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Loss of inhibin alpha uncouples oocyte-granulosa cell dynamics and disrupts postnatal folliculogenesis

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ABSTRACT

Targeted disruption of the inhibin α gene (*Inha*$^{-/-}$) in mice results in an ovarian phenotype of granulosa cell tumors that renders the animals infertile. Little is known about the reproductive defects prior to tumor development. Here, we report novel data on early follicle dynamics in *Inha*$^{-/-}$ mice, which demonstrate that inhibin α has important consequences upon follicle development. Morphological changes in both germ and somatic cells were evident in postnatal day 12 ovaries, with *Inha*$^{-/-}$ mice exhibiting numerous multilayered follicles that were far more advanced than those observed in age-matched controls. These changes were accompanied by alterations in follicle dynamics such that *Inha*$^{-/-}$ ovaries had fewer follicles in the resting pool and more committed in the growth phase. Absence of inhibin α resulted in advanced follicular maturation as marked by premature loss of anti-Müllerian hormone (AMH) in secondary follicles. Additionally, gene expression analysis revealed changes in factors known to be vital for oocyte and follicle development. Together, these data provide key evidence to suggest that regulation of the inhibin/activin system is essential for early folliculogenesis in the prepubertal mouse ovary.

Introduction

Inhibins, activins and other related dimeric members of the transforming growth factor β (TGFβ) family are renowned for their pleiotropic roles in various developmental and physiological processes. Development of the ovarian follicle (folliculogenesis) is a complex event orchestrated by intraovarian growth factors, pituitary gonadotropins, and steroidogenic hormones (Matzuk et al., 2002). The elucidation of many of the specific roles of locally derived growth factors during folliculogenesis has been revealed through the analysis of transgenic mouse models (Matzuk and Lamb, 2008; Myers and Pangas, in press; Pangas and Matzuk, 2008), which have provided key tools in dissecting the molecular pathways involved in follicular growth and development. Particular examples of targeted deletions of members belonging to the TGFβ family include anti-Müllerian hormone (AMH), growth differentiation factor 9 (GDF9) and activin; all of which show alterations in folliculogenesis (Dong et al., 1996; Durlinger et al., 1999; Pangas et al., 2007). Unlike the aforementioned growth factors, the targeted disruption of the inhibin α gene (*Inha*$^{-/-}$) results in granulosa cell tumors, rendering the mice infertile and ultimately resulting in a cachexia-related death (Matzuk et al., 1992, 1994).

Initially discovered for their opposing abilities to regulate pituitary follicle stimulating hormone (FSH), activins and inhibins have also been shown to have diverse endocrine, paracrine and autocrine functions in both gonadal and extragonadal systems (Chang et al., 2002; Pangas and Matzuk, 2008). These two members of the TGFβ family are well characterized for their mutually antagonistic properties; in part, this is a consequence of their related protein structure. The generation of an α subunit (encoded by the *Inha* gene) combined with a β subunit (encoded by the *Inhba* or *Inhbb* gene) makes inhibins (α:βA, α:βB), whilst homodimers (βA;βA, βB;βB) or heterodimers (βA;βB) of the β subunits make activin. The major gonadal sites of inhibin synthesis are the Sertoli cells in males and the granulosa cells in females. Reports thus far on the *Inha*$^{-/-}$ mouse model confirm that there are important roles for inhibin in normal follicle recruitment and gonadal tumor development (Matzuk et al., 1996; Wu et al., 2004). Because of the cancer phenotype, much of the published data on the *Inha*$^{-/-}$ mouse has focused upon the role of inhibin as a tumor-suppressor (Andreu-Vieyra et al., 2007; Brown et al., 2000; Burns et al., 2003; Cipriano et al., 2001; Kumar et al., 1996; Li et al., 2007; Nagaraja et al., 2008) rather than its role in fertility.

Although many studies have shown activins and inhibins to be critical regulators of gonadal function, little is known about their

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specific roles during the early stages of folliculogenesis. Emerging evidence in both mouse and human systems suggests that the inhibin/activin system is important for both cell proliferation (germ and somatic) and follicular assembly (Bristol-Gould et al., 2006; Martins da Silva et al., 2004). We therefore hypothesized that loss of inhibin in the *Inha*$^{-/-}$ mouse, or alternatively, the gain of unopposed activins, would affect early postnatal folliculogenesis, as the homeostatic relationship between these related dimeric glycoproteins is disrupted. Our findings demonstrate that ovaries from *Inha*$^{-/-}$ mice display a precocious follicular development phenotype that is evident within the first two weeks of life and markedly apparent in the subsequent weeks of postnatal life. *Inha*$^{-/-}$ ovaries exhibit alterations in follicular dynamics with evidence of early follicle recruitment and strikingly advanced follicular growth. Analysis of candidate genes revealed the alterations in intraovarian factors notorious for playing key roles in oocyte and follicle development. Additionally, a marked defect in cell-to-cell communication between the germ and somatic cell types further contributes to the perturbed ovarian phenotype in *Inha*$^{-/-}$ mice. Our findings highlight the importance of the inhibin/activin system during early folliculogenesis and illustrate how a refined balance of growth factors is required for proper follicle growth and development.

Materials and methods

Experimental animals

Experimental *Inha*$^{tm1Zuk/tm1Zuk}$ (herein called *Inha*$^{-/-}$) mice were maintained on a C57BL/6J;129S5/SvEvBrd mixed genetic background and kept in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. *Inha*$^{-/-}$ mice were generated as previously described (Matzuk et al., 1992) and genotyped by PCR analysis of genomic tail DNA using the primers E2-2 (5′-GGTCTCCTGCGGCTTTGCGC-3′), HPRT (5′-GGATATGCCCTTGACTATAATG-3′), and Intron (5′-CCTGGGTGGAGCAGGATATGG-3′) to determine whether the mouse was wildtype, heterozygous or homozygous null at the inhibin α locus.

Tissue collection

Mice were anesthetized by isoflurane inhalation (Abbott Laboratories, Abbott Park, IL) and euthanized by decapitation. Ovaries were collected from mice at postnatal day 3, 6 and 12 with the day of birth considered as postnatal day 0. Ovarian tissue was either fixed in Bouin's solution (Sigma, St. Louis, MO) or stored in RNAlater (Ambion, Austin, TX) at −80 °C for subsequent use.

Histology, follicle counting and morphometric measurements

Tissue processing and embedding were performed by the Department of Pathology Core Facility (Baylor College of Medicine) using standard techniques. Ovaries were serially sectioned into 5 μm sections with approximately five sections per slide. Sections were stained with periodic acid Schiff's reagent and hematoxylin. For follicle counting, three to five ovaries were assessed per genotype for each age group and every 5th section was counted. Follicles were classified as follows; primordial, an oocyte surrounded by squamous pregranulosa cells; primary, an oocyte surrounded by predominantly cuboidal granulosa cells; secondary, an oocyte surrounded by two to four layers of granulosa cells, or an oocyte surrounded by more than four layers of granulosa cells without an antrum. To avoid double counting secondary follicles, only oocytes with a visible nucleus were counted. Data were expressed as percentage of follicle types per total follicles. For oocyte and follicle measurements, the shortest and longest diameter of both the oocyte and follicle were taken and the mean was recorded. Oocytes were grouped in 10 μm increments. Graphical representation of oocyte size vs. mean follicle diameter was plotted with SEM for follicle diameters on the *Y*-axis.

Immunohistochemistry

Immunohistochemistry was performed using the Vectorstain ABC method (Vector Laboratories, Burlingame, CA) as previously described (Pangas et al., 2007). Goat polyclonal anti-AMH (1:1500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) was used on at least three different sections from five animals of each genotype. Immunoreactivity was visualized by diaminobenzidine (Vector Laboratories) and counterstained with hematoxylin.

FSH serum assay

Blood was recovered from mice at postnatal day 3, 6 and 12. Due to limited volume of blood in younger mice, pooled serum (for all age groups) from three to five animals was used, and six independent pools were assayed. Blood was collected and serum separated by centrifugation in Microcontainer tubes (Sarstedt, Nümbrecht, Germany) and stored at −20 °C until use. FSH serum measurements were performed by The University of Virginia Ligand Core Facility (Specialized Cooperative Centers Program in Reproduction Research NICHD/NIH U54HD28934). The mouse FSH RIA has a sensitivity of 2 ng/ml and an average intraassay coefficient of 10.1% and an interassay coefficient variation of 13.3%.

