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Anti-Müllerian hormone overexpression restricts preantral ovarian follicle survival

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Abstract

Anti-Müllerian hormone (AMH) is an ovarian regulator that affects folliculogenesis. AMH inhibits the developmental activation of the dormant primordial follicles and the oocyte within. In more mature follicles, AMH reduces granulosa cell sensitivity to follicle-stimulating hormone (FSH). We examined the effects of AMH overexpression on the stages of ovarian folliculogenesis, and the development of embryos, with a transgenic mouse that overexpresses human AMH in central nervous system neurons under the control of the mouse *Thy1.2* promoter (*Thy1.2-AMH^{Tg}* mice). These mice are severely sub-fertile, despite relatively normal ovulation rates. The embryos of *Thy1.2-AMH^{Tg}* females exhibited delayed preimplantation development and extensive mid-gestation fetal resorption. Young *Thy1.2-AMH^{Tg}* mouse ovaries exhibited only a slight reduction in the rate of primordial follicle activation but large declines in the number of developing follicles surviving past the primary stage. It was expected that *Thy1.2-AMH^{Tg}* mice would retain more primordial follicles as they aged, but at 5 months, their number was significantly reduced relative to wild-type females. These data indicate that moderate elevations in AMH levels can severely restrict reproductive output and the number of developing follicles in the ovary. This evidence suggests that early antral follicles are a target for AMH signaling, which may regulate early follicle survival.

Key Words

- ▶ anti-Müllerian hormone
- ▶ ovary
- ▶ folliculogenesis
- ▶ oogenesis
- ▶ embryo

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Introduction

Mammalian ovaries are endowed with a finite number of oocytes during early development. The dormant oocytes are encapsulated by pre-granulosa cells in structures known as primordial follicles. Primordial follicles are released from dormancy during activation, which is the initial stage of folliculogenesis. The rate of activation determines the size of the developing follicle pool and the lifetime of the ovarian reserve of primordial follicles (Faddy *et al.* 1992, Depmann *et al.* 2015). The developing follicle pool contains more oocytes than are required for

ovulation. The excess follicles undergo a degenerative process known as atresia leaving only a fraction to be selected for ovulation (Monniaux *et al.* 2014).

Anti-Müllerian hormone (AMH) is produced by granulosa cells within developing follicles and regulates ovarian reserve depletion by inhibiting primordial follicle activation (Durlinger *et al.* 1999, 2002, Carlsson *et al.* 2006). The activation of primordial follicles leads to transition to the primary stage. Subsequent progression through the preantral stages is facilitated by oocyte growth and

proliferation of the associated granulosa and theca cells. The antral stages are characterized by the acquisition of a fluid-filled cavity and an increasing reliance on pituitary-derived follicle-stimulating hormone (FSH) for survival (McGee & Hsueh 2000). AMH expression peaks at the small antral stage (Weenen *et al.* 2004) and the ability of AMH to counteract the effects of FSH (Visser & Themmen 2014) suggests these follicles are an AMH signaling target. Larger, preovulatory follicles are unlikely to be influenced by AMH due to reduced expression of AMH and its type 2 receptor, AMHR2 (Baarends *et al.* 1995, Weenen *et al.* 2004).

Amh^{-/-} mice have increased rates of primordial follicle activation, increased numbers of developing follicles and accelerated depletion of the ovarian reserve (Durlinger *et al.* 1999). However, atresia is proportionately increased in *Amh*^{-/-} mice and the number of preovulatory follicles available at the time of ovulation is not different to *Amh*^{+/+} mice (Visser *et al.* 2007). This suggests that AMH has strong effects on primordial follicle activation but is less important in the regulation of follicles at later stages of development. However, experimental reduction in AMH levels in rhesus macaques can cause an increase in the number of eggs ovulated (Xu *et al.* 2016), indicating that AMH can influence the later stages of folliculogenesis in some circumstances.

