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FSH inhibits AMH to support ovarian estradiol synthesis in infantile mice

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Abstract

Anti-Müllerian hormone (AMH) regulates ovarian function in cyclic females, notably by preventing premature follicle-stimulating hormone (FSH)-mediated follicular growth and steroidogenesis. Its expression in growing follicles is controlled by FSH and by estradiol (E2). In infantile females, there is a transient increase in the activity of the gonadotrope axis, as reflected by elevated levels of both gonadotropins and E2. We previously demonstrated in mice that elevated FSH concentrations are necessary to induce E2 production by preantral/early antral follicles through the stimulation of aromatase expression without supporting their growth. However, whether this action of FSH could involve AMH is unknown. Here, we show that *Amh* mRNA and protein abundance and serum AMH levels are elevated in infantile mouse females, compared with those in adults. By experimentally manipulating FSH and E2 levels in infantile mice, we demonstrate that high FSH concentrations lower *Amh* expression specifically in preantral/early antral follicles, whereas E2 has no effect. Importantly, treatment of infantile ovaries in organotypic cultures with AMH decreases FSH-mediated expression of *Cyp19a1* aromatase, but it does not alter the expression of cyclin D2-mediating granulosa cell proliferation. Overall, our data indicate that the infantile elevation in FSH levels suppresses *Amh* expression in preantral/early antral follicles, thereby favoring *Cyp19a1* aromatase expression and E2 production. Together with recent discoveries that AMH can act on both the hypothalamus and the pituitary to increase gonadotropin levels, this work suggests that AMH is a critical regulator of the gonadotrope axis during the infantile period, thereby contributing to adult reproductive function programming.

Key Words

- ▶ ovary
- ▶ steroidogenesis
- ▶ follicular growth
- ▶ gonadotropins
- ▶ mini-puberty

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Introduction

In mammals, the infantile period is characterized by a transient elevation in the levels of the two gonadotropins LH (luteinizing hormone) and FSH (follicle-stimulating hormone) shortly after birth (Döhler & Wuttke 1975, Dullaart *et al.* 1975, Winter *et al.* 1975, Chellakooty *et al.* 2003, Kuiri-Hänninen *et al.* 2014). This process

is accompanied by substantial gonadal activity as demonstrated by high sex steroid production in both sexes (estrogens and androgens in females and males, respectively) and by follicular growth in females. The resulting stimulation of gonadal activity, through sex steroid production, may be critical for the maturation

of the genital tract and for brain sexualization, thereby contributing to the programming of adult reproductive function (Stewart & Cygan 1980, Clarkson *et al.* 2009, Brock *et al.* 2011, Kuiri-Hänninen *et al.* 2013, Rey 2014).

The ovary is already receptive to gonadotropins during the infantile period, with FSH receptors (FSHR) expressed in granulosa cells and LH receptors (LHR) present in thecal cells (Fortune & Eppig 1979, White & Ojeda 1981, O'Shaughnessy *et al.* 1997). Similarly as in adult cyclic females, LH stimulates androgen synthesis in thecal cells, whereas FSH promotes the conversion of thecal-derived androgens into estrogens by aromatase in granulosa cells (Fortune & Eppig 1979, White & Ojeda 1981, O'Shaughnessy *et al.* 1997). In immature females, FSH would stimulate follicular growth from the primary stage onward, as suggested by rodent models (Halpin *et al.* 1986, Balla *et al.* 2003). We previously showed in the female mouse that circulating FSH levels are dramatically high during the infantile period at ~12–14 days postnatal (dpn), reaching about five-fold those found in adult cycling females (François *et al.* 2017). We demonstrated that such FSH concentrations are necessary to induce *Cyp19a1* aromatase expression in immature follicles at the preantral/early antral stage, thereby permitting estradiol (E2) production. These follicles belong to the first follicular waves, which started growing immediately after their formation at birth (Hirshfield & DeSanti 1995). Notably, we showed that FSH does not support their growth due, at least in part, to its incapacity at high concentrations to induce the expression of the cell cycle regulator cyclin D2 (*Ccnd2*) and the proliferation of granulosa cells (François *et al.* 2017). Hence, the infantile elevation in FSH would contribute to E2 production from the first follicular waves without boosting their growth. However, the mechanisms underlying the preferential action of FSH on E2 biosynthesis over follicular growth during this period is unknown.

In the ovary, FSH regulates the expression of the anti-Müllerian hormone (AMH, also called Müllerian-inhibiting substance or MIS) either positively (Taieb *et al.* 2011) or negatively (Baarends *et al.* 1995, Hardy *et al.* 2016, Roy *et al.* 2018), depending on the experimental model. AMH is a member of the transforming growth factor β family, which in the ovary is specifically expressed in granulosa cells of growing follicles from the primary to the antral stage. The physiological actions of AMH in the ovary has been first demonstrated by analyses on *Amh*-knockout mice, which display premature ovarian failure owing to acceleration of follicular growth and primordial follicle recruitment into the growing follicle pool (Durlinger *et al.* 2001).

These data have led to the idea that AMH inhibits the recruitment of primordial follicles. Additional *in vivo* and *in vitro* studies have indicated that AMH may keep follicles insensitive to FSH-dependent growth (Durlinger *et al.* 2001) and to FSH-mediated steroidogenesis by inhibiting the expression and activity of aromatase (Grossman *et al.* 2008, Chang *et al.* 2013, Prapa *et al.* 2015, Sacchi *et al.* 2016). Hence, in growing follicles from adult cyclic females, the loss of AMH expression would allow FSH-dependent growth and subsequent maturation associated with E2 synthesis (Visser & Themmen 2014).

