10.1071/RD13372_AC

Supplementary Material: *Reproduction, Fertility and Development*, 2015, 27(7), 985–999.

Supplementary Material

Follistatin is essential for normal postnatal development and function of mouse oviduct and uterus

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Study 2: Expression of FST in WT and tghFST315 uteri

Methods

Prior to considering *FST* expression in transgenic animals, we examined the basal expression of *Fst315* and *Fst288* and related genes (*Inhba* and *Inha*) along the length of the mouse reproductive tract, throughout the estrous cycle (age 6–8 wk) and following superovulation (age 5 wk) in C57/CBA mice. To analyse expression of *Fst315* and *Fst288* along the tract, tissue was collected from the ovary, oviduct (2 segments), uterine horn (4 segments), cervix and vagina (3 segments) (n = 5 animals, at estrus). To study cyclical changes (uterus only), vaginal smears were performed to determine stage of the estrous cycle (Marcondes *et al.* 2002). Mice were dissected at diestrus at 11:00 (D 11), proestrus at 11:00 (P 11),

proestrus/estrus at 20:00 (P/E 20), estrus at 02:00 (E 02) and estrus at 11:00 (E 11) (n = 3–8 per stage).

Gene expression was also examined following superovulation. Three groups of pre-pubertal (5 wk) C57/CBA mice (n = 8 per group) were examined: In Group 1, mice were given one intraperitoneal (ip) injection of pregnant mare's serum gonadotropin (PMSG) (5 IU). After 48 h the mice were injected (ip) with human chorionic gonadotropin (hCG) (5 IU) in saline (0.9% (w/v) NaCl) and culled 12 h later. Group 2 were injected (ip) with PMSG (5 IU) only and culled 48 h later. Group 3 controls were treated with vehicle solution (phosphate buffered saline) (1 mL), followed by an injection (ip) of saline 48 h later and culled 12 h later.

Following reproductive tract dissection, tissues were snap frozen in an isopropanol/dry ice slurry and stored at –80°C. Blood was collected by cardiac puncture. Frozen tissues or blood underwent RNA or protein isolation for qRT-PCR or RIA/ELISA analyses, respectively.

Results

Uterine *Fst* expression was unchanged during the WT estrous cycle

In normal cycling female mice, *Fst288* and *Fst315* mRNA expression levels were 10-fold higher in the ovaries compared to all other parts of the tract examined (oviduct (2 segments), uterine horn (4 segments), cervix and vagina) (P = ≤0.0001) (Fig. S1a–b). There was no difference in *Fst* isoform mRNA expression between the oviduct, uterus, cervix or vagina (Fig. S1a–b). There was no significant change in uterine mRNA expression of *Fst288* (Fig. S2a), *Fst315* (Fig. S2b) or *Inha* (Fig. S2d) through the estrous cycle. However, uterine *Inhba* mRNA expression was significantly increased in P/E20 compared to both D11 and E11 (P/E20: 7.7 ± 3.0, D11: 1.0 ± 0.2, E11: 0.8 ± 0.4, $F_{(4,29)}$ = 3.937, P = 0.013) (Fig. S2c). Total FST protein concentrations were unchanged throughout the estrous cycle in uterine homogenates (Fig. S2e) and sera (Fig. S2f) from WT mice. In contrast, uterine activin A

protein levels were significantly elevated during P/E20 compared to D11 and P11 (P/E20: 0.07 ± 0.01 ng/mg of protein, D11: 0.03 ± 0.01 ng/mg of protein, P11: 0.02 ± 0.003 ng/mg of protein, $F_{(4,32)}$ = 4.639, P = 0.005) (Fig. S2g). There was no change in activin A protein concentration in WT mouse serum during the estrous cycle (Fig. S2h).

Fst288, *Fst315*, *Inhba* and *Inha* mRNA were all expressed in pre-pubertal, non-cycling mouse uteri (Fig. S3a–d). Superovulation was induced in these mice to stimulate the development and ovulation of a cohort of follicles to magnify the hormonal effects on the uterus. However, superovulation with either PMSG alone, or PMSG followed by hCG, failed to induce any significant change in uterine mRNA expression of *Fst288, Fst315, Inhba* or *Inha* compared to vehicle treated mice (Fig. S3a–d). Similarly, activin A protein (uterine tissue and serum) and total FST protein (serum) were not changed significantly by the superovulation protocol (Fig. S3g–h, f). In contrast, total FST protein measured from uterine homogenates was significantly elevated in both the PMSG and PMSG plus hCG treatment groups compared to vehicle (vehicle 4.8 ± 0.9 ng/mg of protein, PMSG 15.4 ± 3.6 ng/mg of protein, PMSG + hCG 14.7 ± 2.0 ng/mg of protein, $F_{(2,21)}$ = 6.043, P = 0.008) (Fig. S3e).

