Follicle-stimulating hormone increases primordial follicle reserve in mature female hypogonadal mice

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Abstract

Ovarian primordial follicle reserve is considered hormonally independent or subject to depletion by FSH-driven follicle recruitment. To explore specific *in vivo* effects of FSH on early follicle populations in the absence of luteinizing hormone (LH) activity, we examined mature hypogonadal (*hpg*), gonadotrophin-deficient mice expressing transgenic (tg) human FSH. Sustained expression of tg-FSH (5.3 ± 0.3 IU/l) increased ovary weights fourfold and significantly elevated total primordial follicle numbers twofold in tg-FSH *hpg* (4209 ± 457) relative to non-tg *hpg* (2079 ± 391) and wild-type (2043 ± 195) age-matched ovaries. Absolute primary follicle numbers in tg-FSH *hpg* ovaries were similar to non-tg *hpg* and wild-type ovaries. Furthermore, tg-FSH quantitatively increased secondary and antral follicles in *hpg* ovaries

to numbers equivalent to wild-type, but did not induce ovulation, indicating a selective FSH response without LH. Circulating inhibin B and inhibin A levels were significantly increased in tg-FSH *hpg* females compared with *hpg* controls, and inhibin B correlated with antral number, consistent with FSH-driven antral follicle formation. These findings revealed that sustained pituitary-independent FSH activity, in the absence of endogenous gonadotrophins, promotes an increase in primordial follicle reserve despite also stimulating follicular growth in mature females. Therefore, the tg-FSH *hpg* ovary presents a novel paradigm to evaluate specific gonadotrophin effects on follicle reserve and recruitment.

Journal of Endocrinology (2006) **188**, 549–557

Introduction

Follicle-stimulating hormone (FSH) is critical for ovarian folliculogenesis and female fertility. FSH plays a key role in antral follicle development and, in combination with luteinizing hormone (LH), stimulates preovulatory follicular growth (reviewed in McGee & Hsueh 2000, Hillier 2001). In contrast, the primordial follicles are generally thought to be FSH-independent. Primordial follicles are present and develop up to the late preantral stage in the ovaries of mice lacking functional gonadotrophin-releasing hormone (GnRH) (Halpin *et al.* 1986, Allan *et al.* 2001), FSH beta-subunit (Kumar *et al.* 1997) or FSH receptor (Dierich *et al.* 1998, Abel *et al.* 2000).

While small preantral follicular development may occur independently of FSH, the total number of early growing follicles is increased by FSH action (Halpin & Charlton 1988, McGee *et al.* 1997, Oktay *et al.* 1998). Furthermore, FSH-mediated follicle growth may also indirectly accelerate the age-dependent depletion of the finite primordial pool initially established after birth. For example, increased basal serum FSH in young adult rats after

unilateral ovariectomy is associated with an increased loss of primordial follicles (Meredith *et al.* 1992, Anzalone *et al.* 2001). Advancing our understanding of FSH effects on ovarian development and its capacity to regulate early folliculogenesis has implications for follicle preservation and/or recovery during or after chemotherapy (Blumenfeld 2002) or *in vitro* fertilization (IVF) treatment (Huirne *et al.* 2004), or for the relationship between elevated FSH and follicle depletion during human reproductive ageing (Hansen *et al.* 2005), particularly in light of the potential for sustained primordial follicle renewal throughout life (Johnson *et al.* 2004). However, investigating the direct, selective *in vivo* effects of FSH in the ovary is difficult in human subjects, while suitable animal models are limited.