AMH is already expressed in infantile ovaries in mammals (Baarends *et al.* 1995, Mazaud *et al.* 2002, Hagen *et al.* 2010, Kuiri-Hänninen *et al.* 2011, Torres-Rovira *et al.* 2016, François *et al.* 2017). Given the known role of AMH and its regulation by FSH in mature ovaries, we made the hypothesis that there is already an interplay between FSH and AMH to regulate ovarian activity in immature females. Therefore, the present study aimed at addressing two issues: (1) could FSH already regulate AMH expression in growing follicles during the infantile period when gonadotropin levels and ovarian activity are both dramatically elevated; and (2) could AMH influence FSH action on the growth and steroidogenesis of prepubertal growing follicles? For that purpose, we analyzed the ontogenesis of AMH expression in prepubertal mice, and we investigated the possible regulatory action of FSH on the expression of AMH in infantile ovaries, by performing *in vivo* studies and organotypic cultures. We also determined whether AMH could modulate the action of FSH in the infantile ovary.

Materials and methods

Animals and treatments

Studies were conducted on C57BL/6JrJ mice aged 7 to 27 dpn and in adult cycling females that were born at the animal facility from genitors purchased at Janvier Labs (Le Genest St Isle, France). Mouse care and handling was performed as previously described (François *et al.* 2017). Ten micrograms of GnRH antagonist (Ganirelix, Orgalutran, N.V. Organon, Puteaux, France) or saline was subcutaneously injected twice on prepubertal female mice at 12 and 13 dpn, as previously described (François *et al.* 2017). In addition to Ganirelix, 13 dpn mice received an intraperitoneal injection of 5 IU of equine chorionic gonadotropin (eCG) (N.V. Organon), estradiol (8 pg/female, Sigma) or vehicle. Mice were then dissected at 14 dpn to collect ovaries. Experiments were performed

in accordance with standard ethics guidelines and were approved by Institutional Animal care and Use committee of the University Paris Diderot and by the French Ministry of Agriculture (agreement #04015.01).

***In situ* studies of *Amh* expression and cell death by TUNEL assay**

At least four prepubertal ovaries per age or treatment group were fixed for 1–2 h in 4% paraformaldehyde (PFA) depending on ovary size, rinsed in PBS and placed in 18% sucrose before being embedded in Optimal Cutting Temperature (Cellpath, Newtown, UK). *In situ* hybridization (ISH) was performed as previously described (Guigon *et al.* 2003) with digoxigenin-11-UTP labeled *Amh* RNA probes (accession number S98336, size: 653 bp) synthesized after cDNA amplification with *Amh* primers, forward 5'-ACCCCTTCCTAGAGACCCTCA and reverse 5'-GGTACGGAACACGTGGTG. Double staining with fibronectin was performed to visualize ovarian morphology and follicles. TUNEL assay was performed after ISH by incubating slides with a mix containing fluorescein-deoxy-UTP (TUNEL label, Roche) and terminal deoxynucleotidyltransferase (TUNEL enzyme, Roche) for 1 h at 37°C. Cells exhibiting nuclear staining were considered as apoptotic. Negative controls lacking the labeling enzyme yielded no reaction product.

Determination of serum AMH levels

Quantification of AMH in the serum was performed using the commercial rat and mouse AMH ELISA kit (Ansh Labs, AL-113) following manufacturer's protocol. Briefly, diluted serum (1/10 for adults and 1/20 for prepubertal females) and standards were added to AMH antibody-coated wells and incubated for 2 h at room temperature. After washing, wells were incubated with biotinylated AMH antibody for 1 h, followed by incubation with streptavidin horseradish peroxidase conjugate for 30 min. Then, the plate was developed using 3, 3', 5, 5' tetramethylbenzidine (TMB) and the light absorbance was read at 450 nm using a microplate reader (FlexStation, Molecular device). Limit of ELISA sensitivity was 0.34 ng/mL. The intra- and inter-assay variability was $1.6 \pm 0.53\%$ and $5.7 \pm 1.6\%$, respectively.

Cultures of postnatal ovaries

Ovaries from 12 to 13 dpn female mice were placed on cell culture inserts (Millicell #PICM01250, Millipore,

Guyancourt, France) on the top of 400 μ L of RPMI culture medium without red phenol containing fetuin (Sigma, #F2379), insulin (Sigma, #I0516), transferrin (Sigma, #T8158), sodium selenite (Sigma, #S5261) and BSA (Euromedex, Souffelweyersheim, France) in 24-well plates for 24 h. Then, the culture medium was replaced with 400 μ L of fresh medium containing purified ovine pituitary FSH (50, 200 or 500 ng/mL) with or without the 25 kDa C-terminal form of AMH (400 ng/mL or 16 nM; R&D, #1737-MS-010), which can bind AMHRII and is a bio-active form (di Clemente *et al.* 2010). After 8 h of treatment, ovarian explants were snap-frozen in liquid nitrogen and stored at -80°C for RNA extraction or fixed in 4% PFA for histology, as described before (François *et al.* 2017).

RNA extraction, reverse transcription and quantitative real-time PCR

Single frozen ovaries were lysed with TissueLyser II (Qiagen) and RNA was obtained using RNeasy mini kit (#74106, Qiagen), following manufacturer's protocol, as previously described (François *et al.* 2017). Total RNA (100 to 200 ng) was reverse transcribed using random primers and Superscript II (Invitrogen) following manufacturer's instructions. Primers used for quantitative real-time PCR are listed in Table 1. Real-time PCR was performed with Lightcycler 480 SYBR Green I Master and Lightcycler 480 instrument (Roche Molecular Biochemicals, La Rochelle, France), as previously described (François *et al.* 2017). Experiments were performed with at least four different ovarian samples/age or treatment group, with each sample run in triplicates.