Quantitative PCR

RNA was isolated using the QIAGEN (Valencia, CA) RNeasy Micro kit. RNA concentration was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Wilmington, DE). cDNA was reverse transcribed from 200 ng of total RNA in a 20 μl reaction using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the Applied Biosystems Prism 7500 Sequence Detection System, Taqman Master Mix and Gene Expression Assays. The following Taqman (Applied Biosystems) Assays used for qPCR: mouse glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (Pangas et al., 2007) as an endogenous control; *Amh*, Mm03023963_m1; *Bmp15*, Mm00437797_m1; *Ccnb1*, Mm00838401_g1; *Ccnd2*, Mm00438071_ml; *Cyp19*, Mm00484049_m1; *Fshr*, Mm00442819; *Gdf9*, Mm00433565_m1; *Inhba*, Mm00434338_ml; *Inhbb*, Mm03023992_ml; *Kitl*, Mm00442972_ml. All qPCR data was analyzed by the ΔΔ cycle threshold method using the ABI 7500 System Software (version 1.2.3) and normalized to the endogenous reference (*Gapdh*). One control sample was randomly chosen as the calibrator sample and the mean and SEM were calculated. Results were plotted using the relative expression of each target gene with each sample compared to the control.

Statistics

Statistical analysis was carried out using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Two-tailed unpaired *t*-tests were used for single comparisons. At least three independent experiments were carried out at all times and $P<0.05$ was considered statistically significant.

Results

Loss of inhibin α results in marked morphologic changes in the postnatal ovary at day 12

During the first few weeks of life, there is significant organisation and development within the postnatal mouse ovary (Pepling,

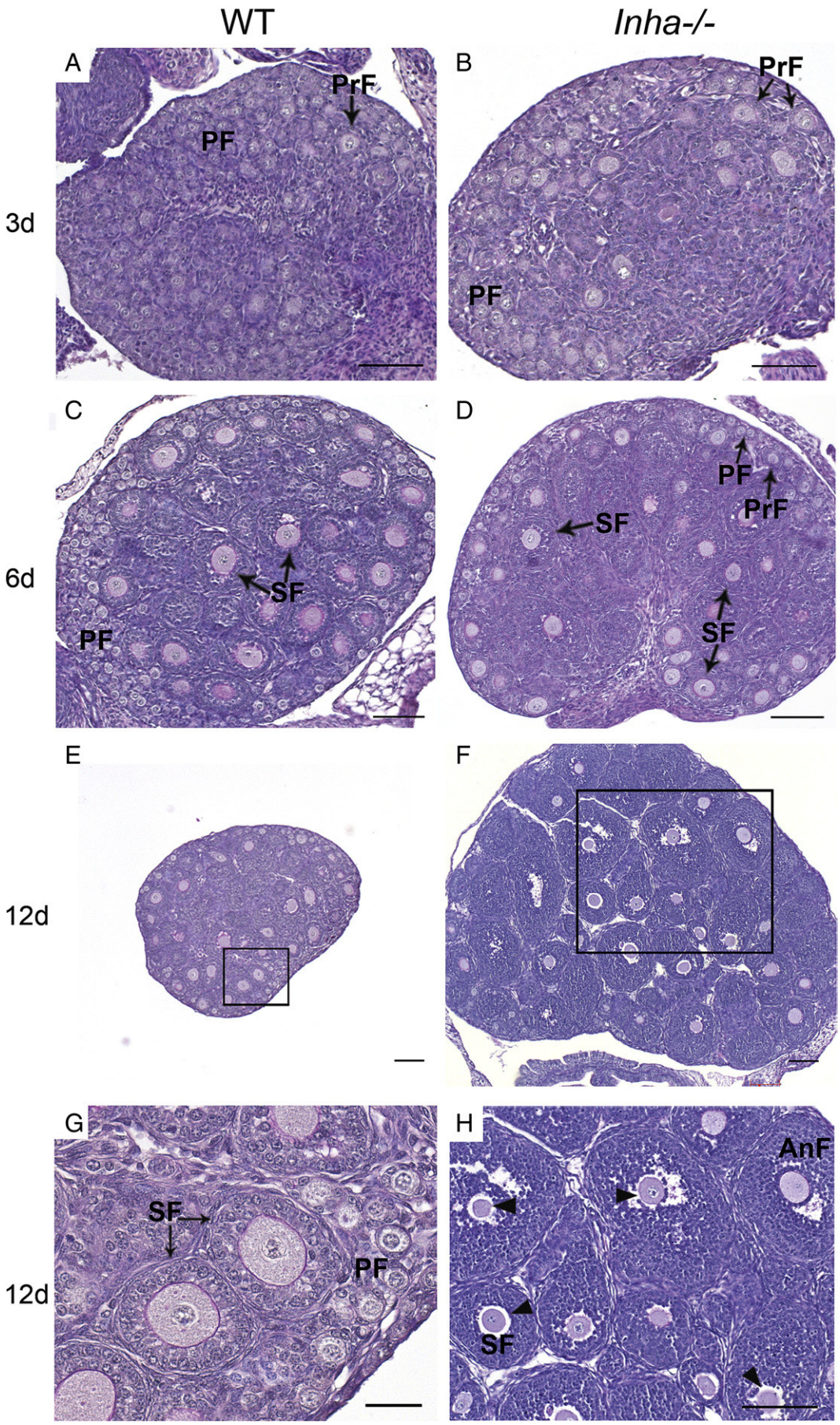
WT
Inha-/-
A
B
C
D
E
F
G
H
3d
6d
12d
12d
PrF
PF
SF
AnF

Table 1
Summary of gene expression from postnatal day 12 WT and *Inha*$^{-/-}$ ovaries.

Gene	Synonym	Fold change in *Inha*$^{-/-}$ ovaries (qPCR)
Inhba	Inhibin/activin βA	+2.58[a]
Inhbb	Inhibin/activin βB	+3.28[a]
Ccnb1	Cyclin B1	No change

Data were analyzed using unpaired *t*-tests for single comparisons.
[a] Denotes statistical difference between *Inha*$^{-/-}$ and control ($P<0.01$).

2006). This is the time of primordial follicle formation and by postnatal day 3, the majority of oocytes are encapsulated by somatic cells (Pepling, 2006). Ovaries from WT (Figs. 1A, C, E, G) and *Inha*$^{-/-}$ (Figs. 1B, D, F, H) mice were examined at postnatal day 3, 6 and 12. At postnatal day 3, there were no obvious gross morphological differences between WT and *Inha*$^{-/-}$ animals with both ovaries consisting predominantly of primordial follicles and some one layer follicles with growing oocytes (primary follicles) (Figs. 1A, B). By postnatal day 6, multiple two layer follicles (secondary) were evident in both genotypes. Although *Inha*$^{-/-}$ ovaries appeared to have more growing multilayer secondary follicles at this age (Figs. 1C, D), morphometric measurements of oocyte size vs. mean follicular diameter did not reveal any major changes (Supplementary Fig. 1). By postnatal day 12, there was a striking difference in size between the WT (Fig. 1E) and *Inha*$^{-/-}$ ovaries. Histological images of the ovaries taken at the same magnification showed more than a 2-fold increase in size of *Inha*$^{-/-}$ ovaries (Fig. 1F). Although WT littermates did not exhibit follicles with more than 2-4 layers of granulosa cells (Fig. 1G), *Inha*$^{-/-}$ ovaries had numerous multilayered follicles (Figs. 1F, H), some of which had greater than 10 layers of granulosa cells. Small antral-like cavities were also observed in many of the large multilayered follicles in *Inha*$^{-/-}$ ovaries (Fig. 1H). Such features indicated an advanced follicular phenotype in *Inha*$^{-/-}$ ovaries.