This study describes the ovarian phenotype in a transgenic AMH-overexpressing (*Thy1.2-AMH*^{Tg}) mouse model with AMH overexpression that was originally created to examine the effects of AMH in the brain (McLennan *et al.* 2017). The *Thy1.2-AMH*^{Tg} mice express a human AMH transgene under the murine *Thy1.2* gene promoter, which is active in a large proportion of central nervous system neurons. Adult *Thy1.2-AMH*^{Tg} mice have circulating AMH levels at approximately 1 nmol/L (McLennan *et al.* 2017) and the endogenous murine proteases are capable of converting the inactive AMH proprotein into the cleaved, receptor-binding isoform (AMH_{N,C}) (Pankhurst *et al.* 2016). The elimination of AMH_{N,C} is biphasic with a fast-phase that is consistent with rapid dispersal into interstitial fluid (Pankhurst *et al.* 2016), suggesting that tissue and circulating AMH concentrations reach equilibrium. For context, circulating AMH levels are ~200 pmol/L in wild-type female mice (Kevenaar *et al.* 2006) and follicular fluid AMH concentrations in humans can be as high as 6.4 nmol/L (Jeppesen *et al.* 2013). In the present study, *Thy1.2-AMH*^{Tg} females were found to be severely sub-fertile, with evidence of abnormal preimplantation embryo development. Primordial follicle activation was modestly reduced but a large reduction in

the preantral follicle population suggests that preantral follicles are more susceptible to the effects of elevated AMH concentrations than previously thought.

Materials and methods

Animals

The *Thy1.2-AMH* transgenic mice express a human AMH transgene in central nervous system neurons in multiple brain nuclei under the control of the murine *Thy1.2* gene promoter, beginning at approximately 21 days of age (McLennan *et al.* 2017). Breeding colonies were maintained on a C57Bl6/J background with wild-type females (*Thy1.2-AMH*^{0/0}) paired to hemizygous transgenic males (*Thy1.2-AMH*^{Tg/0}). Experimental females were *Thy1.2-AMH*^{0/0} (wild type) and *Thy1.2-AMH*^{Tg/0} (denoted as *Thy1.2-AMH*^{Tg}) litter mates derived from these pairings. All stud males used in the experiments had wild-type genotypes (*Thy1.2-AMH*^{0/0}) unless otherwise mentioned. Genotypes were determined by PCR using primers directed to the *Thy1.2* promoter region (5'-TGT AGC TTT CCC CAC CAC AGA-3') and the human *AMH* transgene (5'-AGG GCC TCA GTC CCC AGC AGA-3'). The mice were housed with access to food and water, *ad libitum* under 12:12 light-darkness cycles. All experiments were approved by the University of Otago Animal Ethics Committee or the University of Melbourne Animal Ethics Committee.

Mid-gestation fetal survival

Thy1.2-AMH^{Tg} and wild-type female mice were mated with wild-type studs and the females were examined daily, 2 h after the onset of the light phase. Embryonic day 0 (E0) was recorded when a copulatory plug was observed. The females were euthanized at E10 and the uteri were examined for the presence of growing fetoplacental units. Fetuses were categorized as unhealthy if they were hemorrhagic, had lost turgor or were overtly small relative to their adjacent fetuses.

Preimplantation embryo examination

Thy1.2-AMH^{Tg} and wild-type female mice were housed in an animal house with a shifted light cycle. Females were paired with wild-type studs 8 h after the onset of the darkness phase for a period of 2 h. The female mice were then removed from the stud-cage and examined

for the presence of a copulatory plug. The process was repeated until the female became pregnant. The mated females were killed 74–76 h after copulation and the embryos were flushed from the uteri with Hanks balanced salt solution (ThermoFisher Scientific, cat# 14175-095) driven through a 27 gauge needle attached to a 1 mL syringe. Embryos were examined under a phase-contrast microscope to record the stage of development according to previously established criteria (Kaufman 1992). A morphological abnormality was recorded if embryos exhibited asymmetric cell division cellular degeneration/fragmentation or failure to progress beyond the 8-cell stage. Single (uncleaved) cells within the zona pellucida were recorded as unfertilized oocytes/arrested zygotes as it was not possible to distinguish between the two.

Preimplantation embryo culture

Embryos were obtained from 25-day-old C57BL/6 × CBA F1 hybrid mice, which were superovulated with 5 IU pregnant mare serum gonadotropin (Folligon; MSD Animal Health, Bendigo East, Australia), IP at the mid-point of the light phase. 5 IU human chorionic gonadotropin (hCG) (Chorulon; MSD Animal Health) was administered 48 h later and the female was paired with a C57BL/6 × CBA F1 stud male. The mated females were killed 22 hours after hCG injection, embryos were dissected from the oviducts and prepared for culture as described previously (Gardner & Lane 2007, 2014). Pronucleate oocytes were pooled and allocated randomly to the culture treatments. Single embryos were cultured in 2 µL G1 medium (Vitrolife, Göteborg, Sweden) under paraffin oil (Ovoil; Vitrolife) in a humidified multi-gas incubator at 37°C with 5% O₂ and 6% CO₂. Embryos were cultured in media containing 0 pmol/L (control), 20, 200 or 2000 pmol/L of the 25 kDa recombinant human AMH C-terminal fragment (AMH_C, R&D systems, cat# 1737-MS). The treatment media was replaced after 24 h of culture and again at 48 h when the media was changed to G2 (Vitrolife). Embryo development was assessed at embryonic days 1.5 and 2.5, and afternoon of day 3.5 of culture (46, 70 and 98 h post hCG).