Western blot

Pooled ($n=2$ ovaries at 7 dpn) or single frozen ovaries were lysed with TissueLyser II (Qiagen) as described (François *et al.* 2017). Thirty micrograms of lysates were denatured under reducing conditions and used for the determination of AMH (antibody from R&D Systems, #AF1446), aromatase (Sigma, #A7981), cyclin D2 (Santa-Cruz, #sc-181) and GAPDH (Santa-Cruz, #sc-25778). Western blot were performed as described (François *et al.* 2017).

Determination of intra-ovarian estradiol content

The mass spectrometry coupled with gas chromatography (GC-MS) procedure was used to determine E2 content in the infantile ovary. A single frozen ovary was

Table 1 List of primers used for real-time PCR.

Gene	Forward	Reverse	Amplicon size (bp)
<i>Cyp19a1</i>	5'-TACTTCATGTTACTTCTCGTCGC-3'	5'-TATCCTCGATCTTTATGTCTCTGTCAC-3'	103
<i>Ccnd2</i>	5'-CAGAAGGACATCCAGCCGTAC-3'	5'-TCGGGACTCCAGCCAAGAA-3'	136
<i>Fshr</i>	5'-CTGGCATTCTTGGGCTCG-3'	5'-GGGCGGAATCTCGGTCA-3'	101
<i>Amh</i>	5'-TGCTAGTCCTACATCTGGCTGA-3'	5'-GTCCAGGGTATAGCTAACAGG-3'	120
<i>Foxo1</i>	5'-CTTCAAGGATAAGGGCGACA-3'	5'-GACAGATTGTGGCGAATTGA-3'	66
<i>Glut1</i>	5'-CATCCTTATTGCCAGGTGTTT-3'	5'-GAAGACGACACTGAGCAGCAGA-3'	82
<i>Hprt</i>	5'-AGGACCTCTCGAAGTGT-3'	5'-ATTCAAATCCCTGAAGTATCAT-3'	111

introduced into a Lysing Matrix D tube (MP Biomedicals, Illkirch-Graffenstaden, France) with cold saline water and deuterated methanol internal standard working solution. The sample was homogenized with Ribolyser (Hybaid, Teddington, Middlesex, UK). After centrifugation, the liquid phase with ceramic spheres was collected. The tissue residuals in Lysing Matrix tube were recovered in cold saline water and methanol (HPLC grade). After centrifugation, the organo-aqueous supernatant containing E2 was collected and evaporated. It was then purified in C18 Hypersep mini-columns (Thermo Fisher) and eluted with ethyl acetate (HPLC grade). The organic phase was evaporated to dryness, then reconstituted in saline water and extracted with 1-chlorobutane. After centrifugation, the organic phase was purified on conditioned silica mini-columns (LC-Si SPE columns, Varian, Les Ulis, France). E2 was derivatized with pentafluorobenzoyl chloride (103772-1G, Aldrich, Steinheim, Germany). The final extracts were reconstituted in isooctane and transferred into conical vial for injection into the GC system (6890N, Agilent Technologies). A quadrupole mass spectrometer equipped with a chemical ionization source and operating in single ion monitoring mode was used for detection (HP5973, Agilent Technologies). The linearity of steroid measurement was confirmed by plotting the ratio of the E2 peak response/internal standard peak response to the concentration of E2 for calibration standard. Lower limit of quantification was 1.15 pg. The intra- and inter-assay variability of low limit of quantification CVs was 13.3–20%.

Statistical analyses

Experiments were performed from at least four different samples per age/treatment group and data were analyzed by Prism 6 (version 6.0, GraphPad Software). Statistical analyses used Student *t*-test (comparison of two groups) or one-way ANOVA (>two groups) when data showed normal distribution as assessed by the Shapiro–Wilk test,

and if they did not pass this test, with a Kruskal–Wallis non-parametric test. Data are shown as means $\pm$ S.E.M. A *P* value <0.05 was considered as significant.

Results

High *Amh* expression in the prepubertal ovary, with a transient downregulation in preantral/early antral follicles of the first waves

Ontogenesis analysis of the relative intra-ovarian abundance of *Amh* mRNA was monitored at different ages in infantile (7–21 dpn), juvenile (27 dpn) and in adult cycling females (Fig. 1A). *Amh* mRNA levels progressively increased during the infantile period to become maximal between 14 and 21 dpn. It was then ~2–3-fold higher than that in adult females during reproductive life (Fig. 1A). We also studied AMH expression by Western blot (Fig. 1B). AMH is synthesized as a homodimeric glycoprotein (AMH precursor, 140 kDa) cleaved into a 25-kDa C-terminal dimer and a 110-kDa N-terminal dimer, which remain associated as a non-covalent complex (cleaved AMH) (di Clemente *et al.* 2010). Using an antibody recognizing AMH precursor form, we observed an expected band at ~70 kDa under reducing conditions (Fig. 1B). Although these data are qualitative, a similar trend as that for mRNA was observed for the protein. To determine whether prepubertal *Amh* synthesis resulted in detectable circulating AMH levels, we monitored serum AMH levels in females during the infantile and juvenile periods, as well as in adult cycling females at different stages of the estrus cycle (P, proestrus, E, estrus; D, diestrus) (Fig. 1C). There was no correlation between intra-ovarian *Amh* transcript and protein abundance and AMH serum levels. Indeed, circulating AMH levels were very low at 7 dpn, and they progressively increased during the infantile and juvenile periods. Notably, circulating AMH levels were ~2–3 fold more elevated in late infantile (17, 21 dpn) and juvenile females (27 dpn) than in adult cycling females (Fig. 1C).