FSH serum levels remain unchanged in the absence of inhibin α in postnatal day 3, 6 and 12 mice

Folliculogenesis prior to the antral stage is thought to be independent of the gonadotropins (Kumar et al., 1996). There is however, a peak of FSH that is reported to occur at approximately postnatal day 10 (Weng et al., 2006). Because inhibin/activin β subunits are up regulated in *Inha*$^{-/-}$ ovaries (Table 1), we wanted to determine if the increased follicle maturation in *Inha*$^{-/-}$ mice was a consequence of increased circulating FSH. Serum FSH levels remained unchanged between WT and *Inha*$^{-/-}$ female mice at postnatal day 3 (15.11 ± 1.23 ng/ml vs. 13.88 ± 1.62), postnatal day 6 (22.98 ± 1.47 vs. 26.51 ± 3.47 ng/ml) and postnatal day 12 (29.98 ± 1.50 vs. 27.53 ± 1.98 ng/ml) (Fig. 2A). To assess if the advanced follicular growth was the result of increased FSH sensitivity, we also measured the mRNA levels of the FSH receptor (*Fshr*) and the cytochrome P450 aromatase (*Cyp19*) a well-known target gene of FSH action. There was a non-significant trend for *Fshr* expression to increase and no change in *Cyp19* (FSH target gene) expression in *Inha*$^{-/-}$ ovaries (Figs. 2B, C). To assess any changes in granulosa cell proliferation, we also investigated cell cycle genes, cyclin D2 (*Ccnd2*) (Fig. 2D) and cyclin B1 (*Ccnb1*) (Table 1). *Ccnd2*, a known target gene of activin or GDF9 (Ogawa et al., 2003), and activin and FSH (Ogawa et al., 2003; Park et al., 2005) was significantly increased in *Inha*$^{-/-}$ ovaries (Fig. 2D). There was no change in the mRNA levels of *Ccnb1* (Table 1). Together, these data suggest that the acceleration of follicle growth in *Inha*$^{-/-}$ mice at postnatal day 12 is not secondary to increased circulating FSH or increased FSH sensitivity, but more likely a result of locally produced growth factors.

Loss of inhibin α does not change the number of follicles endowed in the neonatal mouse but results in an acceleration of follicle growth from the resting pool into the growth phase

The formation of primordial follicles is central to a female's reproductive lifespan and is dependent upon the process of pregranulosa cells encapsulating a single oocyte. To determine if the number of oocytes within the *Inha*$^{-/-}$ neonatal ovary, or moreover whether the formation of primordial follicles was altered, we investigated postnatal day 3 *Inha*$^{-/-}$ mice. There was no difference in the total number of oocytes counted in *Inha*$^{-/-}$ ovaries compared with age-matched WT controls, and this was true for both primordial and primary follicle types (Fig. 3A). To further determine if the loss of inhibin α had an effect upon follicles during the initial waves of folliculogenesis, we assessed *Inha*$^{-/-}$ and WT ovaries at postnatal day 12. The numbers of primordial, primary and secondary follicles were assessed. Secondary follicles were broken down into three categories, two to four layers of granulosa cells; more than four layers of granulosa cells and; more than four layers of granulosa cells with an antral cavity) (Fig. 3B). Analysis of follicle dynamics revealed that *Inha*$^{-/-}$ mice exhibit an acceleration in the number of follicles leaving the resting pool (primordial follicles) and an increase in the number of follicles in the growing pool (primary and secondary stages). There was approximately a 30% reduction in the number of primordial follicles in *Inha*$^{-/-}$ ovaries compared to WT littermates. Concomitantly, the reduction of primordial follicles in *Inha*$^{-/-}$ ovaries was associated with an increase of follicles at all growing stages, demonstrating that the follicles in *Inha*$^{-/-}$ mice are developing at an accelerated rate compared to WT mice.

Alterations in the intraovarian growth factor AMH suggests precocious follicular development in Inha$^{-/-}$ *mice*

AMH protein expression is present in non-atretic preantral and small antral follicles of postnatal ovaries (Baarends et al., 1995; Hirobe et al., 1992). Once a follicle develops to a more advanced stage and becomes FSH sensitive, AMH protein expression is lost (Durlinger et al., 2002b). As expected, both adult (8 weeks) and prepubertal (postnatal day 12) WT ovaries showed positive staining for AMH protein in primary, secondary and early antral follicles (only present in 8 week old WT ovary) (Figs. 4A, B). Expression was not evident in primordial follicles (Fig. 4A inset) or corpora lutea (Fig. 4A). Although there was immunoreactivity for AMH in some secondary follicles in *Inha*$^{-/-}$ ovaries, staining patterns were variable with many two to four layered secondary or large multilayered

Fig. 1. Histological analysis of the ovaries of prepubertal *Inha*$^{-/-}$ and WT mice. (A) Ovaries from postnatal day 3 WT mice demonstrate many primordial follicles (PF), which contain oocytes encapsulated by pregranulosa somatic cells. Whilst most of the follicles are in the form of primordials, few have entered into the growth phase and developed into primary follicles (PrF). (B) *Inha*$^{-/-}$ ovaries at postnatal day 3 were similar to those of WT mice with primordial follicles (PF) clusters and some follicles progressing to a primary follicle stage (PrF). (C) By postnatal day 6 in the WT ovary, primordial follicles (PF) were predominantly localized to the ovarian cortex and many of the follicles had progressed to a secondary follicle (SF) stage, with two to four layers of cuboidal granulosa cells. (D) Ovaries from *Inha*$^{-/-}$ mice also had primordial (PF) and primary follicles (PrF) residing in the cortical region however there appeared to be more growing secondary follicles (SF) in these ovaries compared to WT ovaries. (E, F) By postnatal day 12 there were marked differences between ovaries from WT (E) and *Inha*$^{-/-}$ (F) animals, the most striking being the large difference in the size of the ovaries upon gross inspection. Histological images taken at the same magnification show that *Inha*$^{-/-}$ ovaries were more than 2-fold larger than WT ovaries at postnatal day 12. (G) An inset of postnatal day 12 WT ovary in panel E shows primordial follicles (PF) around the cortex of the ovary and secondary follicles (SF) with no more than two to three layers of granulosa cells. (H) *Inha*$^{-/-}$ ovaries at higher magnification show many multilayered secondary follicles (SF), some of which have greater than 10 layers of granulosa cells, not seen in WT ovaries. Many of these follicles progressed to antral follicles (AnF) exhibiting small cavities of follicular fluid. Several large follicles in *Inha*$^{-/-}$ ovaries also displayed evidence oocyte–granulosa cell uncoupling (arrowheads). Scale bars for A–G = 100 μm, H = 50 μm.

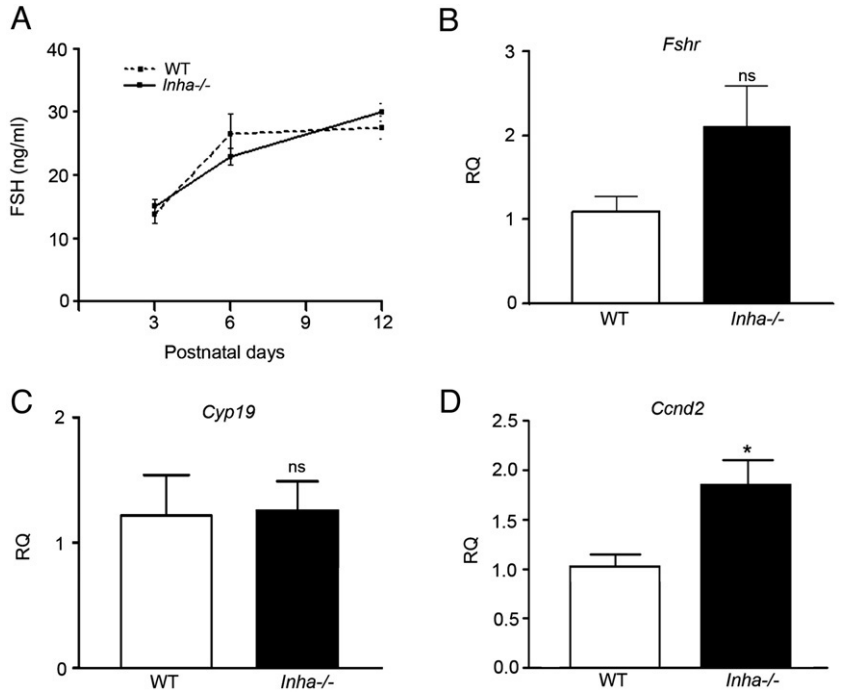

Fig. 2. Serum FSH and *Fshr* expression in *Inha*$^{-/-}$ mice. (A) Serum FSH did not change between WT (dashed line) or *Inha*$^{-/-}$ (solid line) ovaries at any of the time points assessed, $n=6$. (B) Expression levels of *Fshr* show a trend to increase in *Inha*$^{-/-}$ ovaries, however this was not statistically significant. (C) Expression levels of the aromatase (*Cyp19*) gene remained unchanged in WT and *Inha*$^{-/-}$ ovaries (D) Cyclin D2, a regulator of the cell cycle was increased in *Inha*$^{-/-}$ ovaries. qPCR results are expressed as relative quantity (RQ).