Oviduct mRNA reverse-transcriptase qPCR

Thy1.2-AMH^{tg} and wild-type female mice were paired with wild-type studs 8 h after the onset of the dark phase of the light cycle for a period of 2 h. Females were then checked for copulatory plugs and were killed 35–37 h after copulation at a time point 1 h before the onset of the dark phase (concurrent with the diurnal prolactin

peak). A blood sample was obtained by cardiac puncture in mice anesthetized with 225 mg/kg Ketamine (Phoenix Pharm, Auckland, New Zealand) and 3 mg/kg Domitor (Medetomidine, Pfizer Animal Health, Auckland, New Zealand), and the oviducts were dissected out and incubated in RNA later (ThermoFisher Scientific, cat# AM7020). Oviducts were frozen and stored at –80°C before they were homogenized in TRI reagent (Sigma-Aldrich) to isolate the RNA. 1 µg of mRNA was treated with 2 units of DNase (TurboDNase, ThermoFisher Scientific, cat# AM2238) for 30 min at 37°C followed by inactivation at 75°C for 5 min. RNA was converted to cDNA with the Revertaid reverse-transcription kit (ThermoFisher Scientific, cat# K1622) using random hexamer primers. The qPCR was conducted with a LightCycler480 (Roche) cycling through 95°C for 20 s, 60°C for 20 s and 72°C for 30 s, and then data acquisition was done. Relative mRNA expression of each transcript was calculated according to the formula $2^{-\Delta C_p}$ with β -actin (*Actb*) used as the reference gene.

Prolactin assays

Heparinized plasma samples were collected during the oviduct mRNA qPCR experiments (above). Prolactin was assayed according to a previously published procedure (Brown *et al.* 2010). The primary antibody (NIDKK-rabbit anti-oPRL-2, NIH, batch# AFP131078) was used at a final concentration of 1:400,000. A mouse prolactin standard (NIDDK-mouse PRL, batch# AFP6476C) was used to generate the standard curve and was labeled with ¹²⁵I via the chloramine-T method. Iodine-labeled prolactin was added to each tube at 20,000 cpm followed by 24-h incubation at room temperature. The antibodies were precipitated with 5% polyethylene glycol and the radioactivity of the pellet counted and prolactin concentrations were interpolated from the standard curve.

Estrous cycling

Female mice were introduced to a cage containing soiled bedding from a male mouse cage to provide male pheromones. Vaginal lavage fluid (5 µL of water) was collected daily and dried onto a microscope slide. Slides were stained with toluidine blue and estrus stages were determined by vaginal cytology. Proestrus was characterized by clumps of epithelial cells with sparsity of cornified epithelial cells. Estrus was characterized by a predominance of cornified epithelial cells and proportionately fewer non-cornified cells. Metestrus and

diestrus were analyzed together and represent the only two stages with an abundance of polymorphonuclear leukocytes present. Estrus cycles were tracked across three proestrus stages to determine the length of 2 cycles per mouse. Monitoring was ceased after 16 days if 2 cycles had not been recorded.