Figure 1

Ontogenesis of AMH expression in prepubertal female mice. (A) The relative intra-ovarian abundance of *Amh* transcripts in infantile (7–21 dpm), juvenile (27 dpm) and adult cycling females was determined by quantitative real-time RT-PCR and normalized to the mRNA levels of *Hprt*. The number of samples is indicated under parentheses. (B) AMH protein expression was analyzed in immature (7–27 dpm) and adult (A) ovaries by Western blot, with GAPDH used as a loading control. Representative immunoblots are shown. The star shows a non-specific upper band. (C) Serum AMH levels were measured by ELISA assay during the infantile (7–21 dpm) and the juvenile periods (27 dpm), and in adult cycling females (P, proestrus; E, estrus; D, diestrus) with the number of serum samples for each group shown under parentheses. (D) *In situ* hybridization with *Amh* probes (purple color digitally converted into green) and detection of fibronectin by immunofluorescence (red color) showing the distribution of *Amh* transcripts in the ovary during the infantile (8, 14 and 21 dpm) and the juvenile periods (27 dpm). Pictures in a', b', c' and d' are enlargement of the rectangles located on pictures a, b, c and d, respectively. Arrows show *Amh*-positive preantral/early antral follicles, whereas stars show *Amh*-negative preantral/antral or medium/large antral follicles. Triangles show large antral follicles expressing *Amh* in cumulus cells. Due to the large size of the ovaries at 21 and 27 dpm, the pictures in c and d are reconstitution obtained from microphotographs taken in different parts of the same ovarian section. (E) Co-detection of apoptotic cells by TUNEL assay (green color digitally converted into blue), *Amh* transcripts by *in situ* hybridization (purple color digitally converted into green) and fibronectin by immunofluorescence (red color) at 14 (a), 21 (b) and 27 (c and d) dpm. Arrows show apoptotic cells located either outside (a, b) or inside (c) follicles. In graphs, bars are the means $\pm$ S.E.M. Data were analyzed by one-way ANOVA (A) or Kruskal–Wallis (C) tests. Distinct letters indicate significant differences between ages. In (D) and (E), scale bars: 100 μ m.

During the prepubertal period, the ovary is endowed by primordial follicles and by growing follicles at different stages of development. There is a clear regionalization of these different categories of follicles during the neonatal and infantile periods: the most mature follicles, i.e., the first follicular waves, are present in the center, whereas subsequent waves and primordial follicles are located in the periphery (Guigon *et al.* 2003, Mork *et al.* 2012, Zheng *et al.* 2014). These two populations of growing follicles exhibit distinct functional characteristics (Guigon *et al.* 2003, François *et al.* 2017) and emerge from different granulosa cell lineage (Mork *et al.* 2012, Zheng *et al.* 2014). To closely monitor *Amh* ontogenesis in these two populations, we performed *in situ* hybridization (ISH) studies in infantile (8–21 dpn) and juvenile (27 dpn) females. At 8 dpn, *Amh* was expressed in granulosa cells of all growing follicles present, from the primary to the preantral stage (Fig. 1Da and inset in a'). Notably, at 14 dpn, *Amh* expression became low in the most mature follicles of the first waves, then reaching the preantral/early antral stage (Fig. 1Db and inset in b'). Its expression was restricted to growing follicles present in the ovarian periphery (Fig. 1Db and inset in b'). In contrast, in older infantile (21 dpn) and juvenile females (27 dpn), *Amh* was abundantly expressed in preantral/early antral follicles, including in those located in the center (Fig. 1Dc, d, and insets in c' and d', respectively). It was absent in mural granulosa cells from follicles at the medium and large antral stages, but still expressed in cumulus cells, as reported (Fig. 1Dc, d) (Baarends *et al.* 1995). To determine whether the observed transient *Amh* downregulation in preantral/early antral follicles at 14 dpn could result from follicular atresia, we monitored granulosa cell apoptosis by TUNEL assay on the same tissue sections as those used for ISH. We could establish that at 14 dpn, preantral/early antral follicles with low *Amh* expression had no apoptotic granulosa cells (Fig. 1Ea). In contrast, a subset of medium to large antral follicles in late infantile and juvenile females with low *Amh* expression exhibited apoptotic granulosa cells, while others did not, as expected (Baarends *et al.* 1995) (Fig. 1Eb–d).

FSH-induced downregulation of *Amh* expression in preantral/early antral follicles of the first follicular waves

FSH can regulate AMH expression either positively or negatively in granulosa cells, depending on follicular stage, species and experimental approaches (Baarends *et al.* 1995, Taieb *et al.* 2011, Hardy *et al.* 2016, Roy *et al.* 2018).

From our ontogenesis studies, no clear relationship could be observed during the prepubertal period between circulating FSH levels becoming maximal at 12–14 dpn (François *et al.* 2017) and either serum AMH levels or intra-ovarian *Amh* mRNA and protein abundance (Fig. 1A, B and C). However, the observation that the transient loss in *Amh* expression in preantral/early antral follicles occurs at the time when serum FSH levels become markedly elevated led us to determine whether FSH could be involved in the regulation of *Amh* expression in these follicles. For that purpose, we treated female mice with the GnRH receptor antagonist Ganirelix to decrease gonadotropin levels between 12 and 14 dpn. With this protocol, there is a reduction by ~80% for FSH and by ~95% of LH levels (François *et al.* 2017). Some of these mice were supplemented with the exogenous gonadotropin eCG that exhibits pronounced FSH activity in rodents (Combarnous *et al.* 1984) (Fig. 2A). We recently demonstrated that these treatments do not alter the number and the size of growing follicles (François *et al.* 2017), and hence, they probably do not affect ovarian cellular composition. Ganirelix treatment induced a ~2-fold decrease in relative mRNA abundance of *Fshr*, as previously observed for two other FSH-regulated genes, i.e., *Cyp19a1* and *Ccnd2* mRNA. This effect was partially reverted by eCG co-administration (Fig. 2B). Notably, analyses of *Amh* mRNA by real-time RT-PCR revealed that Ganirelix induced a significant increase by ~30% in their relative intra-ovarian levels; this increase was prevented by the co-administration of eCG (Fig. 2C). *In situ* examination of *Amh* expression pattern in the ovary showed that Ganirelix treatment clearly restored *Amh* expression in preantral/small antral follicles located in the core of the ovary, whereas co-treatment with eCG prevented it (Fig. 2D, areas outlined by dotted lines).