follicles that had either totally lost expression of AMH or lost it with the exception of the granulosa cell layer closest to the oocyte (Figs. 4C, D). Similar to the WT ovary, positive staining for AMH was also evident in the early primary follicles of *Inha*$^{-/-}$ ovaries (Fig. 4E), and although AMH expression is not detected in WT primordial follicles (Durlinger et al., 2002b), the occasional primordial follicle located at periphery of the *Inha*$^{-/-}$ stained positive. We hypothesize that these follicles represent a population of primordial follicles that have more advanced granulosa cells and are probably in transition. Consistent with the early loss of AMH protein expression in *Inha*$^{-/-}$ ovaries, transcript levels of *Amh* were also significantly reduced in comparison to WT ovaries (Fig. 4F).

The growth dynamics between germ cells and somatic cells are altered Inha$^{-/-}$ *ovaries*

During early folliculogenesis, oocyte and granulosa cell proliferation occurs in a synchronous manner and is dependent upon locally produced growth factors such as GDF9, BMP15 and KITL. When viewed at high magnification, it became evident that many of the follicles in *Inha*$^{-/-}$ ovaries had lost this synchronous relationship. There were many small oocytes (20–30 μm) contained in abnormally large follicles (Figs. 5B, C). These small oocytes enclosed in these large *Inha*$^{-/-}$ secondary follicles were comparable in size to those of WT primary follicles. Graphical representation of oocyte diameter vs. follicle

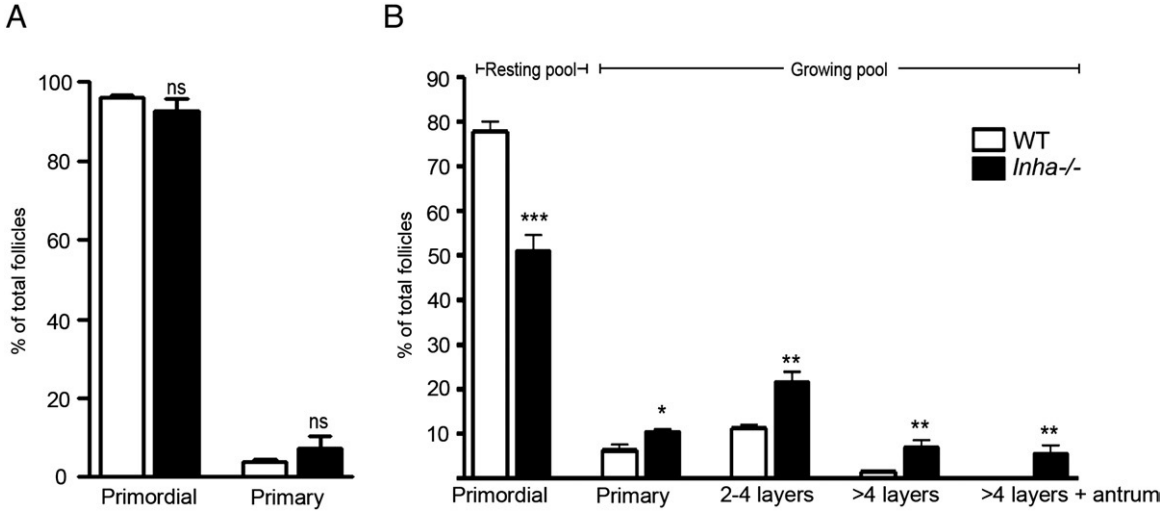

Fig. 3. Alterations in the follicle dynamics in *Inha*$^{-/-}$ ovaries. (A) Assessment of follicle numbers in postnatal day 3 ovaries revealed that the number of primordial or primary follicles was unchanged between WT (clear bar) and *Inha*$^{-/-}$ (solid bar) ovaries. (B) Changes in follicle numbers became evident by postnatal day 12 with a 30% reduction in the number of primordial follicles in *Inha*$^{-/-}$ ovaries compared to WT littermates. Consistent with the decline in primordial follicles, *Inha*$^{-/-}$ ovaries also had a significant increase in the number of follicles at each developmental stage.

