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Actions of anti-Müllerian hormone on the ovarian transcriptome to inhibit primordial to primary follicle transition

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Abstract

The oocytes found within the primordial follicles of mammalian ovaries remain quiescent for months to years until they receive the appropriate signals to undergo the primordial to primary follicle transition and initiate folliculogenesis. The molecular mechanisms and extracellular signaling factors that regulate this process remain to be fully elucidated. The current study investigates the mechanisms utilized by anti-Müllerian hormone (AMH; i.e. Müllerian inhibitory substance) to inhibit the primordial to primary follicle transition. Ovaries from 4-day-old rats were placed into organ culture and incubated in the absence or presence of AMH, either alone or in combination with known stimulators of follicle transition, including basic fibroblast growth factor (bFGF), kit ligand (KITL), or keratinocyte growth factor (KGF). Following 10 days of culture, the ovaries were sectioned, stained, and morphologically evaluated to determine the percentage of primordial versus developing follicles.As previously demonstrated, AMH treatment decreased primordial to primary follicle transition. Interestingly, AMH inhibited the stimulatory actions of KITL, bFGF, and KGF. Therefore, AMH can inhibit the basal and stimulated development of primordial follicles. To investigate the mechanism of AMH actions, the influence AMH has on the ovarian transcriptome was analyzed. AMH treatment when compared with controls was found to alter the expression of 707 genes. The overall effect of AMH exposure is to decrease the expression of stimulatory factors, increase the expression of inhibitory factors, and regulate cellular pathways (e.g. transforming growth factor β signaling pathway) that result in the inhibition of primordial follicle development. Analysis of the regulatory factors and cellular pathway altered by AMH provides a better understanding of the molecular control of primordial follicle development.

Introduction

Primordial follicles in mammalian ovaries are composed of an oocyte surrounded by squamous (i.e. flattened) pre-granulosa cells. The oocytes in primordial follicles are arrested in the diplotene stage of prophase I of meiosis. These arrested follicles remain quiescent for months or years until they receive the necessary signals to grow and develop. The initiation of primordial follicle development is termed primordial to primary follicle transition. Although recent studies have identified several factors that regulate primordial to primary

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follicle transition (Nilsson & Skinner 2001, Fortune 2003, Skinner 2005, Visser & Themmen 2005), this vital process for mammalian reproduction remains to be fully elucidated.

Primordial to primary follicle transition involves pre-granulosa cells around the oocyte becoming cuboidal epithelial granulosa while the oocyte increases in diameter. As the follicle grows, it gains successive layers of granulosa cells, a theca cell layer, and eventually a fluid-filled antrum. Once the primordial to primary follicle transition is complete, the follicle will either continue to develop until the oocyte is ovulated or undergo atresia and regress (Peters *et al.* 1975, Cran & Moor 1980, Hirshfield 1991, Rajah *et al.* 1992). Primordial follicles gradually develop and leave the arrested pool over the course of a female's reproductive lifespan. Once the follicle pool is depleted, reproduction ceases and women enter menopause (Gosden *et al.* 1983, Richardson *et al.* 1987, Faddy *et al.* 1992, Faddy & Gosden 1996, Faddy 2000).The size of the primordial follicle pool is fixed early in life (Hirshfield 1991). Although recent studies speculate that new oocytes might be formed in adulthood (Johnson *et al.* 2004, 2005), the initial size of the primordial follicle pool is a major determinant of reproductive lifespan (Hirshfield 1994).

Primordial to primary follicle transition is regulated by both inhibitory and stimulatory growth factors acting locally in a paracrine and/or autocrine manner.The only two extracellular signaling molecules that have been identified as inhibiting follicle transition are anti-Müllerian hormone (AMH; Durlinger *et al.* 1999, 2002b) and stromal-derived factor 1 (SDF1 or CXCl12; Holt et al. 2006). SDF1 is a member of the large family of chemokine molecules and binds to the G-protein-coupled receptor CXCR4 (Feng *et al.* 1996). A recent study revealed that SDF1 is expressed in the oocytes of primordial and primary follicles. Mouse ovaries cultured in the presence of SDF1 showed an increase in follicle density and a decrease in follicle diameter compared with controls, suggesting an inhibition of primordial to primary follicle transition (Holt *et al.* 2006).