Ovarian follicle stereological counts

Ovarian tissue was obtained at the proestrus stage of the estrous cycle. A younger age-group was collected between 45 and 58 days of age and an older group at 150–162 days. Ovaries were dissected from the mice and immersion-fixed in Bouins fixative for 4 h at room temperature. The tissue was embedded in paraffin wax and serially sectioned at 5 μ m and stained with hematoxylin and eosin. Ovarian follicles were counted with methods adapted from Myers *et al.* (2004). Every follicle from the secondary stage onwards was counted by sampling serial sections at 25 μ m intervals in both ovaries. Preantral follicles included secondary follicles, with at least 2 layers of granulosa cells at any part of the follicle perimeter. Follicles were considered as antral if a cavity was observed at any point in the granulosa cell layer. A follicle was considered to be preovulatory if it had a diameter greater than 250 μ m. Follicles with oocytes lacking a continuous cumulus granulosa cell layer adjacent to the oocyte were considered to be overtly atretic and were not included in counts. Primordial follicles were counted in a single ovary from each mouse by physical dissector stereology using every 10th section as the reference section and every 11th section as the look-up section. Primary follicles were counted with every 10th section used as the reference section and every 12th section used as the look-up section. Primordial follicles were counted across the entire ovary section if the nucleus was present in the reference section but not in the look-up section. Primary follicles were counted if the cytoplasm was visible in the reference section but not in the look-up section. A follicle was considered to be primordial if all granulosa cells in the reference section displayed flattened/squamous morphology. The presence of a single cuboidal granulosa cell was an indication that the follicle had begun the process of activation; hence, the follicle was counted as a primary follicle. Formulae for stereological counts are listed in (Oorschot 1994). Section area was determined using the Gaussian blur (pixel radius=20.0) and color thresholding functions of ImageJ software (NIH) to identify and measure the area within the section boundary. Cell counting was conducted with the observer blinded to the genotype.

Statistical analysis

Statistical analysis was carried out with SPSS 24.0 (IBM) and Prism 7.0 (Graphpad Software). Student's *t*-test was used to compare means between the wild-type and *Thy1.2-AMH*^{tg} females with Levene's test used to determine homogeneity of variances. One-way ANOVA was used for comparisons of three or more groups. Proportional data (percentages) were analyzed with Fisher's exact test. All data except proportional data are expressed as means $\pm$ S.E.M.

Results

Prior investigation into the *Thy1.2-AMH*^{tg} mice revealed that the transgenic females rarely give birth when paired with males and in the rare occasions that the females gave birth, the litter size was less than half of the mean for wild-type dams (McLennan *et al.* 2017). The murine analogue of miscarriage is typically embryo resorption, which allows removal of nonviable fetuses without affecting viable fetuses. Fetal resorption was apparent in both wild-type and *Thy1.2-AMH*^{tg} females at embryonic day 10 (E10) (Fig. 1A and B). The prevalence of resorbing fetuses was increased relative to wild-type females (Fig. 1C), but no differences in the number of oocytes was observed on the day of ovulation (Fig. 1D). The mean ($\pm$ S.E.M., *n*=6) number of healthy fetuses remaining in *Thy1.2-AMH*^{tg} females was 4.3 ± 1.4 . By contrast, wild-type females had an average of 10.3 ± 1.9 healthy fetuses at E10. Fetal resorption does not appear to be caused by expression of the transgene within the fetus because stud *Thy1.2-AMH*^{tg} males sire litters with the expected 1:1 Mendelian ratios when paired with wild-type females (Fig. 1E). Furthermore, the survival of fetuses sired by *Thy1.2-AMH*^{tg} males is not influenced by fetal sex (Fig. 1F). Collectively, this suggests that AMH overexpression has a maternal effect that reduces survival of the female's fetuses.

Fetal miscarriage can arise due to oocyte defects present prior to fertilization and in preimplantation embryos (Richardson *et al.* 1987). The development of preimplantation embryos of wild-type and *Thy1.2-AMH*^{tg} females was examined 74–76 h after mating (Fig. 2A, B, C, D, E and F). In this experiment, the number of embryos and unfertilized oocytes/arrested zygotes was reduced in some *Thy1.2-AMH*^{tg} females but others had counts that were equivalent to their wild-type counterparts (Fig. 2G). We have previously shown that *Thy1.2-AMH*^{tg} females rarely give birth after mating (McLennan *et al.* 2017), but the present data suggest that failure to ovulate is not the cause.

Figure 1

Thy1.2-AMH^{Tg} dam uteri exhibit signs of embryo resorption at mid-gestation. The reproductive tracts of pregnant wild-type (A) and *Thy1.2-AMH^{Tg}* (B) dams at embryonic day 10 (E10) both contain fetuses but *Thy1.2-AMH^{Tg}* dams more frequently exhibited signs of fetal resorption (arrowheads). The location of the ovaries are indicated by arrows, grid scale = 1 cm. The percentage of fetuses at E10 that appeared healthy and showed no overt signs of degeneration (C) was lower in *Thy1.2-AMH^{Tg}* dams ($n=43$) compared to wild-type dams ($n=64$, Fisher's exact test). There was no difference in the number of ovulated oocytes (cumulus-oocyte complexes) obtained from the oviducts (D) of wild-type dams and *Thy1.2-AMH^{Tg}* dams on the first morning of estrus (Student's *t*-test, $n=8-10$, error bars = S.E.M.). Offspring derived from wild-type dams and *Thy1.2-AMH^{Tg}* studs ($N=254$) display the expected 1:1 Mendelian ratios in both *Thy1.2-AMH* genotype (E) and sex (F). A full color version of this figure is available at <https://doi.org/10.1530/JOE-18-0005>.