To further specify the effect of FSH on the expression of *Amh*, we used an *ex vivo* culture system (Fig. 2E). Ovaries from 12-dpn mice were cultured in serum-free medium, and then treated for 8 h with different concentrations of purified FSH (50, 200 and 500 ng/mL) (Fig. 2E). In control conditions (no FSH), *Fshr* transcripts were detected in the ovary; their relative abundance increased by ~2-fold following treatment with the highest used FSH concentration mimicking the elevated infantile levels (Fig. 2F). Importantly, FSH induced a dose-dependent decrease in *Amh* mRNA levels that became substantial at the highest used concentration (Fig. 2G). No apparent change in preantral/early antral follicle size was observed between control and FSH-treated group, as expected for a short-term treatment (Fig. 2H). It is therefore unlikely that the observed alterations in gene expression result from a change in ovarian cellular composition.

Figure 2

High FSH concentrations repress *Amh* expression in the first follicular waves. (A) Schematic representation of the procedure used to experimentally manipulate FSH levels between 12 and 14 dpn in mice. (B) Relative intra-ovarian contents of *Fshr* mRNA in the ovaries of infantile females treated with saline (controls), Ganirelix (+) or Ganirelix + eCG (+eCG) determined by quantitative real-time RT-PCR after normalization by the levels of *Hprt* mRNAs. The number of used samples is indicated under parentheses. (C) Relative intra-ovarian contents of *Amh* mRNA in the ovaries of infantile females treated with saline (controls), Ganirelix (+) or Ganirelix + eCG (+eCG) determined by quantitative real-time RT-PCR after normalization by the levels of *Hprt* mRNAs. The number of used samples is indicated under parentheses. (D) Distribution pattern of *Amh* mRNA by *in situ* hybridization with specific probes (purple color digitally converted into green) and fibronectin immunofluorescence (red color) in the ovaries of control or Ganirelix-treated mice supplemented or not with eCG *in vivo*. The area containing follicles of the first follicular waves is delimited by dotted lines. (E) Schematic representation of the procedure used for ovarian culture. (F) Effect of increasing FSH concentrations (50–500 ng/mL) on the relative intra-ovarian levels of *Fshr* mRNA in organotypic culture. Data were obtained by quantitative real-time RT-PCR and normalized by the levels of *Hprt* mRNAs. The number of used samples is indicated under parentheses. (G) Effect of increasing FSH concentrations (50–500 ng/mL) on the relative intra-ovarian levels of *Amh* mRNA in organotypic culture. Data were obtained by quantitative real-time RT-PCR and normalized by the levels of *Hprt* mRNAs. The number of used samples is indicated under parentheses. (H) Representative histological sections of ovaries cultured in control (CTR) or FSH (500 ng/mL)-supplemented medium. Right panels show enlargement of the largest follicle seen on the left panels. In graphs, bars are the means $\pm$ s.e.m. Data were analyzed by one-way ANOVA (B and G) or Kruskal–Wallis (C and F) tests. Distinct letters indicate significant differences between ages. In (D) and (H), scale bars: 100 μ m.

FSH repressive action on *Amh* expression in preantral/early antral follicles is not mediated by estradiol

FSH stimulates the production of E2, which in turn would suppress *Amh* expression through estrogen receptor β (ER β)

in vitro, as shown in luteinized or tumoral granulosa cells (Grynbeg *et al.* 2012). In infantile mice, we previously showed that the increased levels of FSH induced a significant elevation in serum E2 levels (François *et al.* 2017). Thus, we next sought to investigate whether FSH-induced repression of *Amh* expression observed in

infantile ovaries could, in fact, rely on E2 action through ER β , since this receptor is already expressed in growing follicles of the immature mouse ovary (Balla *et al.* 2003).

To analyze the possible involvement of E2, we took advantage of the infantile Ganirelix-treated mouse model, which displays a ~2-fold decrease in E2 serum levels (François *et al.* 2017). We first measured the intra-ovarian E2 contents in both groups to determine the appropriate amount of E2 to administer to Ganirelix-treated females to restore E2 levels close to those of control females. The observed quantity (controls: 5.54 ± 0.94 pg/ovary vs Ganirelix: 1.36 ± 0.39 pg/ovary, $n=6-7$ ovaries/group, $P<0.01$) led us to administer 8 pg/female, following the schedule shown in Fig. 3A. This amount of E2 efficiently increased uterus weights in Ganirelix-treated mice as compared with the two other groups (Fig. 3B). We then analyzed the relative intra-ovarian *Amh* expression by both quantitative and *in situ* approaches. Notably, *Amh* transcript levels in Ganirelix-treated females were not altered by E2, and they remained higher than in control females (Fig. 3C). In line with these findings, preantral/early antral follicles in the center of the ovary maintained *Amh* expression in Ganirelix-treated females despite E2 treatment (Fig. 3D, virtual area outlined by dotted lines). Thus, FSH may directly regulate *Amh* expression, without implying estrogen signaling at this stage of development.