For selective examination of gonadal FSH actions, we previously generated transgenic (tg) mice that express human FSH independently of GnRH (Allan *et al.* 2001). This features pituitary-independent tg-FSH expression, which, when combined with the gonadotrophin-deficient background of hypogonadal (*hpg*) mice, allows investigation of FSH activity in isolation of LH effects

(Allan *et al.* 2001, 2004). The *hpg* mouse is complementary to models with selective disruption of LH- β (Ma *et al.* 2004) or receptor (Lei *et al.* 2001, Zhang *et al.* 2001) genes, although free from secondary effects on FSH secretion that confound interpretation of the later model of permanent LH insensitivity. The immature ovaries of *hpg* females exhibit follicle arrest at the preantral stage but remain hormonally responsive (Halpin *et al.* 1986, Allan *et al.* 2001). We previously showed that tg-FSH dose-dependently increased ovary weights and circulating inhibin B levels in *hpg* mice (Allan *et al.* 2001). This tg-FSH *hpg* paradigm is now used to explore the effect of FSH upon follicle dynamics in young, mature females, revealing unexpected upregulation by FSH of the primordial follicle stock.

Materials and Methods

Transgenic FSH mice

Transgenic $\alpha\beta$.6 mice expressing human (h)FSH (tg-FSH) independently of GnRH were previously described (Allan *et al.* 2001). Females expressing tg-FSH on a gonadotrophin-deficient hypogonadal background (*Gnrh1*^{-/-}) were obtained by crossing tg animals heterozygous for the *Gnrh1* gene deletion (*Gnrh1*^{+/-}) as determined by detection of wild-type or disrupted *Gnrh1* gene or tg PCR products, as previously described (Singh *et al.* 1995, Allan *et al.* 2001). Animals were housed under controlled conditions (12-h light-dark cycle, 19–22 °C) with free access to food and water. Mature mice (9–11 weeks old) were used for ovary collection. Littermates or age-matched non-tg *Gnrh1*^{-/-} (denoted *hpg*) or wild-type (*Gnrh1*^{+/+} or *Gnrh1*^{+/-}) females served as controls. Animal procedures were approved by the University of Sydney animal ethics committee and performed in accordance with the National Health and Medical Research Council Code of Practice for the Care and Use of Animals and the NSW Animal Research Act 1985. Blood was collected from terminally anaesthetized mature females, and serum was stored at -20 °C.

Serum hormone assays

Serum hormone levels were measured in duplicate in all assays. Serum levels of human FSH were determined by species-specific, two-site immunofluorometric assay as previously described (Allan *et al.* 2001). Inhibin A and B levels were determined with human inhibin assays and serially diluted mouse follicle culture media as standard (Wang *et al.* 2005). Inhibin levels were interpolated from mouse standards, using arbitrary units (AU/ml) to avoid non-parallelism between human standards and mouse samples (Wang *et al.* 2005). Detection limits for inhibin A and B were 7.8 and 8.0 AU/ml respectively.

Ovary fixation and follicle counting

Ovaries were removed and incubated overnight in 4% paraformaldehyde in PBS (pH 7.4), washed with 70% ethanol and then embedded in hydroxymethylmethacrylate resin (Technovit 7100; Kulzer, Friedrichsdorf, Germany) as recommended by the manufacturer. With one ovary per mouse, tissue sections were cut with the Polycut S microtome (Reichert Jung, Nossloch, Germany). Thin sections (5 μ m) were stained with 0.5% toluidine blue, and thick sections (25 μ m) for follicle counting were consecutively stained with periodic acid-Schiff, haematoxylin and Scott's blue solution. Morphological identification and estimation of total primordial (oocytes with partial or complete layer of squamous granulosa cells (Meredith *et al.* 2000)), primary (single layer of cuboidal granulosa cells), secondary (two or more layers of cuboidal granulosa cells but no visible antrum), or antral (one or multiple visible fluid filled antral cavities) follicles were performed with a light microscope with microcator to monitor section depth and CAST-GRID software (Olympus, Albertslund, Denmark). Follicle nuclei (primordial) or nucleoli (primary-antral) were used as reference counting points. In serial sections, the first sections were selected randomly, then all primordial and primary follicles were counted ($\times 40$ oil objective) to a depth of 18 μ m in every fourth 25 μ m section, and then whole ovary estimates were calculated by Cavalieri's principle and accounting for section ($\times 4/1$) and depth ($\times 25/18$) sampling. Section areas flanking the 18 μ m counting depth allowed confirmation of follicle classification (e.g. primordial vs primary) to avoid potential capping effects. Total secondary and antral follicle numbers were counted in every 25 μ m serial section from each ovary ($\times 10$ objective).