Zygote cleavage rates per pregnant female (Fig. 2H) and the number of 2-stage embryos that reached the morula stage or further were not significantly different between the two genotypes (Fig. 2I). However, significantly fewer embryos had developed to the blastocyst stage in *Thy1.2-AMH^{Tg}* females compared to wild-type females (Fig. 1J). The embryos from *Thy1.2-AMH^{Tg}* females also displayed a significantly higher incidence of morphological abnormalities (Table 1). Abnormal embryos were present in 7 of 12 *Thy1.2-AMH^{Tg}* females but only 1 of 11 wild-type females. The effects of AMH overexpression appear to be indirect because embryos cultured from the zygote stage in the presence of recombinant AMH_C have normal developmental progression (Fig. 2K) and normal cell numbers in the trophectoderm and inner cell mass (Supplementary Table 1, see section on supplementary data given at the end of this article). The highest AMH_C dose (2 nmol/L) is equivalent to circulating AMH concentrations in the *Thy1.2-AMH^{Tg}* mice (McLennan *et al.* 2017).

Preimplantation embryo development is regulated by maternal production of growth factors and growth inhibitors. Transcripts for murine *Amh* mRNA and the AMH receptor subunits (*Amhr2*, *Acvr1*, *Bmpr1a*, *Bmpr1b*) were all detected in the oviducts at 35–37 h post-coitus

(Supplementary Table 2). We tested whether overexpression of AMH affects the expression of well-characterized growth promoters (*Egf*, *Csf2*, *Il6*, *Pdgfa*, *Kitlg* and *Faah*) and growth inhibitors (*Csf1*, *Tnfsf10*, *Ifng*). No significant differences were observed in the expression of any transcript between *Thy1.2-AMH^{Tg}* and wild-type females (Supplementary Table 2) suggesting that AMH does not induce global changes in oviductal growth factor profiles.

AMH can modulate the hypothalamic neurons that regulate gonadotropin secretion (Cimino *et al.* 2016) and a severely defective hypothalamic-pituitary-gonadal axis in *Thy1.2-AMH^{Tg}* was expected to affect estrus cycling. No significant difference was observed in the time-to-copulation of wild-type and *Thy1.2-AMH^{Tg}* females when paired with male studs (Fig. 3A). Two of 10 *Thy1.2-AMH^{Tg}* females failed to get pregnant within 20 days of pairing, but became pregnant within 5 days when swapped to a new male. The mean estrus cycle length and the length of each stage as a proportion of the full cycle were not significantly different between the two genotypes (Fig. 3B and C). A single *Thy1.2-AMH^{Tg}* female remained at the diestrus stage for 16 days and upon euthanasia her ovary was found to be abnormally small (see below). In early pregnancy, the rodent hypothalamus and pituitary orchestrate twice daily prolactin pulses that are needed

Figure 2

Delayed and abnormal development becomes evident in embryos from *Thy1.2-AMH^{Tg}* dams between the morula and blastocyst stage. The embryos flushed from wild-type and *Thy1.2-AMH^{Tg}* dams displayed a range of developmental stages at 3.25 days post-coitus, including morula (A), compacted morula (B) and blastocyst (C) stages. Abnormal forms were found in both genotypes, including embryos with asymmetric cell division (D, arrowhead) or cellular degeneration (E), often accompanied by developmental arrest. Single cell entities that could represent either unfertilized oocytes or growth-arrested zygotes were also observed in both genotypes (F). The number of oocytes or concepti per dam was significantly lower (Student's *t*-test) in *Thy1.2-AMH^{Tg}* dams (G) but the percentage of oocytes that underwent the first cleavage per dam (H) was not significantly different between wild-type and *Thy1.2-AMH^{Tg}* dams ($P=0.313$, Student's *t*-test, $n=11-12$, error bars = s.e.m.). Of concepti from wild-type ($n=79$) and *Thy1.2-AMH^{Tg}* ($n=57$) dams that reached the 2-cell stage or greater, the percentage that developed to the morula stage or greater by 3.25 days post-coitus (I) was not significantly different between the genotypes ($P=0.071$, Fisher's exact test). However, significantly fewer *Thy1.2-AMH^{Tg}* dam embryos reached the blastocyst stage than wild-type dam embryos (Fisher's exact test) by 3.25 days post-coitus (J). Recombinant AMH treatment had no significant effect on the development of cultured zygotes (K). Development was assessed by the number of embryos reaching the 2-cell stage by day 1.5, the morula stage by day 2.5 and the blastocyst stage by day 3.5 *in vitro* ($n=80-81$, Pearson's chi-square test). A full color version of this figure is available at <https://doi.org/10.1530/JOE-18-0005>.