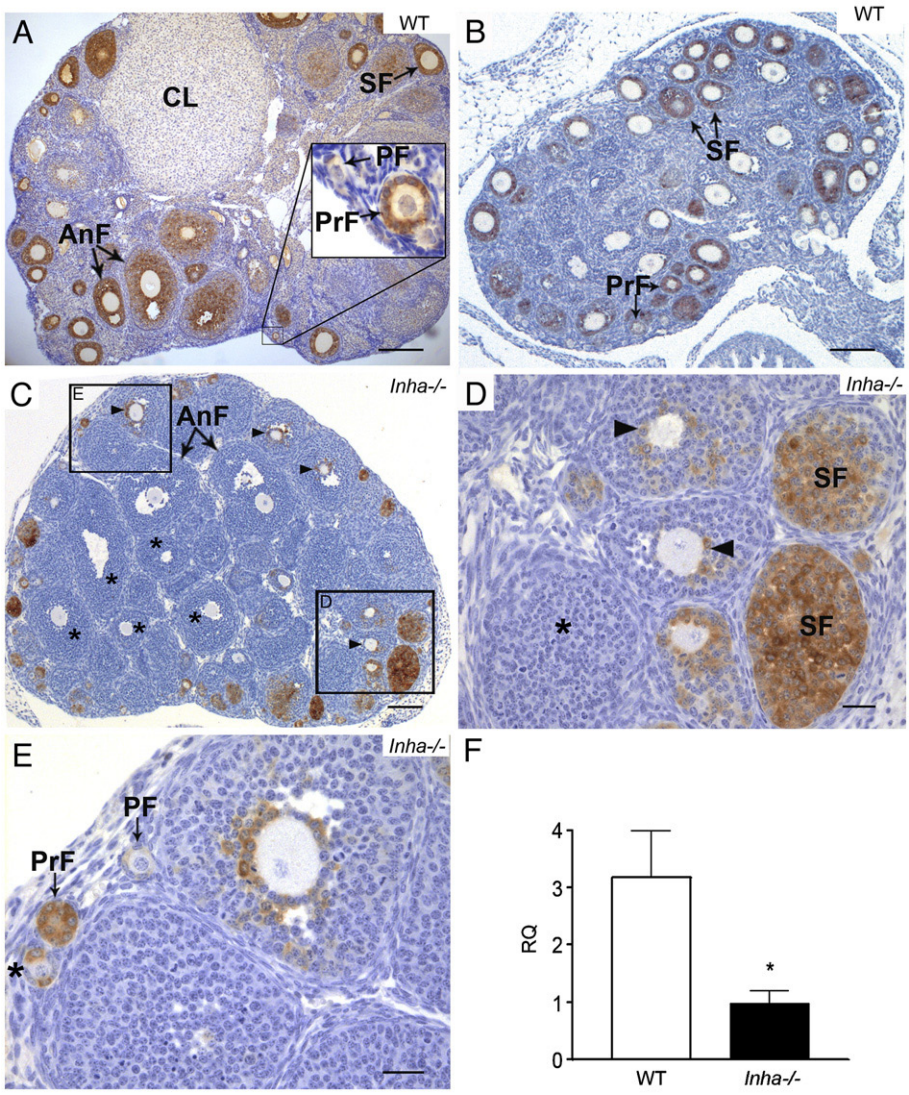

Fig. 4. Premature loss of AMH in *Inha*$^{-/-}$ follicles. (A) Immunoreactivity of AMH in an adult (8 week old) WT ovary demonstrates localization of the protein in the growing follicle population, which includes secondary (SF) and early antral (AnF) follicles. The corpus luteum (CL) is negative for AMH. The inset shows a higher magnification image of a primordial follicle (PF) that does not express AMH until they transition into primary follicles (PrF). (B) Similar to the expression pattern in adult ovaries, postnatal day 12 WT ovaries also exhibit follicles from the primary (PrF) to secondary stage (SF) that are positive for AMH. (C) *Inha*$^{-/-}$ ovaries show follicles positive for AMH around the ovarian cortex, whilst the expression pattern in secondary (*) and antral (AnF) follicles is variable. Most of the growing follicles within the ovary are AMH-negative or have only retained expression in their cumulus cells (arrowhead). (D, E) show insets of panel C. (D) Higher magnification of secondary follicles from an *Inha*$^{-/-}$ ovary shows immunoreactivity in some secondary follicles (SF) whilst expression is either mostly (arrowhead) or totally (*) lost in others. (E) Inset of panel C shows high magnification of a primordial follicle (PF) negative for AMH expression and a positive primary follicle (PrF). Interestingly, few of the squamous cells on one of the primordials (*) in an *Inha*$^{-/-}$ ovary is positive for AMH. (F) Compared to WT levels, the transcript levels for AMH in *Inha*$^{-/-}$ ovaries were reduced. Scale bars for A–C = 100 μm, D–E = 25 μm. qPCR results are expressed as relative quantity (RQ).

diameter further reiterated the loss of synchronous oocyte-follicle growth in *Inha*$^{-/-}$ ovaries (Fig. 5D) at postnatal day 12, that remained unchanged at postnatal day 6 (Supplementary Fig. 1). Investigation into the expression of factors known to be involved in oocyte growth and development revealed that the expression of *Gdf9* was significantly increased in *Inha*$^{-/-}$ ovaries whilst *Bmp15* and *Kitl* were significantly reduced (Figs. 5D–E). These results suggest that the loss of synchronous growth between germ and somatic cells in *Inha*$^{-/-}$ follicles may be due to changes in the expression of growth factors that maintain oocyte-granulosa cell development.

Discussion

Inhibins and activins play integral roles during various stages of folliculogenesis. We describe novel data on the early follicle dynamics in the postnatal *Inha*$^{-/-}$ mouse prior to tumor development. This mouse model provides an excellent *in vivo* tool to investigate the physiological and mechanistic roles for inhibin during the early stages of follicle formation and their subsequent entry into the growth phase. Unfortunately, with respect to folliculogenesis, the *Inha*$^{-/-}$ model is limited to development prior to puberty because beginning at around 4 weeks and with the onset pituitary gonadotropin secretions, *Inha*$^{-/-}$ mice begin to develop sex-cord stromal tumors (Kumar et al., 1996; Matzuk and Lamb, 2002; Nagaraja et al., 2008). In the current study, we reveal that the targeted deletion of inhibin α results in precocious follicle development, involving the disruption of oocyte-granulosa cell interactions and the generation of aberrant oocyte and somatic cell gene expression patterns.

The formation of the earliest follicles, the primordials, is a critical stage in female fertility and in the mouse, occurs during the first few days of postnatal life (Pepling and Spradling, 2001). Utilizing the *Inha*$^{-/-}$ mouse model, we wanted to address questions regarding the roles of the inhibin/activin system during early follicular development because activins are reported to play important roles in the proliferation and survival of germ and somatic cells proceeding primordial follicle formation in the human (Coutts et al., 2008;

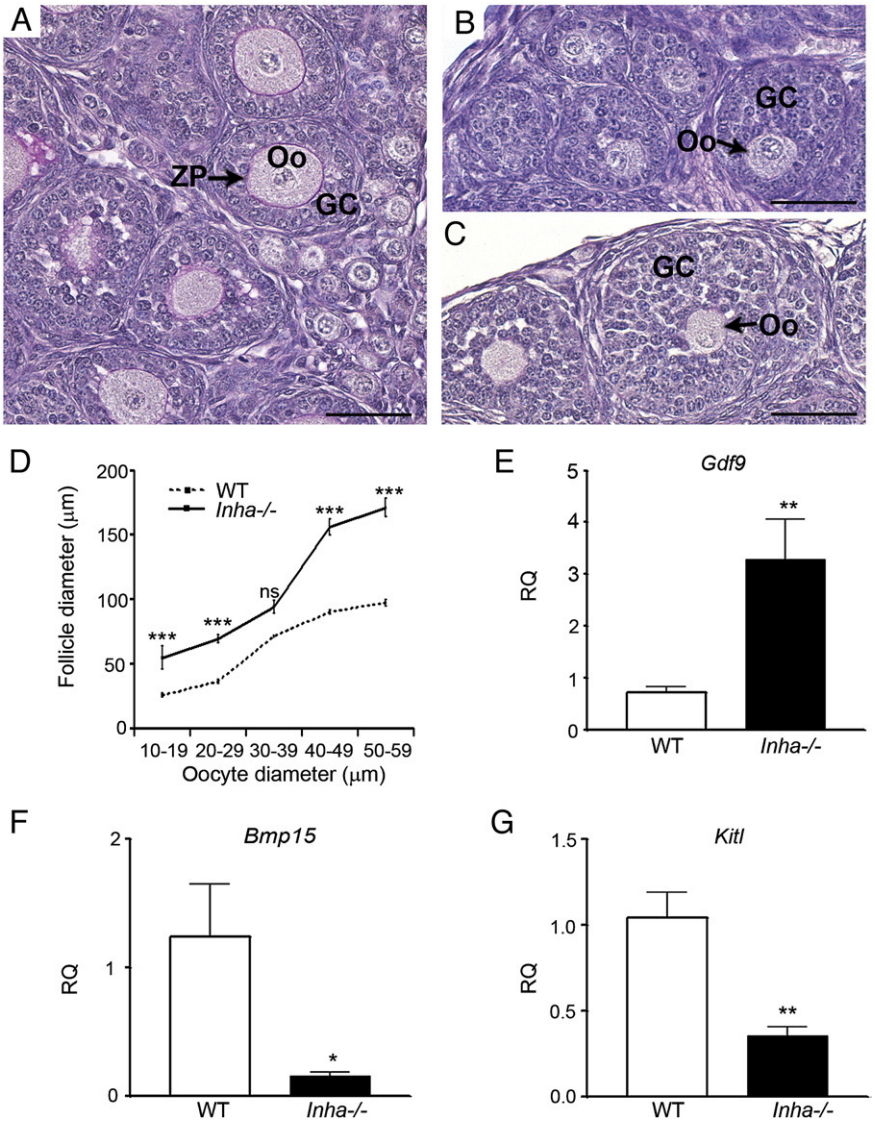

Fig. 5. Alterations in the growth dynamics between germ and somatic cells in *Inha*$^{-/-}$ follicles. (A) A typical secondary follicles in a postnatal day 12 WT ovary consists of an oocyte (Oo, diameter = 43 μm), covered by a zona pellucida matrix (ZP) and two to three layers of granulosa cells (GC). (B) In contrast, the *Inha*$^{-/-}$ ovary has numerous secondary follicles with many of them exhibiting oocytes (Oo, diameter = 30 μm) of very small diameters and multiple layers of granulosa cells (GC). (C) Another example of a small oocyte (Oo, diameter = 23 μm) contained within multilayers of granulosa cells (GC). (D) Measurements from *Inha*$^{-/-}$ and WT ovaries show that oocytes in *Inha*$^{-/-}$ ovaries are associated with much larger follicles than those from WT littermates. This became particularly evident once the oocyte diameters were greater than 40 μm and were contained in very large multilayered follicles that had larger diameters than any follicle type in the WT ovary. (E) Compared to WT ovaries, the **e**xpression of *Gdf9* was significantly increased in *Inha*$^{-/-}$ ovaries, (F) whilst *Bmp15* and (G) *Kitl* were significantly reduced. Scale bars for A–C = 50 μm. qPCR results are expressed as relative quantity (RQ).