Statistical analysis

Statistical analysis was performed with SigmaSat statistical software (SPSS Version 11.0; Chicago, IL, USA). Data were analysed by unpaired *t*-test, Fisher's test, Pearson correlation or one-way ANOVA; significance (differences, linear association) was defined as $P < 0.05$. Samples with undetectable hormones were assigned zero. Alternative assignment to detection limit values did not alter any significant difference observed. All data are presented as mean $\pm$ S.E.M.

Results

Body and ovary weights

Body weights of 9–11-week-old tg-FSH and non-tg *hpg* females were equivalent, but reduced (15–18%) in comparison to age-matched, non-tg, wild-type controls (Fig. 1). Compared with *hpg* controls, the tg-FSH *hpg* females

Figure 1 Body (A) and left ovary (B) weights of non-tg ($n=14$, white bars) or tg-FSH *hpg* ($n=17$, black bars) females, as well as corresponding data for age-matched, non-tg, wild-type females ($n=7$, grey bars) for comparison. Significant differences from wild-type are indicated, * $P<0.05$.

exhibited significantly increased ovary weights (fourfold), which remained threefold smaller than wild-type age-matched ovaries (Fig. 1).

Ovary histology

Qualitative histological examination of non-tg *hpg* ovaries from 9–11-week-old females showed follicular development progressed beyond primary follicle stage, and sparse

numbers of early antral follicles were detected, as shown in Fig. 2. Examination of tg-FSH *hpg* ovaries revealed that follicular development had progressed to advanced antral stage, as Fig. 2 shows. No corpora lutea were observed in any tg-FSH or non-tg *hpg* ovary examined.

Follicle numbers

Total primordial follicle numbers in 9–11-week-old tg-FSH *hpg* females were significantly higher (twofold, $P<0.05$) than in both non-tg *hpg* and wild-type females. The total number of primordial follicles was equivalent in age-matched, gonadotrophin-deficient *hpg* relative to wild-type ovaries (Fig. 3). The absolute number of primary follicles in tg-FSH *hpg* ovaries was not significantly different from non-tg *hpg* or wild-type ovaries (Fig. 3). However, secondary follicles were significantly increased ($P<0.001$) and restored to normal levels in tg-FSH *hpg* mice (Fig. 3), although the total number of secondary ovarian follicles in *hpg* mice was only 25% of wild-type numbers. Non-tg *hpg* control ovaries contained limited numbers of early antral follicles with small emerging antral spaces, and total antral follicle numbers of just 4.5% of wild-type numbers. Expression of tg-FSH significantly increased ($P<0.001$) total antral follicles more than 15-fold compared with non-tg *hpg* controls, restoring numbers to wild-type levels (Fig. 3).

Hormone levels

Mature (9–11-week-old), transgenic, $\alpha\beta.6$ line *hpg* females expressed serum hFSH levels of 5.4 ± 0.2 IU/l ($n=14$), whereas non-tg females had undetectable hFSH levels consistent with previous findings (Allan *et al.* 2001).

Serum inhibin B was detectable (≥ 8.0 AU/ml) in significantly more tg-FSH (14/14) than non-tg (3/16) *hpg* females ($P<0.001$, Fisher's test), and tg-FSH *hpg* mice had significantly higher ($P<0.001$) serum inhibin B levels (Fig. 4). There was no difference between serum inhibin B levels in tg-FSH *hpg* and mature, wild-type females. Furthermore, there was a significant correlation between inhibin B levels and secondary and antral follicle numbers in the tg and non-tg *hpg* ovaries examined, whereas there was no correlation with primordial and primary follicle numbers (Fig. 5). Partial regression showed that the strongest inhibin B relationship was with the antral follicles and that the relationship with secondary follicles was derived from that with antral follicles.

