *Pcsk6^{tm1Rob}* ovaries.

We further identified a dramatic decrease in ovarian endothelial lipase (*Lipg*) RNA levels in *Pcsk6^{tm1Rob}* ovaries (0.08-fold expression) at 6 mo, and hypothesized that as the animals age, the loss of *Lipg* expression compromises cholesterol metabolism contributing to aberrant cell physiology. It is unclear how a loss of PCSK6 activity may result in decreased endothelial lipase RNA expression in the ovary. Endothelial lipase expression can be regulated through peroxisome proliferator-activated receptor (PPAR) α activity (Ahmed *et al.* 2006, Rakhshandehroo *et al.* 2007). Ahmed *et al.* (2006) have demonstrated that PPAR ($\alpha > \gamma > \delta$) activity is increased by endothelial lipase-mediated HDL hydrolysis. While there is an abundance of evidence to support a role for PPARs in normal reproduction (Froment *et al.* 2006), there is currently no evidence linking PCSK6 protein activity with PPAR activity. This will be the focus of future research. Decreased endothelial lipase expression was not restricted to the *Pcsk6^{tm1Rob}* ovary alone. We also measured decreased expression in abdominal adipose tissue; however, there was no change in endothelial lipase mRNA levels in the liver compared with *Pcsk6^{+/+}* mice, suggesting that the mechanisms regulating endothelial lipase activity are tissue specific (QPCR data not shown).

We discovered that the expression of *Pcsk4*, *Pcsk6*, and *Pcsk9* are altered by exogenous eCG, strongly

suggesting that PC expression is regulated during the process of follicle maturation. Given the myriad of changes in PC substrate production that occurs during the process of follicle maturation, e.g. growth factors, growth factor receptors, and integrins, it is logical to expect that PC expression is capable of responding to changes in substrate levels. There is precedence for PC levels changing in response to endocrine signals, or for substrate levels changing when PCs are inactivated. In rats, it was shown that anterior pituitary *Pcsk6* expression is dependent on thyroid status, low under hypothyroid conditions and high with excess thyroid hormone (Johnson *et al.* 1994). Male and female *Pcsk4* knockout mice exhibit increased RNA levels of propituitary adenylate cyclase-activating peptide, a known PCSK4 substrate (Li *et al.* 2000). Our research shows that changes in *Pcsk6* expression result in alterations in the basal level of ovarian *Pcsk4* and *Pcsk9* RNA at 6 mo, and we believe that these changes are due to either an alteration in PCSK6 substrate activity or a change in the number or activity of the cells expressing these enzymes. PCSK4 production is localized to ovarian theca, interstitium, and corpora luteal cells (Tadros *et al.* 2001). Importantly, female *Pcsk4* knockout mice are subfertile and show reduced increase in ovarian weight and P₄ production following eCG treatment (Tadros *et al.* 2001). Thus, the reduced *Pcsk4* expression profile observed in *Pcsk6*^{tm1Rob} mice at 6 mo is one more indication of compromised ovarian health. We hypothesize that with age, the loss of *Pcsk6* activity establishes an imbalance in substrate activity for which the ovarian cells cannot compensate leading to detrimental changes in ovarian cell biology.

The mechanism(s) underlying how the loss of PCSK6 protease activity results in altered gene expression, hormone production, and tissue morphology remains to be determined. This initial investigation lays the foundation to assess the relationship between PCSK6 expression and these molecular and cellular processes. This study shows that the *Pcsk6*^{tm1Rob} mouse provides a platform to examine ovarian pathologies. A rise in serum FSH is the most sensitive and widely accepted early marker for ovarian failure (Conway 2000). The observed alterations in FSH secretion coinciding with reduced fertility and decreased follicle number suggest that PCSK6 may also contribute to human diseases including infertility and premature ovarian insufficiency. The *Pcsk6*^{tm1Rob} mouse offers a platform to experimentally investigate mechanisms of reproductive failure, including the contributions of the oocyte and somatic cells to maintain follicle health, the identity and bioactive state of the substrates that regulate these cellular activities, and the signaling pathways or functional activities that are affected via disruption of substrate processing.