Martins da Silva et al., 2004) and during primordial follicle formation in the mouse (Bristol-Gould et al., 2006). Surprisingly, we found that loss of inhibin α had no detrimental effect upon the formation of primordial follicles. At postnatal day 3, *Inha*$^{-/-}$ ovaries had similar morphology to their WT counterparts and had no differences in numbers of primordial or primary follicles. While injection of exogenous activin formation of primordial follicles (Bristol-Gould et al., 2006), our studies suggest that loss of inhibin α has no effect upon the number of follicles at postnatal day 3. Although the *Inha*$^{-/-}$ mouse provides a unique model that allows an *in vivo* insight into the effects of unopposed activin upon germ cell endowment and follicle formation, the potential roles of other growth factors should also be considered.

By postnatal day 6, the there was no apparent difference between WT and *Inha*$^{-/-}$ ovaries however, by day 12, there was a striking difference. Follicles in postnatal day 12 *Inha*$^{-/-}$ mice had progressed to very large multilayered structures, consisting of more than 10 layers of granulosa cells. Both activins and FSH are known regulators of granulosa cell proliferation (Hillier, 2001; Miro and Hillier, 1996), with activins in particular stimulating granulosa cell proliferation in small follicles that are FSH-independent (Miro and Hillier, 1996). The pituitary-derived gonadotropins, FSH and luteinizing hormone (LH), are major regulators of mammalian gonadal function (Halpin et al., 1986; Kumar et al., 1999), and are essential modulators for further follicle development and subsequent ovulation (Kumar et al., 1997; Peters et al., 1975), once the follicle has acquired an antral cavity. Because inhibins suppress pituitary FSH, and adult mice display increased serum FSH levels comparison to WT mice (Matzuk et al., 1992), we investigated serum FSH in prepubertal animals. However, there were no discernible differences in serum FSH in prepubertal *Inha*$^{-/-}$ mice at any of the postnatal days investigated (days 3, 6 and 12). We propose that during these early developmental stages prior to puberty and before the requirement of a gonadotropin-surge, inhibin does not have an endocrine effect upon FSH production. This coupled with no significant changes in the expression of the FSH receptor or CYP19, a well-known target gene of FSH action (Hillier et al., 1994),

infer that the acceleration in granulosa cell proliferation in *Inha*$^{-/-}$ prepubertal animals is not a major direct effect of FSH, but more likely a consequence of intraovarian growth factors signaling.

We report a regulatory role for inhibin in primordial follicle activation and also at the rate at which they entered into the growth phase. Because AMH is a known regulator of primordial follicle recruitment, we postulate that the premature loss of AMH in *Inha*$^{-/-}$ ovaries contributes to the advanced rate of primordial follicle recruitment at postnatal day 12. Although mice genetically deficient in *Amh* do not display a reduction in the number of primordial follicles at postnatal day 25 (Durlinger et al., 1999), *in vitro* evidence suggests that AMH can act as an inhibitory growth factor in neonatal ovaries (Durlinger et al., 2002a). These data imply that AMH is one of many regulators of primordial follicle recruitment and functional redundancies are likely to exist between many of these growth factors. Together, our data suggests that inhibins have inhibitory roles upon primordial follicle recruitment (possibly via influencing AMH signaling) and a regulatory role that controls the rate that follicles mature (by inhibiting positive-acting growth factors such as activin and GDF9).

Although many follicles within *Inha*$^{-/-}$ ovaries had progressed to very large multilayered structures, many had abnormal interactions between their germ and somatic cell components. A striking feature of the postnatal day 12 *Inha*$^{-/-}$ ovary was the dysregulation or loss of synergy between the oocyte and granulosa cell growth dynamics in many of the growing follicles. A number of the oocytes contained in the multilayered follicles in *Inha*$^{-/-}$ ovaries had unusually small diameters for follicles of such a size; diameters that were comparable to WT primary follicles. To our knowledge, the observed follicular phenotype in the prepubertal *Inha*$^{-/-}$ mouse model is unique, and with respect to oocyte morphology displays a phenotype opposite to other observed transgenic models, whereby loss of a germ cell factor (i.e., GDF9) or loss of a somatic cell factor (i.e., FOXO3A) causes enlargement of the oocyte (Dong et al., 1996; Liu et al., 2007). Future investigations into whether these small oocytes are growth restricted (as explained below) or are simply too immature for follicles of such diameters will offer further insight into key regulators of oocyte growth.

Analysis of genes integral for oocyte-follicle dynamics demonstrated that important intraovarian growth factors such as *Kitl*, *Bmp15* and *Gdf9* were altered in *Inha*$^{-/-}$ ovaries. We found that whilst the expression of *Gdf9* was increased, both *Bmp15* and *Kitl* were significantly reduced. Although both oocyte-derived growth factors (GDF9 and BMP15) have important roles within the ovary, only GDF9 appears to be essential for female fertility in the mouse (Dong et al., 1996; Yan et al., 2001). Signaling pathways that regulate GDF9 are unclear, and it is possible that important intrafollicular signaling dynamics exist between GDF9 and the inhibin/activin system because the removal of the inhibin α gene overcomes the block at the primary follicle stage in *Gdf9*$^{-/-}$ mice (Wu et al., 2004). In contrast to *Gdf9* expression, we found reduced transcripts of oocyte-derived *Bmp15* in prepubertal ovaries. Although the expression patterns of BMP15 have been reported in adult mice (Dube et al., 1998), the precise role and temporal expression patterns of BMP15 in the prepubertal mouse are still unclear. This coupled with the reported differences between poly and mono-ovular species (Dong et al., 1996; Juengel et al., 2002, 2004; McNatty et al., 2005; Yan et al., 2001) make the role of BMP15 hard to decipher. This may suggest that the synergistic or compensatory roles for these oocyte-derived factors are limited to the gonadotropin-dependent or at least the later stages folliculogenesis involving the development of the oocyte-cumulus cell complex (Yan et al., 2001). Subsequent investigations will further determine the relevance and potential roles for BMP15 in early mouse folliculogenesis.

Granulosa cell-derived KITL is negatively regulated by both activin (Coutts et al., 2008; Pangas et al., 2007) and oocyte-derived GDF9 (Elvin et al., 1999; Wu et al., 2004). In light of its functional importance for oocyte growth and development (Thomas and Vanderhyden, 2006), we postulate that the reduction of *Kitl* in *Inha*$^{-/-}$ ovaries is a likely contributor to the small oocyte phenotype. Our working model hypothesizes that in the absence of inhibin, activin and/or GDF9 suppress *Kitl,* resulting in the loss of a key trophic signal required for oocyte growth. Simultaneously, the adjacent granulosa cells are responding to increased activin and GDF9 as positive regulators of their own proliferation (Fig. 6). Thus, in *Inha*$^{-/-}$ ovaries, alterations in these important growth factors are likely contributors to the loss of synchronous oocyte-follicle growth. This further suggests that activin and GDF9 at times may be functionally redundant (i.e., such that they can both inhibit the

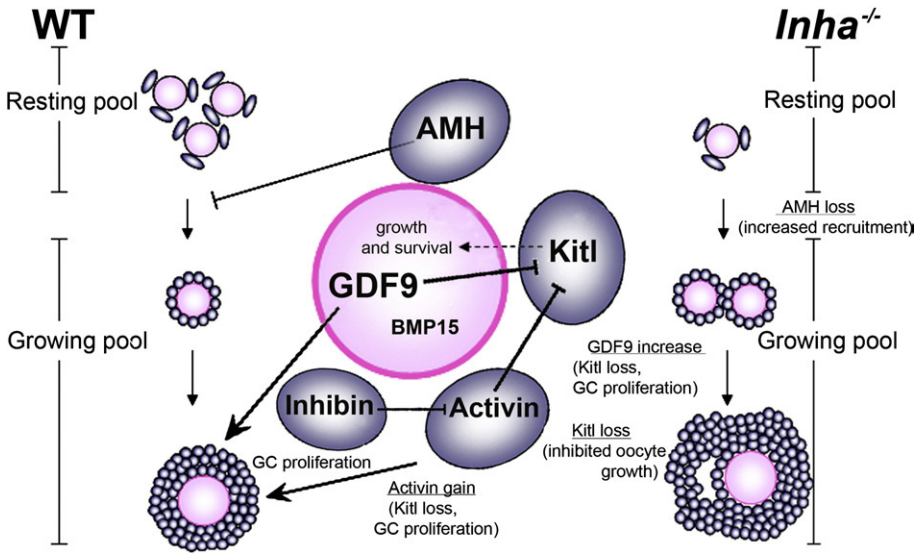

Fig. 6. A working model of some of the intraovarian factors involved in early folliculogenesis. *Inha*$^{-/-}$ mice (right panel) show defects in both the resting and growing pool of follicles. AMH is an inhibitory factor that suppresses the recruitment of resting follicles in WT ovaries (left panel). Oocyte-derived GDF9 and granulosa cell-derived activin suppress KITL, which acts on the oocyte to promote growth and survival. In *Inha*$^{-/-}$ ovaries, GDF9 and activin are overexpressed, potentially contributing to the lack of oocyte development and the excessive granulosa cell proliferation. The prepubertal *Inha*$^{-/-}$ mouse provides a valuable means to dissecting out the roles of intraovarian factors that are important during early folliculogenesis. Redundancy or non-redundancy of some of these growth factors in early folliculogenesis remains to be determined.