Likewise, serum inhibin A was detected (≥ 7.8 AU/ml) in significantly more tg-FSH (10/14) than non-tg (2/16) *hpg* females ($P<0.005$, Fisher's test), and mean levels of serum inhibin A were significantly higher ($P<0.05$) in the tg-FSH group (Fig. 4). Serum inhibin A levels were lower in tg-FSH *hpg* than in mature, wild-type females, and there was no correlation between serum inhibin A and

Figure 2 Ovarian development in tg-FSH *hpg* female mice. Left panels show a typical ovary section and maximal level of follicle development observed in non-tg *hpg* control females, which exhibited few follicles with very early antral cavity formation, as shown below. The right panels demonstrate the folliculogenesis and maximal antral follicle development found in tg-FSH *hpg* ovaries.

different follicle types present in the tg and non-tg *hpg* ovaries examined (data not shown).

Discussion

We have investigated the potential effect of FSH upon early follicle dynamics, using a mouse model exhibiting isolated FSH-driven follicle development. Transgenic expression of pituitary-independent FSH in gonadotrophin-deficient *hpg* mice allowed the selective *in vivo* investigation of the FSH ovarian response in the absence of LH activity. Unexpectedly, primordial follicle numbers were higher in tg-FSH *hpg* than non-tg *hpg* females, suggesting that FSH may have a role in the positive regulation of ovarian primordial follicle reserve.

Total primordial follicle numbers in tg-FSH *hpg* mice were also greater than the numbers in age-matched wild-type mice, suggesting that tg-FSH activity increased the overall development and/or survival of primordial

follicles. The precise mechanism of the FSH-stimulated increase in total primordial follicles in tg *hpg* ovaries remains undefined, but may involve increased initial accrual, decreased atresia or reduced developmental progression of the primordial follicle population. The observation of equivalent numbers of primordial follicles in mature non-tg *hpg* and wild-type ovaries is consistent with an early report of similarly 'non-growing' follicle numbers in immature and adult *hpg* and wild-type mice (Halpin *et al.* 1986). Mouse primordial follicle numbers are maximal during early postnatal development (Peters 1969, Hirshfield 1991, Johnson *et al.* 2004) and thereafter rapidly decline via atresia (Faddy *et al.* 1983, 1987, McGee & Hsueh 2000). Therefore, the larger primordial pool in mature tg-FSH *hpg* ovaries relative to normal (or non-tg *hpg*) may reflect a reduced rate of atresia. Alternatively, the rate at which primordial follicles enter the growth phase may be reduced in tg *hpg* mice by the presence of more secondary/antral follicles (Faddy *et al.* 1987). However, we did not observe decreased numbers of downstream

Total follicle numbers / ovary

primary follicles in tg *hpg* ovaries compared with controls, consistent with studies in *hpg* females treated with exogenous FSH for 4–20 days (Halpin & Charlton 1988, Wang *et al.* 2005). Instead, primary follicle numbers were equivalent to controls, suggesting that reduced primordial recruitment may not explain our results.