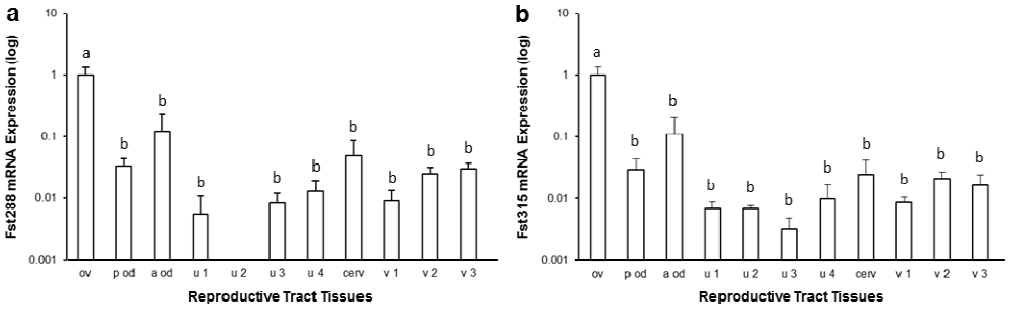

Fig. S1. Basal expression of *Fst* variants in mouse reproductive tract. Relative expression of (a) mouse *Fst288* and (b) *Fst315* mRNA across the female reproductive tract. Samples sizes were n = 5 per tissue. mRNA data are displayed as back-transformed logarithmic means ± 95% confidence intervals. Data were compared by one-way ANOVA followed by Tukey's

post-hoc tests and bars that do not share a common letter are significantly different ($P < 0.05$). *ov*, ovary, *p od*, posterior oviduct, *a od*, anterior oviduct, *u 1-4*, uterus segment 1-4, *cerv*, cervix, *v 1-3*, vagina segment 1-3.