production of KITL and enhance granulosa cell proliferation). Both growth factors work via the same intracellular SMAD signaling proteins [SMAD2/3 (Gilchrist et al., 2006; Kaivo-Oja et al., 2003; Pangas and Matzuk, 2008; Roh et al., 2003)], in some instances stimulate or inhibit common genes (Eramaa et al., 1995; Kaivo-Oja et al., 2003) and have recently been shown to act in a co-operative manner to regulate key components of the activin signaling pathway (Shi et al., 2009).

In summary, the prepubertal *Inha*$^{-/-}$ mouse ovary provides a unique *in vivo* genetic system to study the dynamics of the inhibin/activin system during early follicle development. Unlike adult *Inha*$^{-/-}$ mice, prepubertal *Inha*$^{-/-}$ animals do not show evidence of tumor development. They are however, subjected to perturbed folliculogenesis. We show that in the absence of inhibin, the prepubertal ovary displays precocious follicle growth and alterations in intraovarian factors that are essential for folliculogenesis (Fig. 6). We propose that loss of inhibin α results in more granulosa cell-derived activin signaling (i.e. all of the β subunits form activin with no inhibin to antagonize activin signaling) and oocyte-derived GDF9, which then suppress *Kitl* expression in the granulosa cells. The reduction in KITL signaling to the oocyte via its receptor KIT has detrimental effects upon oocyte growth and contributes to the small oocyte phenotype observed in many of the *Inha*$^{-/-}$ follicles. Additionally, the loss of inhibin α inhibits the production of factors that play important roles in regulating the rate at which follicles enter the growth phase, including AMH. We hypothesize that in addition to being a tumor-suppressor, inhibin also plays a very important role in regulating follicle dynamics such that the ovary is not subjected to precocious development. These studies emphasize the need for further research of inhibin/activin system in clinical diseases such as premature ovarian failure.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.08.001.

References

Andreu-Vieyra, C., Chen, R., Matzuk, M.M., 2007. Effects of granulosa cell-specific deletion of Rb in Inha-alpha null female mice. Endocrinology 148, 3837–3849.

Baarends, W.M., Uilenbroek, J.T., Kramer, P., Hoogerbrugge, J.W., van Leeuwen, E.C., Themmen, A.P., Grootegoed, J.A., 1995. Anti-mullerian hormone and anti-mullerian hormone type II receptor messenger ribonucleic acid expression in rat ovaries during postnatal development, the estrous cycle, and gonadotropin-induced follicle growth. Endocrinology 136, 4951–4962.

Bristol-Gould, S.K., Kreeger, P.K., Selkirk, C.G., Kilen, S.M., Cook, R.W., Kipp, J.L., Shea, L. D., Mayo, K.E., Woodruff, T.K., 2006. Postnatal regulation of germ cells by activin: the establishment of the initial follicle pool. Dev. Biol. 298, 132–148.

Brown, C.W., Houston-Hawkins, D.E., Woodruff, T.K., Matzuk, M.M., 2000. Insertion of Inhbb into the Inhba locus rescues the Inhba-null phenotype and reveals new activin functions. Nat. Genet. 25, 453–457.

Burns, K.H., Agno, J.E., Sicinski, P., Matzuk, M.M., 2003. Cyclin D2 and p27 are tissue-specific regulators of tumorigenesis in inhibin alpha knockout mice. Mol. Endocrinol. 17, 2053–2069.

Chang, H., Brown, C.W., Matzuk, M.M., 2002. Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr. Rev. 23, 787–823.

Cipriano, S.C., Chen, L., Burns, K.H., Koff, A., Matzuk, M.M., 2001. Inhibin and p27 interact to regulate gonadal tumorigenesis. Mol. Endocrinol. 15, 985–996.

Coutts, S.M., Childs, A.J., Fulton, N., Collins, C., Bayne, R.A., McNeilly, A.S., Anderson, R.A., 2008. Activin signals via SMAD2/3 between germ and somatic cells in the human fetal ovary and regulates kit ligand expression. Dev. Biol. 314, 189–199.

Dong, J., Albertini, D.F., Nishimori, K., Kumar, T.R., Lu, N., Matzuk, M.M., 1996. Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature 383, 531–535.

Dube, J.L., Wang, P., Elvin, J., Lyons, K.M., Celeste, A.J., Matzuk, M.M., 1998. The bone morphogenetic protein 15 gene is X-linked and expressed in oocytes. Mol. Endocrinol. 12, 1809–1817.

Durlinger, A.L., Kramer, P., Karels, B., de Jong, F.H., Uilenbroek, J.T., Grootegoed, J.A., Themmen, A.P., 1999. Control of primordial follicle recruitment by anti-Mullerian hormone in the mouse ovary. Endocrinology 140, 5789–5796.

Durlinger, A.L., Gruijters, M.J., Kramer, P., Karels, B., Ingraham, H.A., Nachtigal, M.W., Uilenbroek, J.T., Grootegoed, J.A., Themmen, A.P., 2002a. Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 143, 1076–1084.

Durlinger, A.L., Visser, J.A., Themmen, A.P., 2002b. Regulation of ovarian function: the role of anti-Mullerian hormone. Reproduction 124, 601–609.

Elvin, J.A., Yan, C., Wang, P., Nishimori, K., Matzuk, M.M., 1999. Molecular characterization of the follicle defects in the growth differentiation factor 9-deficient ovary. Mol. Endocrinol. 13, 1018–1034.

Eramaa, M., Hilden, K., Tuuri, T., Ritvos, O., 1995. Regulation of inhibin/activin subunit messenger ribonucleic acids (mRNAs) by activin A and expression of activin receptor mRNAs in cultured human granulosa-luteal cells. Endocrinology 136, 4382–4389.

Gilchrist, R.B., Ritter, L.J., Myllymaa, S., Kaivo-Oja, N., Dragovic, R.A., Hickey, T.E., Ritvos, O., Mottershead, D.G., 2006. Molecular basis of oocyte-paracrine signalling that promotes granulosa cell proliferation. J. Cell. Sci. 119, 3811–3821.

Halpin, D.M., Charlton, H.M., Faddy, M.J., 1986. Effects of gonadotrophin deficiency on follicular development in hypogonadal (hpg) mice. J. Reprod. Fertil. 78, 119–125.

Hillier, S.G., 2001. Gonadotropic control of ovarian follicular growth and development. Mol. Cell. Endocrinol. 179, 39–46.

Hillier, S.G., Whitelaw, P.F., Smyth, C.D., 1994. Follicular oestrogen synthesis: the 'two-cell, two-gonadotrophin' model revisited. Mol. Cell. Endocrinol. 100, 51–54.

Hirobe, S., He, W.W., Lee, M.M., Donahoe, P.K., 1992. Mullerian inhibiting substance messenger ribonucleic acid expression in granulosa and Sertoli cells coincides with their mitotic activity. Endocrinology 131, 854–862.