To determine whether the lack of E2 action on *Amh* expression results from the relative insensitivity of those follicles to E2, we performed a small-scale analyses of documented E2 target genes in granulosa cells of growing follicles, i.e., *Cyp19a1*, *Foxo1* (Forkhead box protein 1) and *Glut-1* (Glucose transporter 1) (Richards *et al.* 2002). Surprisingly, none of the studied genes exhibited alterations in their expression levels following E2 treatment in Ganirelix-treated females as compared with those administered with Ganirelix alone (Fig. 3E). These findings suggest that the ovary would not be sensitive to E2 during the infantile period.

AMH prevents FSH-mediated induction of *Cyp19a1* aromatase without altering *Fshr* and *Ccnd2* expression in the infantile ovary

The present study reveals that FSH represses *Amh* expression specifically in the first follicular waves, wherein we previously showed that FSH induces a consistent *Cyp19a1* aromatase expression without altering that of *Ccnd2* (François *et al.* 2017). Since other studies reported that AMH prevents FSH-induced E2 synthesis (Grossman *et al.* 2008, Chang *et al.* 2013, Prapa *et al.* 2015,

Sacchi *et al.* 2016), possibly by down-regulating FSHR expression (Pellatt *et al.* 2011), and that it regulates FSH-mediated follicular growth (McGee *et al.* 1997, Durlinger *et al.* 2001), we next sought to investigate whether the observed decrease in *Amh* expression could facilitate FSH-mediated *Cyp19a1* expression and explain the loss of FSH-mediated *Ccnd2* expression in preantral/early antral follicles. We therefore tested the effect of a treatment by AMH on the expression of *Cyp19a1*, *Fshr* and *Ccnd2* in the infantile ovary. We used an *ex vivo* culture system instead of administering AMH *in vivo*, since we and others have reported the occurrence of extra-gonadal actions of AMH that could increase both LH and FSH levels, thereby indirectly altering ovarian function (Cimino *et al.* 2016, Garrel *et al.* 2016). We cultured infantile ovaries from mice aged 12 dpn with either FSH (500 ng/mL) or AMH alone or combined, for 8 h (Fig. 4A). Histological analyses of ovarian explants showed no visible alteration in the size of preantral/early antral follicles (shown for FSH and FSH+AMH treatment in Fig. 4B). We then determined the relative abundance of *Cyp19a1*, *Fshr* and *Ccnd2* mRNA. Under these experimental conditions, treatment with AMH alone had no significant effect on either basal *Cyp19a1*, *Fshr* or *Ccnd2* expression levels (Fig. 4Ca–c). FSH significantly upregulated *Cyp19a1* and *Fshr* mRNA levels, while it had no effect on those of *Ccnd2*, as reported before (François *et al.* 2017) (Fig. 4Ca–c). Notably, in the presence of AMH, FSH did not induce *Cyp19a1* expression, which remained at basal levels (Fig. 4Ca). This inhibitory effect of AMH on FSH action occurred in the absence of alterations in *Fshr* mRNA levels (Fig. 4Cb). Similarly, AMH did not alter the relative abundance of *Ccnd2* mRNAs in the presence of FSH (Fig. 4Cc). Western blot analyses of Cyclin D2 and aromatase proteins recapitulated the findings obtained on their mRNA levels (Fig. 4D).

Discussion

AMH plays a critical role in regulating ovarian function during reproductive life, in particular by limiting FSH actions in small growing follicles to prevent their premature maturation (reviewed in (Visser & Themmen 2014)). This study is, to our knowledge, the first to investigate the interplay between AMH and FSH on the regulation of the infantile ovarian function. Because the infantile ovary is relatively easy to maintain in *ex vivo* culture, this experimental model provided us with the opportunity to decipher in a better physiological context than in luteinized or in tumoral granulosa cell cultures,

Figure 3

Estradiol does not repress *Amh* expression in infantile ovaries. (A) Schematic representation of the procedure used to experimentally alter E2 levels between 12 and 14 dpn in mice. (B) Uterus weights in controls, Ganirelix (+) and Ganirelix + E2-treated (+/E2) female mice. The number of samples for each group is shown under parentheses. (C) Relative intra-ovarian abundance of *Amh* transcripts in controls, Ganirelix (+) and Ganirelix + E2-treated (+/E2) female mice determined by quantitative real-time RT-PCR after normalization by the levels of *Hprt* mRNAs. The number of samples for each group is shown under parentheses. (D) Distribution pattern of *Amh* mRNA by *in situ* hybridization with specific probes (purple color digitally converted into green) and fibronectin immunofluorescence (red color) in the ovaries of control or Ganirelix-treated mice supplemented or not with estradiol (E2) *in vivo*. The area containing follicles of the first follicular waves is delimited by dotted lines. (E) Relative intra-ovarian abundance of E2-target genes, i.e., *Cyp19a1*, *Foxo1* and *Glut1* transcripts, in controls, Ganirelix (+) and Ganirelix + E2-treated (+/E2) female mice, determined by quantitative real-time RT-PCR after normalization by the levels of *Hprt* mRNAs. The number of samples for each group is shown under parentheses. In graphs, bars are the means $\pm$ S.E.M. Data in (B), (C), and (E) were analyzed by Kruskal–Wallis non-parametric test, with distinct letters indicating significant differences between groups ($P < 0.05$). In (D), scale bars: 100 μ m.