AMH, also known as Müllerian inhibitory substance, is a member of the transforming growth factor β family of growth factors and binds to AMH receptor 2 (AMHR2). AMH was first known for its role in causing regression of the Müllerian ducts during fetal development in the male (Lee & Donahoe 1993, Mishina *et al.* 1999). In females, AMH is expressed postnatally by the granulosa cells of developing follicles from the secondary stage to the early antral stage (Durlinger *et al.* 2002*a*, 2002*b*). It is thought that the AMH produced by these early stage developing follicles acts locally on primordial follicles to inhibit the primordial to primary follicle transition (Visser & Themmen 2005). How AMH acts to inhibit primordial follicle transition is unknown, and is the focus of the current study.

Several growth factors have been found to be produced by the different cell types of the developing primordial follicle that can stimulate the primordial to primary follicle transition. Those stimulatory factors that are expressed in the oocytes of developing early stage follicles include platelet-derived growth factor (PDGF; Dube *et al.* 1998, Laitinen *et al.* 1998, Nilsson *et al.* 2006), and basic fibroblast growth factor (bFGF/FGF2; van Wezel *et al.* 1995, Yamamoto *et al.* 1997, Nilsson *et al.* 2001*a*). PDGF appears to act on the adjacent granulosa and theca cells (Nilsson *et al.* 2006). Receptors for bFGF have been found on granulosa cells and oocytes (Shikone *et al.* 1992, Wandji *et al.* 1992, Ben-Haroush *et al.* 2005). Basic FGF

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has been shown to stimulate proliferation of granulosa and ovarian stromal cells, and increase the mRNA expression of another stimulatory growth factor kit ligand (KITL; Lavranos *et al.* 1994, Roberts & Ellis 1999, Nilsson *et al.* 2001*a*, Nilsson & Skinner 2004). How bFGF interacts with AMH to regulate primordial to primary follicle transition is investigated in the current study.

Growth factors that are expressed by granulosa cells which promote primordial to primary follicle transition include leukemia inhibitory factor (Arici *et al.* 1997, Khalifa *et al.* 1997, Nilsson *et al.* 2002) and KITL (Manova *et al.* 1993, Motro & Bernstein 1993, Parrott & Skinner 1999, Nilsson & Skinner 2004). KITL receptors (c-kit) are present on oocytes and theca cells (Horie *et al.* 1991, Manova *et al.* 1993, Motro & Bernstein 1993). KITL acts to recruit thecal cells from surrounding ovarian stroma during the primordial to primary follicle transition (Parrott & Skinner 2000). How KITL interacts with AMH to regulate primordial to primary follicle transition is also examined in the current study.

Growth factors produced by the theca/interstitial cells that surround follicles and promote the primordial to primary follicle transition include bone morphogenic protein 4 (BMP4), BMP7 (Shimasaki *et al.* 1999, Lee *et al.* 2001, Nilsson & Skinner 2003), and keratinocyte growth factor (KGF or FGF7; Kezele *et al.* 2005a). KGF is produced by isolated precursor theca cells surrounding primordial and early stage developing follicles (Kezele *et al.* 2005*a*). Ovaries in culture treated with KGF have an increased expression of KITL mRNA. Conversely, cultured ovaries treated with KITL have increased KGF expression, suggesting a positive feedback loop between these two growth factors to promote the primordial to primary follicle transition (Kezele *et al.* 2005*a*). The interaction of KGF with AMH to regulate primordial to primary follicle transition is also examined in the current study.

The objective of the current study is to investigate the mechanisms of AMH action to inhibit primordial to primary follicle transition. The interactions between AMH and an oocyte-derived growth factor (bFGF), a granulosa-derived growth factor (KITL), and a precursor theca-derived growth factor (KGF) are examined *in vitro*. In addition, the pattern of gene expression (i.e. transcriptome) in AMH-treated ovaries is examined using a microarray analysis to gain insight into what signaling pathways might be involved in AMH regulation of primordial to primary follicle transition. Elucidation of the regulatory mechanisms involved in primordial follicle development will provide potential therapeutic targets to manipulate the primordial follicle pool and treat disease states such as premature ovarian failure.