Our previous research also demonstrated a reduction or absence of *Pcsk6* RNA in human ovarian cancer cell samples (Fu *et al.* 2003), and this observation was

confirmed by Page *et al.* (2007). Indeed, we determined that *PCSK6* gene expression is reduced due to epigenetic modification of the 5'-flanking DNA and first exon in human ovarian cancer patient cell samples (Fu *et al.* 2003). As *Pcsk6*^{tm1Rob} ovaries can develop cellular aberrations and tumors similar to precursor lesions in human tumors, we predict that an alteration in PC activity, increased or decreased, resulting in changes in the activity of proteins contributing to cellular stability is likely to contribute to the formation or progression of human ovarian cancers. Page *et al.* (2007) have shown that increased expression of *Pcsk3* was correlated with decreased survival in a subset of ovarian cancer patients. Although they did not show a correlation between decreased *Pcsk6* RNA levels and survival, this does not preclude the possibility that reduced *Pcsk6* expression contributes to ovarian cancer etiology through reduced bioactivation or inactivation of substrates critical to maintain cellular homeostasis. Indeed, given the myriad of phenotypes displayed by the *Pcsk6*^{tm1Rob} ovary, it is suggested that PCSK6 activity should be examined in human ovarian pathology to ascertain further the clinical role that altered PC activity may contribute to human reproductive disease.

Materials and Methods

Animals

Animals were maintained according to the Canadian Council on Animal Care, and institutional approval for research with animals was received prior to the initiation of these studies (Protocol 09-002). All animals were maintained in a temperature-controlled environment at 22 °C on a 12 h light:12 h darkness schedule and provided with food and tap water *ad libitum*. Generation and genotyping of mice with a targeted disruption of the *Pcsk6* gene was described previously (Constam & Robertson 2000). Crosses of *Pcsk6*^{tm1Rob/+} mice produced progeny in the expected ratio assuming 25% embryonic lethality for *Pcsk6*^{tm1Rob} mice: 27:55:18% (*Pcsk6*^{+/+}:*Pcsk6*^{tm1Rob/+}:*Pcsk6*^{tm1Rob}; n=267).

Histological assessment of ovaries

Ovaries were harvested, fixed for 24 h in 10% acetate-buffered formalin, and then embedded in paraffin. Ovaries were sectioned serially at 4 µm and stained automatically with hematoxylin and eosin using a Thermo Shandon Gemini Stainer by the Centre for Modeling Human Disease (CMHD) Pathology Core at The Toronto Centre for Phenogenomics (Toronto, ON, Canada). All stained serial sections were assessed by a minimum of two independent observers in the Nachtigal laboratory, and by Dr Susan Newbigging, a veterinary pathologist and Director of Pathology at the CMHD Pathology Core. Observers were blinded to the genotype and age of the samples. Definitions of normal and pathological features are included as supplementary information

(Supplementary Figure 3, see section on [supplementary data](#) given at the end of this article).

Breeding analysis

Wild-type *Pcsk6* (*Pcsk6*^{+/+}) and *Pcsk6*^{tm1Rob} female mice were paired at 6 weeks of age with *Pcsk6*^{+/+} male mice. The number of litters and number of offspring were measured for 12 mo after pairing. The data discussed correspond to when the breeding female was 3–9 mo of age, the period of peak cyclicity for female mice (Nelson *et al.* 1982).

Estrous cycle

Virgin female mice were evaluated for estrous cyclicity by daily vaginal smears (taken between 0800 and 1100 h) for a minimum of 4 weeks. Smears were obtained using a sterile saline-moistened cotton-tipped applicator. Slides were allowed to air dry and subsequently stained using DipQuick Stain kit (Jorgenson Laboratories, Inc., Loveland, CO, USA). Vaginal smears were examined and classified into one of seven estrous phases, as described by Nelson *et al.* (1982).