to rescue the corpus luteum (Grattan *et al.* 2008). Serum prolactin levels were assessed 1 h before the darkness phase of the daily light cycle, when the diurnal peak is expected to occur. No significant difference was observed

in prolactin levels between pregnant females of the two strains on day 2 of pregnancy (Fig. 3D). These preliminary experiments suggest that if AMH alters hypothalamic dysfunction, the effects are subtle.

Table 1 Abnormal embryo morphology in pregnant *Thy1.2-AMH^{Tg}* females.

	Wild-type dams	<i>Thy1.2-AMH^{Tg}</i> dams
Number of dams	11	12
Unfertilized oocytes/uncleaved zygotes	6	13
Total embryo count ^{c,d}	79	57
Abnormal embryo count ^{c,d}	4	17
% Abnormal ^c	5.1	29.8 ^b
Number of dams with abnormal embryos ^c	1 (9.1%)	7 (58.3% ^a)

^a $P<0.05$ compared to wild-type group, Fisher's exact test; ^b $P<0.001$ compared to wild-type group, Fisher's exact test; ^cincludes only the concepti/embryos that reached the 2-cell stage or greater; ^dmorphological abnormalities include; asymmetric cell division, failure to progress beyond the 8-cell stage and embryo degeneration commonly including reduced embryo size relative to the zona pellucida volume and an accumulation of cellular debris.

Figure 3

Estrous cycling and the stimulus to rescue the corpora lutea both appear to be functional in *Thy1.2-AMH^{Tg}* female mice. The time-to-copulation for most *Thy1.2-AMH^{Tg}* females, determined by copulatory plugs, was within the range of the wild-type studs following pairing with stud males (A). No significant differences in mean estrous cycle length was observed between wild-type and *Thy1.2-AMH^{Tg}* females (B) with no differences in the relative lengths of the different cycle stages (C, $n=7-8$, error bars=S.E.M.). The percentage of the estrus cycle occupied by the different stages was not significantly different between the two genotypes (C). The diurnal surge in plasma prolactin on the second day of pregnancy (D) showed no significant difference between wild-type and *Thy1.2-AMH^{Tg}* females ($P=0.986$, Student's t -test, $n=10-12$). Error bars=S.E.M.

Histological examination of *Thy1.2-AMH^{Tg}* and wild-type ovaries collected immediately after puberty revealed the presence of developing follicles at all stages, but there were conspicuously fewer preantral follicles in *Thy1.2-AMH^{Tg}* ovaries (Fig. 4G and H). Most *Thy1.2-AMH^{Tg}* ovaries had typical numbers of preovulatory follicles (Fig. 4F). Corpora lutea tissue from prior estrous cycles was present in most *Thy1.2-AMH^{Tg}* females with the exception

of one female that had few preovulatory follicles (Fig. 4I). Quantification of the different follicle classes revealed no differences in absolute numbers of primordial (Fig. 4A) or primary follicles (Fig. 4B). Primordial follicle counts are highly variable between females but correlate with the number of primary follicles (Richardson *et al.* 1987). When primary follicle numbers were standardized to primordial follicle counts a significant reduction in the relative rate of primordial follicle activation was detected in *Thy1.2-AMH^{Tg}* female mice (Fig. 4C). An even larger reduction in preantral and small antral follicle numbers was present in the *Thy1.2-AMH^{Tg}* ovaries relative to wild-type mice (Fig. 4D and E), but there was no significant difference in the number of preovulatory follicles (Fig. 4F). These data indicate that there is a moderate reduction in primary follicle counts in *Thy1.2-AMH^{Tg}* mice relative to wild-type mice but a large reduction in follicle numbers immediately after the primary stage.