the effect of FSH on AMH expression on the one hand, and the action of AMH on FSH-induced regulation on the other hand. We used a complementary approach *in vivo* in the mouse in which gonadotropin levels can be easily manipulated during the infantile period (François *et al.* 2017). Remarkably, our data demonstrate the existence

of a repressive action of FSH on *Amh* expression in preantral/early antral follicles of the first follicular waves. As the main suppliers of E2 during the infantile period, these follicles may play a critical role in the programming of adult reproductive function. Our data support the idea that this action of FSH on *Amh* expression is necessary to

Figure 4

AMH represses the relative abundance of *Cyp19a1* mRNA but not that of either *Fshr* or *Ccnd2*. (A) Schematic representation of the procedure used for ovarian culture. (B) Representative histological sections of ovaries cultured in FSH (500 ng/mL) or FSH+AMH-supplemented medium. The right panels show enlarged magnification of the largest follicles seen on the left panels. Scale bars: 100 μ m. (C) Effect of FSH (500 ng/mL) and AMH (400 ng/mL) alone or combined, on *Cyp19a1* (a), *Ccnd2* (b), and *Fshr* (c) mRNA levels in cultured ovaries. Transcript levels were analyzed by quantitative real-time RT-PCR, and normalized by those of *Hprt* mRNAs. The number of samples for each group is shown under parentheses. (D) Aromatase and Cyclin D2 protein expression was analyzed in ovarian explants by Western blot, with GAPDH used as a loading control. Representative immunoblots are shown. The star shows an upper non-specific band. In graphs, bars are the means $\pm$ s.e.m. Data were analyzed by Kruskal–Wallis non-parametric test with *, $P < 0.05$; **, $P < 0.01$. NS, not significant.

alleviate the inhibitory action of AMH on FSH-dependent E2 biosynthesis in these growing follicles (Fig. 5).

Our *in situ* studies highlight the fact that *Amh* displays a marked alteration in its expression pattern during the prepubertal period. Indeed, initially expressed in all growing follicles at the very beginning of the infantile period, *Amh* was subsequently downregulated in preantral/early antral follicles of the first waves at ~ 14 dpn. This decrease did not result from follicular atresia, since granulosa cell apoptosis was still marginal at this age. In older prepubertal females, *Amh* expression was again observed in preantral/early antral follicles, and it was downregulated in (healthy or atretic) medium to large antral follicles, as described by others (Baarends *et al.* 1995). The observation that *Amh* downregulation in preantral/early antral follicles of the first waves occurred concomitantly to the dramatic rise in FSH levels in infantile females prompted us to test the hypothesis that FSH represses *Amh* expression in these follicles despite their relative immaturity. In our studies, *in vivo* manipulation of gonadotropin levels in infantile

mice strongly suggests that it would be the case. Further supporting this hypothesis, our *ex vivo* studies indicated that FSH dose-dependently repressed *Amh* expression in the infantile ovary. In line with our findings, a previous report suggested that administration of FSH to juvenile rats decreases *Amh* expression in preantral/early antral follicles, albeit this effect has not been quantified (Baarends *et al.* 1995). In addition, *in vitro* studies on cultured preantral follicles collected from infantile mice reported that FSH induces *Amh* downregulation (Hardy *et al.* 2016). In adult and juvenile ovaries, the expression of AMH disappears from mural granulosa cells at the antral stage, when the follicle supposedly requires FSH for its subsequent growth and maturation. The role played by the oocyte-derived factors GDF9 and BMP15 on FSH-mediated repression of *Amh* has been recently highlighted in mouse primary cultures of granulosa cells and in the tumor-derived granulosa KGN cell line (Roy *et al.* 2018). This study indicates that *Amh* expression implies a tight dialog between granulosa cells and the oocyte. It could also

Figure 5

Proposed model for the regulatory loop between FSH and AMH during the infantile period in the mouse. The high FSH levels would enhance FSH receptivity in preantral/small antral follicles of the first waves located in the center of the ovary, and decrease their expression of *Amh*. The loss of *Amh* expression in these follicles may facilitate FSH-induction of *Cyp19a1* aromatase and thus the synthesis of E2 that is required for programming adult reproductive function. The production of AMH by subsequent follicular waves located in the ovarian periphery may target the hypothalamus and the pituitary (Cimino *et al.* 2016, Garrel *et al.* 2016) to increase LH and FSH levels, thereby leading to increased ovarian activity (see text for more explanations). A full-colour version of this figure is available at <https://doi.org/10.1530/JOE-18-0313>.

explain the opposite findings about FSH regulation of *Amh* expression, and warn about culture conditions and experimental models.

The occurrence of FSH-induced *Amh* downregulation in follicles at an earlier developmental stage in the infantile ovary than in older females suggests a precocious acquisition of FSH receptivity in growing follicles of immature females. FSHR starts to be expressed in the mouse ovary as soon as the neonatal period (O'Shaughnessy *et al.* 1997). Cultured infantile preantral/early antral follicles display *Fshr* expression and are responsive to FSH in terms of steroidogenesis-related gene induction (Hardy *et al.* 2016). The apparent lack of FSH-mediated repression of *Amh* expression in growing follicles from the ovarian periphery may reflect their low receptivity to FSH, as suggested by others (Hardy *et al.* 2016). We report here that FSH stimulates the expression of its own receptor in the infantile ovary, as previously suggested (O'Shaughnessy *et al.* 1997). Thus, the high FSH levels of the infantile period may well boost FSH receptivity specifically in the first follicular waves, thereby leading to the premature extinction of *Amh* expression in growing follicles despite their early stage of development. Whether high levels of FSH would have the same effect in the adult ovary remains

to be investigated. In favor of this hypothesis, however, the expression of *Amh* is downregulated in preantral follicles specifically at the estrus stage (Baarends *et al.* 1995), i.e., right after the cyclic elevations in FSH levels.