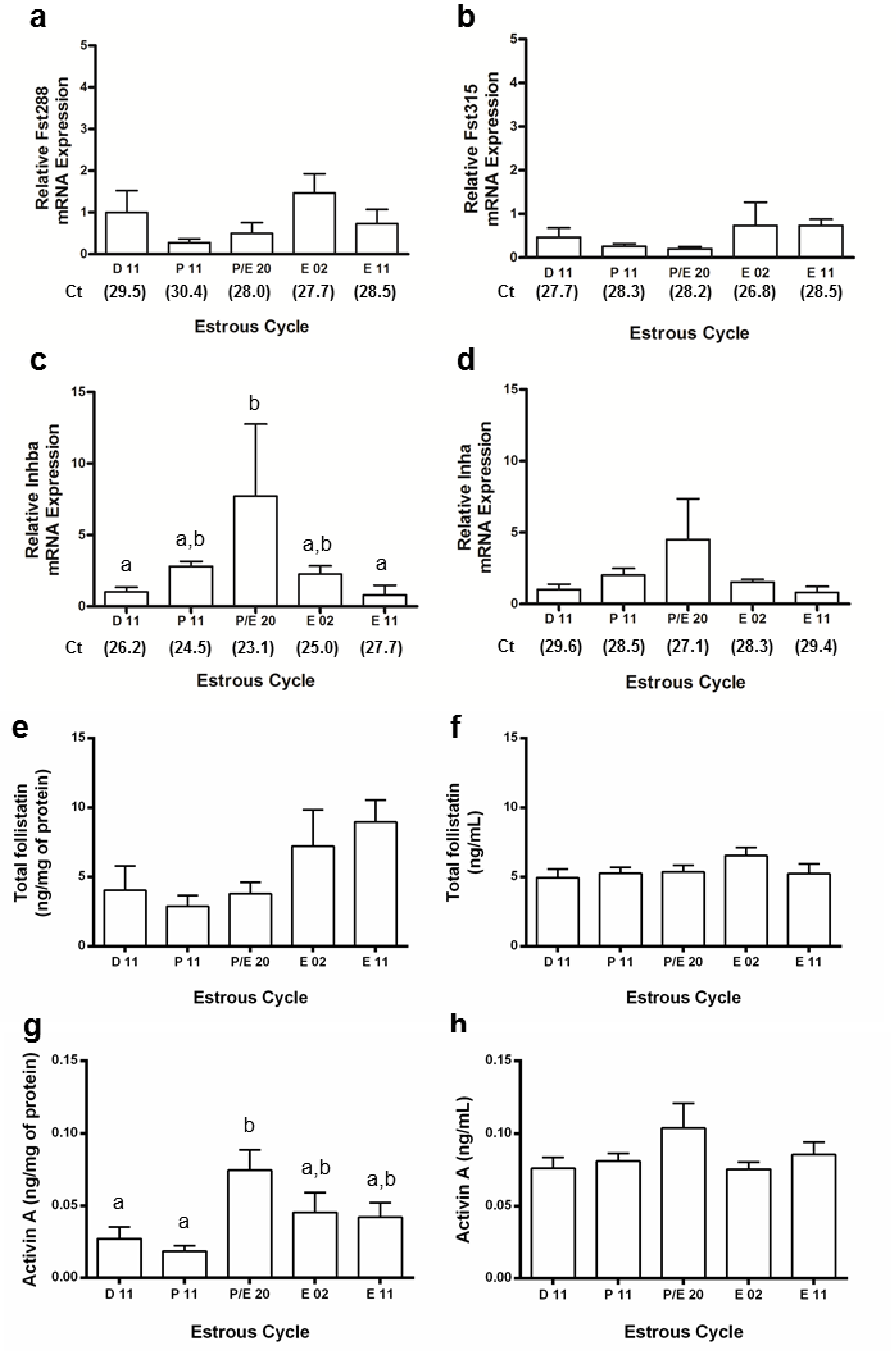

Fig. S2. Uterine *Inhba* mRNA and activin A protein expression change in WT estrous. Relative mRNA expression of (a) *Fst288*, (b) *Fst315*, (c) *Inhba* and (d) *Inha* on diestrus at

11:00 (D11) (Day 1), proestrus at 11:00 (P11) (Day 2), proestrus/estrus at 20:00 (P/E20) (Day 2), estrus at 2:00 (E02) and E at 11:00 (E11) (Day 3). Median Ct values for each stage are listed below the *x*-axis. mRNA data is displayed as back-transformed logarithmic means ± 95% confidence intervals. Protein expression of total FST (e and f) and activin A (g and h) throughout the estrous cycle. Samples sizes were $n = 3–8$ per cycle stage. Graphs e and g represent expression of total FST and activin A from whole uterine tissue homogenates, displayed as ng of follistatin or activin A per mg of total protein. Graphs f and h represent expression of total FST and activin A from sera, displayed as ng/mL. Data from e, g and h are displayed as means ± SEM. Data from f are displayed as back-transformed logarithmic means ± 95% confidence intervals. All data were compared by one-way ANOVA followed by Tukey's post-hoc tests and bars that do not share a common letter are significantly different ($P < 0.05$).