Ovaries from *Thy1.2-AMH^{Tg}* and wild-type mice were examined at 5 months of age to determine if prolonged exposure to high levels of AMH leads to an accumulation of primordial follicles. Surprisingly, the numbers of primary and primordial follicles were significantly lower in *Thy1.2-AMH^{Tg}* ovaries (Fig. 5A and B). There was no longer a difference in the ratio of primary to primordial follicles (Fig. 5C), but the large reduction in preantral and small antral follicles in *Thy1.2-AMH^{Tg}* mice persisted (Fig. 5D and E). There was also a statistically significant reduction in the number of preovulatory follicles of *Thy1.2-AMH^{Tg}* ovaries relative to wild types at this time point (Fig. 5F).

Discussion

The *Thy1.2-AMH^{Tg}* females exhibit severe subfertility caused by mid-gestational fetal resorption, with altered preimplantation embryo development. The delivery of transgenic AMH through the blood is likely to cause an elevation in AMH concentrations ovarian stroma, as there was a moderate inhibition of primordial follicle activation. The reduction of early preantral follicles was disproportionate to the reduction in primary follicle numbers suggesting that elevated AMH levels affect follicle survival at this stage. Young *Thy1.2-AMH^{Tg}* females were still capable of ovulating similar numbers of oocytes to wild-type mice, but the delay in embryonic development might indicate that the developmental competence of the oocytes is impaired but other factors may contribute to the loss of embryos at the fetal stage (see below).

Figure 4

Ovarian follicle development is altered in *Thy1.2-AMH^{Tg}* female mice at 45–58 days of age. Unilateral primordial follicle (A) and primary follicle (B) counts were not significantly different in wild-type (white bars) and *Thy1.2-AMH^{Tg}* mouse ovaries. The ratio of primary follicles to primordial follicles per female was significantly lower in *Thy1.2-AMH^{Tg}* female mice (C). Bilateral preantral follicle (D) and small antral follicle (E) counts were significantly lower in *Thy1.2-AMH^{Tg}* females than in wild types. No significant differences were observed in bilateral preovulatory follicle counts (F). Histological sections from the center of wild-type (G) and *Thy1.2-AMH^{Tg}* ovaries (H, I) reveal a large difference in the size of the ovaries. Large preovulatory follicles are present in the ovaries from both genotypes (arrowheads) but developing preantral follicles (arrows) are conspicuously absent in many sections from *Thy1.2-AMH^{Tg}* ovaries. (Scale bar = 500 μm, $n=7$, error bars = s.e.m., Student's *t*-test.) A full color version of this figure is available at <https://doi.org/10.1530/JOE-18-0005>.

The majority of the follicle loss in *Thy1.2-AMH^{Tg}* mice occurred in the early preantral follicle stage, with little additional attrition at subsequent stages. Larger follicles may be less susceptible because they have greater

sensitivity to the survival-promoting effects of FSH (Abel *et al.* 2000). A reduction in AMHR2 expression in the later antral stages (Baarends *et al.* 1995) may also protect the larger follicles. *Amh*^{-/-} mice have greater rates of primordial

Figure 5

Ovarian follicle development is altered in *Thy1.2-AMH^{Tg}* female mice at 150–162 days of age. Unilateral primordial follicle (A) and primary follicle counts (B) were significantly lower in the *Thy1.2-AMH^{Tg}* female mice. No difference ($P=0.303$) was observed in the ratio of primary follicles to primordial follicles per female (C). Reduced bilateral preantral (D), small antral (E) and preovulatory (F) follicle counts were observed in the *Thy1.2-AMH^{Tg}* females relative to wild-type controls. ($n=6-7$, error bars = s.e.m., Student's *t*-test.)

follicle activation and larger developing follicle pools than *Amh*^{+/+} mice (Durlinger *et al.* 1999) but the number of preovulatory follicles is unaffected (Visser *et al.* 2007). This is likely due to the strong overriding influence of the hypothalamic–pituitary–gonadal axis on the regulation of ovulation. One hypothesis is that acquisition of greater sensitivity to FSH may be capable of protecting antral follicles from the influence of AMH. Few follicles make it to the antral stage in the *Thy1.2-AMH*^{Tg} mice but a high proportion of these follicles survive to ovulation. Another hypothesis is that the developmental increases in follicle expression of gremlin 1 and 2 (Kristensen *et al.* 2014, Nilsson *et al.* 2014) may also protect larger follicles, as these AMH-binding proteins neutralize the effects of AMH (Nilsson *et al.* 2014). Additional unidentified processes may also contribute to the survival of larger follicles when AMH levels are elevated.