We presently do not know the molecular mechanisms whereby FSH downregulates *Amh* expression in the infantile ovary. Since FSH stimulates E2 production, a few studies have proposed that this effect of FSH could be mediated by E2 (Baarends *et al.* 1995, Grynberg *et al.* 2012). Furthermore, *in vitro* studies have shown that it would imply ER β binding to an estrogen response element in the *AMH* promoter (Grynberg *et al.* 2012). Despite ER β expression and significant E2 production in the infantile ovary (Balla *et al.* 2003, François *et al.* 2017), our experiments provide some evidence that E2 has, by itself, no effect on *Amh* expression in preantral/early antral follicles during this period. This contrasts with results obtained in juvenile rats, wherein exogenous E2 treatment could downregulate *Amh* expression in preantral/early antral follicles (Baarends *et al.* 1995). Together with our observations that E2 has no effect on a subset of known E2 target genes in the infantile ovary, our study raises the intriguing possibility that E2 signaling may be inactive in preantral/early antral follicles during this period. Whether the peculiar endocrine environment of the infantile period underlie this feature should be investigated in the future. FSH may, thus, be a direct regulator of *Amh* expression in these follicles. The possibility that it involves FSH-mediated activation of the canonical cAMP/ protein kinase A (PKA) pathway to regulate *Amh* promoter activity is supported by a recent study in primary cultures of mouse granulosa cells and in KGN cells (Roy *et al.* 2018).

In the infantile ovary, our findings showed that AMH treatment significantly repressed FSH-mediated induction of *Cyp19a1* aromatase, as previously described for human luteinized granulosa cells *in vitro* (Grossman *et al.* 2008, Chang *et al.* 2013, Prapa *et al.* 2015, Sacchi *et al.* 2016). This effect unlikely resulted from decreased FSHR expression as described in human tumoral granulosa cells (Pellatt *et al.* 2011), since AMH did not alter *Fshr* transcript abundance, albeit we cannot exclude that it could have affected FSHR protein levels. However, one can hypothesize that AMH could inhibit FSH-stimulated adenylyl cyclase activity, as described in human granulosa cells *in vitro* (Chang *et al.* 2013). *Amh* abundance was low in preantral/early antral follicles of the first waves, which express *Cyp19a1* aromatase (François *et al.* 2017). In contrast, *Amh* expression was high in growing follicles of the subsequent waves, wherein there is little *Cyp19a1*

expression (François *et al.* 2017). Hence, we hypothesize that FSH-induced repression of *Amh* in preantral/early antral follicles of the first waves contributes to FSH induction of E2 production in these follicles. In contrast, although AMH regulates the growth of follicles *in vivo* and *in vitro* (McGee *et al.* 1997, Durlinger *et al.* 2001), we did not observe any effect of AMH on *Ccnd2* expression in infantile ovaries, in the presence or not of FSH. We previously reported that at high levels, FSH could not induce granulosa cell expression of *Ccnd2* and granulosa cell proliferation (François *et al.* 2017). These findings suggest that the lack of FSH action on the growth of the first follicular waves during the infantile period does not rely on *Amh* loss in these follicles. Future studies are warranted to understand how high FSH levels lead to the loss of FSH-mediated induction of *Ccnd2* induction and granulosa cell proliferation.

The consistent elevation in the relative levels of intra-ovarian *Amh* transcript and protein abundance during the infantile period correlates with the previously reported large number of growing follicles as compared with the adult (Pedersen 1969). This profile in intra-ovarian AMH abundance contrasted with that of circulating AMH, which showed a progressive increase during the infantile period to become dramatically elevated in juvenile females. While the reason behind this apparent discrepancy between intra-ovarian and circulating AMH levels is not clear at present, it may simply result from changes in ovarian cellular composition, with juvenile ovaries producing more AMH because of the overall increase in the number of granulosa cells in large antral follicles (Jeppesen *et al.* 2013).

The significant production of AMH by the prepubertal ovary and its detection in the serum raises the point as to whether ovarian AMH could target newly identified extra-gonadal sites, i.e., the hypothalamus and the pituitary (Cimino *et al.* 2016, Garrel *et al.* 2016). In the hypothalamus, AMH binds to its receptor, AMHRII, to activate GnRH neuron activity, and thereby it stimulates LH secretion *in vivo* in the mouse (Cimino *et al.* 2016). In the pituitary, AMH binds to AMHRII to increase FSH synthesis and secretion, specifically during the infantile period in the female rat (Garrel *et al.* 2016). It would be of interest to specify the contribution of ovarian AMH to the transient increase in both GnRH neuron activity and gonadotropin levels observed during the infantile period (Dulka & Moenter 2017, François *et al.* 2017).

In conclusion, the present work leads us to propose that the high levels of gonadotropin hormones during the infantile period prematurely sensitize the first follicular

waves to the action of FSH (Fig. 5). FSH stimulates *Cyp19a1* aromatase expression in these follicles, an effect that may be facilitated by FSH-induced downregulation of *Amh* expression in these follicles (Fig. 5). In contrast, FSH may not act on subsequent follicular waves that still significantly express AMH. Together with recent reports on AMH actions in the hypothalamo-pituitary system (Cimino *et al.* 2016, Garrel *et al.* 2016), the present study highlights the idea that AMH would exert both intra- and extra-ovarian actions and thus be a major regulator of the gonadotrope axis in infantile mice. By eventually contributing to the production of high amounts of E2 in infantile females, AMH would therefore play an important role in reproductive function programming.

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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Author contribution statement

M D, F P, V C, C M F, F G and G G performed experiments. M D and C M F prepared figures and analyzed the data. J C-T read the manuscript. C J G designed the project, analyzed data, prepared figures and wrote the manuscript.

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