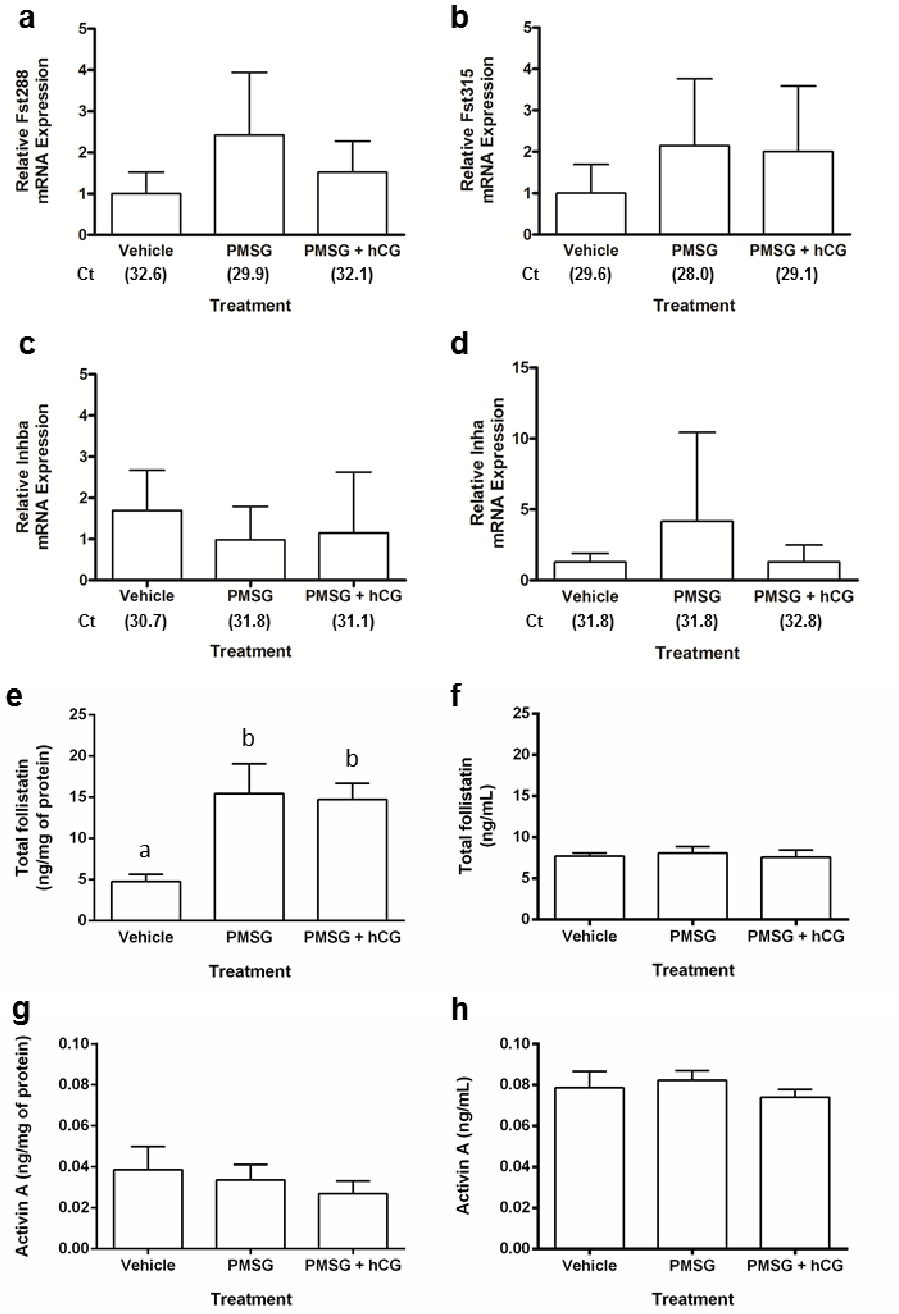

Fig. S3. Uterine total FST protein expression increased WT mice following superovulation. Relative mRNA expression of (a) *Fst288*, (b) *Fst315*, (c) *Inhba* and (d) *Inha* for vehicle, PMSG treatment or PMSG plus hCG treatment. Median Ct values for each stage are listed below the x-axis. Protein expression of total FST (e and f) and activin A (g and h) for vehicle, PMSG treatment or PMSG plus hCG treatment. Graphs e and g represent expression of total FST and activin A from whole uterine tissue homogenates, displayed as ng of FST or

activin A per mg of total protein. Graphs f and h represent expression of total FST and activin A from sera, displayed as ng/mL. Samples sizes were between 4 and 8 per treatment group. mRNA data are displayed as back-transformed logarithmic means ± 95% confidence intervals and protein data are displayed as means ± SEM. Data were compared by one-way ANOVA followed by Tukey's post-hoc tests and bars that do not share a common letter are significantly different ($P < 0.05$).

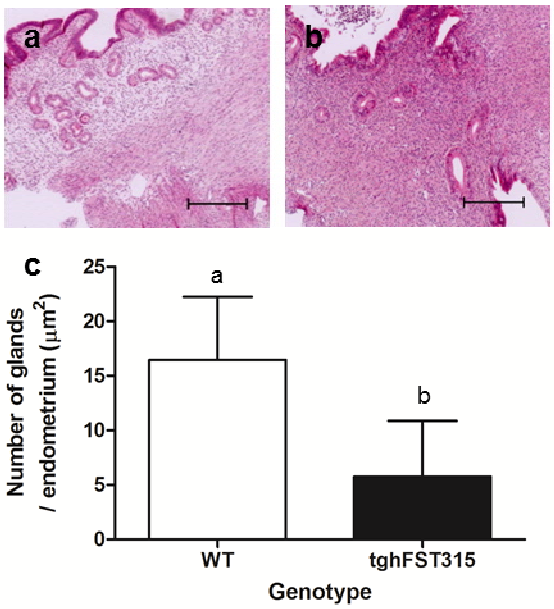

Fig. S4. tghFST315 mice have reduced endometrial glands. Using the vehicle treated animals from the ovariectomy study, gland profiles were counted in the WT and tghFST315 endometrium. Representative histogram of H&E stained 8 week (a) WT and (b) tghFST315 uterine section. (c) Quantification of number of glands per endometrium (μm^2). Samples sizes were n = 6–9 per genotype. Scale bar is equal to 0.1 mm. Data are displayed as mean ± SEM, and were analysed by independent t-test, groups that do not share a common letter are significantly different ($P < 0.05$).