Early antral follicles typically express less AMH than late preantral and small antral follicles (Weenen *et al.* 2004); hence, granulosa cells in early antral follicles may be incapable of high AMH-output. However, the present study raises the possibility that AMH induces early antral follicle atresia, in which case high-output follicles may be rapidly removed from the developing population. Our initial observations suggested that *Thy1.2-AMH*^{Tg} mouse late preantral and small antral follicles express normal levels of AMH, but AMH was not detectable in primary and early secondary follicles (McLennan *et al.* 2017). This may represent a compensatory mechanism or it may be that only follicles with low endogenous expression can survive the transgenic overexpression of AMH. This may have particular relevance in pathological conditions such as polycystic ovary syndrome, where follicular fluid and serum AMH levels are often elevated (Pellatt *et al.* 2007).

The reduced primordial follicle counts in 5-month-old *Thy1.2-AMH*^{Tg} mice was surprising, as numerous *in vitro* studies have demonstrated that short-term AMH treatment (2–10 days) inhibits primordial follicle activation in ovary explant cultures (Durlinger *et al.* 2002, Carlsson *et al.* 2006, Nilsson *et al.* 2007, Rocha *et al.* 2016). However, 4 weeks of AMH treatment has been shown to increase primordial activation rates (Schmidt *et al.* 2005); hence, the effect of AMH may change with the length of exposure. Alternatively, the collapse of the primordial follicle population may be indirect and subsequent to the profound effects on the developing follicle population. However, the possibility that prolonged elevation in AMH is toxic to dormant primordial follicles requires urgent investigation because AMH treatment has been proposed as a reversible contraceptive and a therapeutic inhibitor

of ovarian reserve depletion (Margolskee & Selgrade 2013, Hayes *et al.* 2016, Kong *et al.* 2016, Kano *et al.* 2017). The loss of primordial follicles is thought to be irreversible and the 5-month-old *Thy1.2-AMH*^{Tg} phenotype indicates that more research is needed to determine the safety of long-term AMH treatment.

It is not yet clear whether the *Thy1.2-AMH*^{Tg} ovarian phenotype is related to the subfertility phenotype. The phenotype is consistent with the limited oocyte pool hypothesis, which proposes that a small developing follicle pool imparts reduced negative feedback on the pituitary and hypothalamus leading to increased FSH secretion, accelerated antral follicle development and ovulation of immature oocytes (Warburton 1989). The phenotype is also consistent with an alternative hypothesis proposing that larger developing follicle pools enable greater capacity to select follicles with high-quality oocytes, while excluding oocytes of low-quality (Pankhurst 2017). However, AMH in the hypothalamus can modulate gonadotropin secretion (Cimino *et al.* 2016), and there is scope for the neuronal AMH expression to affect this system, with downstream effects on reproduction. Similarly, AMHR2 has been detected in the endometrium and placenta (Renaud *et al.* 2005, Wang *et al.* 2009, Novembri *et al.* 2015), which raises the possibility that AMH acts directly on the uterus or placenta to induce fetal resorption independently of the ovarian effects. The upregulation of AMHR2 expression in uterine endometrium during pregnancy (Renaud *et al.* 2005) suggests that sensitivity to AMH increases in this organ during pregnancy.

We propose three hypothetical mechanisms of fetal loss in the *Thy1.2-AMH*^{Tg} females; (1) uterine rejection of the fetus, (2) failure of endocrine support of pregnancy from the hypothalamic–pituitary–gonadal axis and/or (3) poor initial oocyte quality, affecting subsequent embryonic and fetal development. The loss of oocytes and concepti at different stages may arise due to a combination of multiple mechanisms acting at different stages. Alternatively, elevated AMH levels may have a single action with varied effects on oocytes or concepti from *Thy1.2-AMH*^{Tg} dams. Such variation could explain why some concepti survive longer than others but our prior experiments indicate that few will survive to birth (McLennan *et al.* 2017).

Conclusion

Very high levels of AMH affect fertility by shutting down primordial follicle activation (Kano *et al.*

2017). The lower levels of AMH in the circulation of *Thy1.2-AMH*^{tg} mice permit primordial follicle activation but still lead to severe subfertility. A large absence of early preantral follicles appears to indicate that this follicle stage is most susceptible to the effects of moderate AMH overexpression. Serum AMH levels naturally show a large degree of variation between individuals; hence, it will be important to determine whether the *Thy1.2-AMH*^{tg} phenotype recapitulates a biological phenomenon or is more relevant to pharmacological doses of AMH.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-18-0005>.

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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