Serum FSH, LH, and steroid measurements

For serum FSH and LH measurements, virgin mice were evaluated over two consecutive cycles prior to harvesting. Mice displaying estrous cyclicity were harvested at ~1700 h on the day of proestrous–estrous, and serum was obtained and stored at –80 °C until analyzed. FSH and LH levels were determined by RIA through custom service from the National Hormone and Peptide Program (NHPP) through the US NIH National Institute of Diabetes and Digestive and Kidney Diseases (www.humc.edu/hormones/material.html). Each sample was examined in duplicate. Mouse FSH was detected using guinea pig anti-mouse FSH (AFP1760191) at a final dilution of 1:200 000. Mouse LH was detected using NIDDK-anti-rat LH-S-11 at a final dilution of 1:1 500 000.

Serum concentrations of 17β-E₂, testosterone, and P₄ were measured by the Endocrine Technology and Support Core Lab at the Oregon National Primate Research Center (Rasmussen *et al.* 1984). Serum steroids were organically extracted, followed by column chromatography using Sephadex LH-20 for separation, and a specific RIA was used to measure each steroid. Hormonal values were corrected for extraction chromatography losses determined by radioactive trace recovery at the same time with sample extraction. The efficiency for steroid recovery was 77% for E₂, 85% for testosterone, and 88% for P₄. Assay sensitivity was 1 pg/tube for the E₂ RIA, 2.5 pg/tube for the testosterone RIA, and 5 pg/tube for the P₄ RIA. Intra-assay variation for the extraction chromatography RIA was estimated at 15.5% for E₂, 18.7% for testosterone, and 16.9% for P₄.

Stimulation with eCG

Female mice at ages 30–48 days were injected i.p. with saline or 10 IU of eCG (NHPP). The age distribution was the same

in WT and KO mice. Blood samples were collected, and ovaries harvested for total RNA extraction 48 or 60 h after eCG injection.

Superovulation studies

Immature 30-day-old (d) and 3-mo-old female mice were injected i.p. with 10 IU of eCG (Folligon; Intervet, Kirkland, QC, Canada) and 48 h later with 10 IU of human chorionic gonadotropin (Chorulon; Intervet). Mice were harvested 26 h after Chorulon injection, and the oocytes were isolated from the ampulla. The number of oocytes was determined by direct count.

RNA isolation and QPCR

Total RNA was isolated from mouse tissue using the GeneElute Total Mammalian RNA MiniPrep Kit (Sigma). From each mouse, one ovary was used to isolate RNA, and the other ovary was used for histological assessment. For QPCR experiments, cDNA was generated from 2 µg of total RNA using Superscript III reverse transcriptase (Invitrogen). QPCR was performed using the Brilliant SYBR Green QPCR Master Mix and the Mx3000P Quantitative PCR machine and analysis software (Stratagene, La Jolla, CA, USA). RNA levels were assessed by QPCR (40 cycles: 30 s at 95 °C, 30 s at 59 °C, 30 s at 72 °C) using primers specific for each mouse cDNA sequence (Supplementary Table 2, see section on [supplementary data](#) given at the end of this article for list of primers). Melting curves were performed on each reaction to confirm the specificity of the QPCR, and PCR amplicons were subcloned and verified by sequencing. Data were analyzed relative to mouse RNA polymerase II (*mrpl1*) and expressed as relative fold change. Fold difference in RNA expression between *Pcsk6*^{+/+} and *Pcsk6*^{tm1Rob} mice was calculated using the normalized C_t value obtained from the Mx3000P analysis software as reported previously (Livak & Schmittgen 2001); expression levels in basal samples from wild-type ovaries was set to 1.

Statistical analyses

All data were analyzed for statistical significance using GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA, USA). For FSH, LH, and calculation of whelping interval, data were analyzed using unpaired *t*-test with Welch's correction, not assuming equal variances. Differences were considered significant at *P* ≤ 0.05. Serum FSH and LH measurements are expressed as the mean ± s.e.m. An unpaired *t*-test was performed for experiments examining serum steroids, gene expression, and ovulatory capacity in response to exogenous hormonal stimulation where significance was set at *P* ≤ 0.05.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-10-0451>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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