Another possibility is that perinatal formation of the primordial population may be enhanced in tg-FSH *hpg* mice. Although this stage was considered FSH-independent due to minimal FSH binding (Dunkel *et al.* 1994) or responsiveness (Peters *et al.* 1973, Sokka & Huhtaniemi 1990) and absence of full-length FSH receptor mRNA (Dunkel *et al.* 1994, Rannikki *et al.* 1995, O'Shaughnessy *et al.* 1996) in perinatal mouse or rat ovaries, recent studies suggest that FSH activity may regulate early folliculogenesis before the arrival of later-stage, FSH-dependent growing antral follicles. In hamsters, an early role for FSH in folliculogenesis is suggested by reduced primordial follicle formation after treatment of mothers with FSH-antiserum during late gestation, and expression of full-length FSH receptor mRNA in the fetal ovary as early as day 13 of gestation (Roy & Albee 2000). Neonatal (2-day-old) mice genetically lacking functional FSH receptor exhibit reduced numbers of early, non-growing follicles (Balla *et al.* 2003). Furthermore, FSH receptor mRNA transcripts are present in 1–5-day-old mouse ovaries (O'Shaughnessy *et al.* 1996), 3-day-old rat ovaries (Dunkel *et al.* 1994) and human early preantral follicles (Oktay *et al.* 1997). Our data combined with the above observations challenge the view of gonadotrophin-independent early folliculogenesis and suggest that FSH activity can significantly influence the primordial and early preantral follicle populations. Whether or not tg-FSH in our model has direct effects on early follicles during (or after) primordial formation, or progressive indirect actions through the growing follicle populations over time remains to be determined. Future analysis of primordial follicle dynamics in postnatal and ageing tg *hpg* mice will enhance our understanding of this unexpected primordial follicular response to FSH.

In contrast to the normal primordial numbers found in *hpg* ovaries, hypophysectomized (Meredith *et al.* 1986) or GnRH-antagonist treated (Meijs-Roelofs *et al.* 1990) rats exhibit a decreased rate of loss of primordial follicles, resulting in higher than normal primordial follicle numbers. The different primordial numbers found in these gonadotrophin-deficient mouse and rat models remains an enigma. One notable difference between

Figure 3 Total ovarian follicle populations in non-tg ($n=8$, white bars) or tg-FSH *hpg* ($n=12$, black bars) females, as well as follicle numbers in age-matched, non-tg, wild-type ovaries ($n=6$, grey bars) for comparison, quantified, as described in Materials and Methods. Significant differences relative to *hpg* are indicated, * $P<0.05$.

Figure 4 Serum inhibin B (A) and inhibin A (B) levels in the tg-FSH ($n=14$, black bars) and non-tg ($n=16$, white bars) *hpg* female mice are shown as arbitrary units, as described in Materials and Methods. For comparison, serum inhibin A and B levels in mature, wild-type females ($n=7$, grey bars) are shown. Significant differences from *hpg* are indicated, $*P<0.05$.

the *hpg* mouse and classical rat models is the delayed postnatal loss of gonadotrophins in 21-day-old hypophysectomized or 6–15-day-old GnRH-antagonist-treated rats, compared with congenital FSH/LH absence in *hpg* mice. Complete absence of postnatal FSH in *hpg* mice may account for the decreased primordial stock relative to the above rat models. In the present study, tg-FSH expression is driven by the rat insulin II gene promoter that confers postnatal expression of heterologous transgenes (Epstein *et al.* 1992, Takamura *et al.* 1998), as confirmed by equivalent serum tg-FSH levels in 10-day-old mice (data not shown). Therefore, the tg-*hpg* model will provide FSH expression during the important postnatal period of follicular development, unlike previous studies of short-term exogenous FSH actions in *hpg* females only after complete postnatal gonadotrophin deficiency (Halpin & Charlton 1988, Wang *et al.* 2005).

Our tg-*hpg* model has provided direct evidence that FSH activity in the absence of LH may markedly affect the development, survival or recruitment of the early follicle population. Previous work showed that mice overexpressing tg LH exhibited an accelerated loss of primordial follicles despite the presence of FSH (Flaws *et al.* 1997). Therefore, increased numbers of primordial follicles in tg-FSH *hpg* females may also reflect the absence of an opposing LH effect on the resting primordial follicle pool. Despite the lack of LH activity in *hpg* ovaries, the expression of tg-FSH increased secondary and stimulated antral follicle development, confirming previous *in vivo* studies demonstrating that antral follicle development is highly regulated by FSH (Halpin & Charlton 1988, Wang & Greenwald 1993, Kumar *et al.* 1997, McGee *et al.* 1997, Dierich *et al.* 1998, Oktay *et al.* 1998, Abel *et al.* 2000).