Juengel, J.L., Hudson, N.L., Heath, D.A., Smith, P., Reader, K.L., Lawrence, S.B., O'Connell, A.R., Laitinen, M.P., Cranfield, M., Groome, N.P., Ritvos, O., McNatty, K.P., 2002. Growth differentiation factor 9 and bone morphogenetic protein 15 are essential for ovarian follicular development in sheep. Biol. Reprod. 67, 1777–1789.

Juengel, J.L., Bodensteiner, K.J., Heath, D.A., Hudson, N.L., Moeller, C.L., Smith, P., Galloway, S.M., Davis, G.H., Sawyer, H.R., McNatty, K.P., 2004. Physiology of GDF9 and BMP15 signalling molecules. Anim. Reprod. Sci. 82-83, 447–460.

Kaivo-Oja, N., Bondestam, J., Kamarainen, M., Koskimies, J., Vitt, U., Cranfield, M., Vuojolainen, K., Kallio, J.P., Olkkonen, V.M., Hayashi, M., Moustakas, A., Groome, N. P., ten Dijke, P., Hsueh, A.J., Ritvos, O., 2003. Growth differentiation factor-9 induces Smad2 activation and inhibin B production in cultured human granulosa-luteal cells. J. Clin. Endocrinol. Metab. 88, 755–762.

Kumar, T.R., Wang, Y., Matzuk, M.M., 1996. Gonadotropins are essential modifier factors for gonadal tumor development in inhibin-deficient mice. Endocrinology 137, 4210–4216.

Kumar, T.R., Wang, Y., Lu, N., Matzuk, M.M., 1997. Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat. Genet. 15, 201–204.

Kumar, T.R., Palapattu, G., Wang, P., Woodruff, T.K., Boime, I., Byrne, M.C., Matzuk, M.M., 1999. Transgenic models to study gonadotropin function: the role of follicle-stimulating hormone in gonadal growth and tumorigenesis. Mol. Endocrinol. 13, 851–865.

Li, Q., Kumar, R., Underwood, K., O'Connor, A.E., Loveland, K.L., Seehra, J.S., Matzuk, M.M., 2007. Prevention of cachexia-like syndrome development and reduction of tumor progression in inhibin-deficient mice following administration of a chimeric activin receptor type II-murine Fc protein. Mol. Hum. Reprod. 13, 675–683.

Liu, L., Rajareddy, S., Reddy, P., Du, C., Jagarlamudi, K., Shen, Y., Gunnarsson, D., Selstam, G., Boman, K., Liu, K., 2007. Infertility caused by retardation of follicular development in mice with oocyte-specific expression of Foxo3a. Development 134, 199–209.

Martins da Silva, S.J., Bayne, R.A., Cambray, N., Hartley, P.S., McNeilly, A.S., Anderson, R.A., 2004. Expression of activin subunits and receptors in the developing human ovary: activin A promotes germ cell survival and proliferation before primordial follicle formation. Dev. Biol. 266, 334–345.

Matzuk, M.M., Lamb, D.J., 2002. Genetic dissection of mammalian fertility pathways. Nat. Cell Biol. (Suppl. 4), s41–s49.

Matzuk, M.M., Lamb, D.J., 2008. The biology of infertility: research advances and clinical challenges. Nat. Med. 14, 1197–1213.

Matzuk, M.M., Finegold, M.J., Su, J.G., Hsueh, A.J., Bradley, A., 1992. Alpha-inhibin is a tumour-suppressor gene with gonadal specificity in mice. Nature 360, 313–319.

Matzuk, M.M., Finegold, M.J., Mather, J.P., Krummen, L., Lu, H., Bradley, A., 1994. Development of cancer cachexia-like syndrome and adrenal tumors in inhibin-deficient mice. Proc. Natl. Acad. Sci. U. S. A. 91, 8817–8821.

Matzuk, M.M., Kumar, T.R., Shou, W., Coerver, K.A., Lau, A.L., Behringer, R.R., Finegold, M.J., 1996. Transgenic models to study the roles of inhibins and activins in reproduction, oncogenesis, and development. Recent Prog. Horm. Res. 51, 123–154 discussion 155-7.

Matzuk, M.M., Burns, K.H., Viveiros, M.M., Eppig, J.J., 2002. Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 296, 2178–2180.

McNatty, K.P., Galloway, S.M., Wilson, T., Smith, P., Hudson, N.L., O'Connell, A., Bibby, A.H., Heath, D.A., Davis, G.H., Hanrahan, J.P., Juengel, J.L., 2005. Physiological

effects of major genes affecting ovulation rate in sheep. Genet. Sel. Evol. 37 (Suppl. 1), S25–S38.
Miro, F., Hillier, S.G., 1996. Modulation of granulosa cell deoxyribonucleic acid synthesis and differentiation by activin. Endocrinology 137, 464–468.
Myers, M., Pangas, S.A., in press. Regulatory roles of transforming growth factor beta family members in folliculogenesis. WIREs Systems Biology and Medicine.
Nagaraja, A.K., Agno, J.E., Kumar, T.R., Matzuk, M.M., 2008. Luteinizing hormone promotes gonadal tumorigenesis in inhibin-deficient mice. Mol. Cell. Endocrinol. 294, 19–28.
Ogawa, T., Yogo, K., Ishida, N., Takeya, T., 2003. Synergistic effects of activin and FSH on hyperphosphorylation of Rb and G1/S transition in rat primary granulosa cells. Mol. Cell. Endocrinol. 210, 31–38.
Pangas, S.A., Matzuk, M.M., 2008. The TGF-β family in the reproductive tract. In: Derynck, R., Miyazono, K. (Eds.), The TGF-β Family. InCold Spring Harbor Laboratory Press, New York, pp. 861–889.
Pangas, S.A., Jorgez, C.J., Tran, M., Agno, J., Li, X., Brown, C.W., Kumar, T.R., Matzuk, M.M., 2007. Intraovarian activins are required for female fertility. Mol. Endocrinol. 21, 2458–2471.
Park, Y., Maizels, E.T., Feiger, Z.J., Alam, H., Peters, C.A., Woodruff, T.K., Unterman, T.G., Lee, E.J., Jameson, J.L., Hunzicker-Dunn, M., 2005. Induction of cyclin D2 in rat granulosa cells requires FSH-dependent relief from FOXO1 repression coupled with positive signals from Smad. J. Biol. Chem. 280, 9135–9148.
Pepling, M.E., 2006. From primordial germ cell to primordial follicle: mammalian female germ cell development. Genesis 44, 622–632.
Pepling, M.E., Spradling, A.C., 2001. Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. Dev. Biol. 234, 339–351.
Peters, H., Byskov, A.G., Himelstein-Braw, R., Faber, M., 1975. Follicular growth: the basic event in the mouse and human ovary. J. Reprod. Fertil. 45, 559–566.
Roh, J.S., Bondestam, J., Mazerbourg, S., Kaivo-Oja, N., Groome, N., Ritvos, O., Hsueh, A.J., 2003. Growth differentiation factor-9 stimulates inhibin production and activates Smad2 in cultured rat granulosa cells. Endocrinology 144, 172–178.
Shi, F., Cheung, A.P., Leung, P.C., 2009. Growth differentiation factor 9 (GDF9) enhances activin A-induced inhibin B production in human granulosa cells. Endocrinology 150, 3540–3546 (Epub 2009 May 7).
Thomas, F.H., Vanderhyden, B.C., 2006. Oocyte-granulosa cell interactions during mouse follicular development: regulation of kit ligand expression and its role in oocyte growth. Reprod. Biol. Endocrinol. 4, 19.
Weng, Q., Wang, H., M, S.M., Jin, W., Xia, G., Watanabe, G., Taya, K., 2006. Expression of inhibin/activin subunits in the ovaries of fetal and neonatal mice. J. Reprod. Dev. 52, 607–616.
Wu, X., Chen, L., Brown, C.A., Yan, C., Matzuk, M.M., 2004. Interrelationship of growth differentiation factor 9 and inhibin in early folliculogenesis and ovarian tumorigenesis in mice. Mol. Endocrinol. 18, 1509–1519.
Yan, C., Wang, P., DeMayo, J., DeMayo, F.J., Elvin, J.A., Carino, C., Prasad, S.V., Skinner, S.S., Dunbar, B.S., Dube, J.L., Celeste, A.J., Matzuk, M.M., 2001. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. Mol. Endocrinol. 15, 854–866.