Increased serum levels of inhibin B and inhibin A in tg-*hpg* females demonstrated that tg-FSH stimulated ovarian inhibin secretion (reviewed in Findlay *et al.* 2001). Elevated inhibin B supports our previous work showing that tg-FSH dose-dependently increased circulating inhibin B in *hpg* females (Allan *et al.* 2001), and confirming the accepted role of FSH in stimulating granulosa cell inhibin B expression. Furthermore, our present work showed a significant correlation between serum inhibin B and predominantly the antral follicle populations. Likewise, elevated circulating inhibin A in tg-FSH *hpg* mice exhibiting antral follicle development, relative to non-tg *hpg* controls with antral arrest, agrees with the reported rise of inhibin A expression in secondary and antral follicles (Meunier *et al.* 1988, Wang *et al.* 2005). Inhibin A detection in 71% of tg-FSH (LH-deficient) *hpg* females in this study was similar to the 63% of LHR-null females expressing detectable inhibin A found by Hirst *et al.* (2004). Overall, these findings support recent studies showing increased serum inhibins B and A in FSH-treated *hpg* females to be associated with antral follicles, although higher inhibin A requires LH activity and the presence of preovulatory follicles (Wang *et al.* 2005).

Figure 5 Correlations between the relative inhibin B levels and the absolute numbers of different follicle types found in corresponding tg and non-tg *hpg* females ($n=16$). Serum inhibin B levels were significantly correlated to the numbers of secondary ($P=0.017$) and antral ($P=0.011$) follicles per ovary, but not the primordial ($P=0.287$) or primary ($P=0.475$) populations.

The tg-FSH *hpg* ovary without LH stimulation would be expected to resemble ovaries in mice lacking functional LH-beta or receptor genes (Lei *et al.* 2001, Zhang *et al.* 2001, Ma *et al.* 2004), which previously provided valuable comparisons for testicular development (Zhang *et al.* 2001, Allan *et al.* 2004, Hirst *et al.* 2004). Indeed, arrested antral follicle development was observed in all these models exhibiting isolated FSH activity. Mice congenitally lacking the LH receptor exhibit elevated circulating gonadotrophins (Lei *et al.* 2001, Zhang *et al.* 2001) that are susceptible to steroidal regulation, which would complicate analysis of FSH actions combined with other hormones. In comparison, ectopic FSH expression in the tg-*hpg* model independent of GnRH and LH (Allan *et al.* 2004, Allan *et al.* 2001), as well as preserved hormone responsiveness, allows further investigation of FSH in isolation or combined with other hormones. The

confounding effects of supraphysiological FSH concentrations previously reported in tg-FSH mice (Kumar *et al.* 1999) were not observed in this tg-FSH *hpg* model, since serum tg-FSH (4–5 IU/l) did not impede the fertility of young non-*hpg* females and was therefore within the limits compatible with physiological ovarian function (paper in preparation, Allan *et al.*).

In summary, our present study shows that FSH may enhance the early follicle stock. In isolation, tg-FSH activity increased the primordial pool relative to normal, despite predicted attrition through concurrent stimulation of antral follicle development and growth to the early preovulatory stage. Thus, the tg-FSH *hpg* ovary provides a valuable platform for exploration of the underlying molecular mechanisms that may orchestrate the development, survival or progression of the primordial follicle population. Future studies using this novel tg-FSH

paradigm will explore the functional consequences of combined LH or steroidal actions upon follicle dynamics.

Acknowledgement

We acknowledge the valued research assistance of the late Adam Koch.

Funding

This research was supported by funding from the National Health and Medical Research Council. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 14 December 2005

Accepted 19 December 2005

Made available online as an Accepted Preprint
21 December 2005