Materials and Methods

Organ culture and treatments

Postnatal 4-day-old rat ovaries were dissected from freshly euthanized Sprague–Dawley rat pups. All animal protocols were approved by the Washington State University Animal Care and Use Committee. Whole ovaries were cultured as previously described (Nilsson *et al.* 2001*a*) on floating filters (0.4 μm Millicell-CM, Millipore, Bedford, MD, USA) in 0.5 ml Dulbecco’s modified Eagle’s medium–Ham’s F-12 medium (1:1 (v/v)) containing 0.1% BSA (Christin-Maitre *et al.* 2002), 0.1% Albumax (Gibco BRL, Gaithersburg, MD, USA),

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27.5 μg/ml transferrin, 200 μg/ml insulin (human recombinant, Sigma), and 0.05 μg/ml L-ascorbic acid (Sigma) in a four-well culture plate (Nunc plate, Applied Scientific, South San Francisco, CA, USA). A culture experiment consisted of several wells in a culture plate with each well receiving a separate treatment. Two or three ovaries from different rats were placed on floating filters into each well and cultured for 10 days. Since each ovary receiving a particular treatment is genetically unique and can respond independently to the treatment, each ovary is considered an experimental unit (n=1). Treatments during organ culture included recombinant human Müllerian-inhibiting substance/AMH (generously provided by Dr Richard Cate at Biogen, Cambridge, MA, USA) at 50 ng/ml, rat KITL (R&D Systems Inc., Minneapolis, MN, USA) at 50 g/ml, recombinant human bFGF (R&D Systems Inc.) at 50 ng/ml, or recombinant human KGF (R&D Systems) at 50 ng/ml. The dose of 50 ng/ml for all the growth factors has previously been shown to be optimal using dose curve analysis (Nilsson *et al.* 2001*a*, Nilsson & Skinner 2004, Kezele *et al. 2005a*). The 50 ng/ml dose gave the same response as higher doses suggesting that 50 ng/ml is adequate. Lower doses were not extensively analyzed, such that the 50 ng/ml may not be the minimal effective dose, but is an optimal dose for stimulation. The AMH dose curve was run in the current study and 50 ng/ml gave the same response as 250 ng/ml, suggesting that 50 ng/ml is optimal. The medium was supplemented with penicillin and streptomycin to prevent bacterial contamination and the culture medium was changed every 2 days. After culture, the ovaries were fixed, sectioned, and stained with hematoxylin and eosin (H&E) for use in the morphological analysis. Alternatively, if mRNA levels were to be measured from cultured ovaries, the ovaries were only cultured for 48 h and then the cultured ovaries from one treatment group were pooled and homogenized in 1 ml Trizol (Gibco BRL) and stored at −20 °C until RNA isolation. Experiments were replicated so that two independent RNA samples, each derived from four to eight ovaries receiving the same treatment, were obtained for each treatment (AMH treated and control). Each individual replicate was derived from ovaries pooled from at least two wells from each of at least two experiments.

Morphological evaluation

Four-day-old rat ovaries were cultured for 10 days in the absence or presence of a treatment and then fixed in Bouin’s solution (0.9% picric acid, 9% formaldehyde, and 5% acetic acid) for 1–2 h. Ovaries were paraffin embedded and sectioned at 3–5 μm. Ovaries were de-paraffinized in xylene and hydrated through an ethanol series. Sections were stained with H&E using standard protocols. The number of follicles at each developmental stage was counted in two serial sections and averaged from the largest cross-sections through the center of the ovary. The oocyte nucleus had to be visible in a follicle in order for the follicle to be counted. Normally, 100–200 follicles were present in a cross-section. Previously, it has been demonstrated that the total follicle number per section does not change after 2 weeks of culture compared with freshly isolated 4-day-old ovaries (Parrott & Skinner 1999). Follicles were classified as either primordial (stage 0), or as one of the developing pre-antral stages (stages 1–4) as previously described (Parrott & Skinner 1999). Briefly, primordial follicles consist of an oocyte partially or completely encapsulated by flattened squamous pre-granulosa cells. Developing (stages 1–4) follicles contain successively more cuboidal granulosa cells in layers around the oocyte (Parrott & Skinner 1999, Nilsson *et al.* 2001*a*). The results of follicle counting are calculated as percentages (percentage of primordial

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follicles or percentage of developing follicles) to account for differences between individual rats in total number of oocytes per section. These percentage data were subjected to an arcsine transformation so that their distribution more closely resembles a standard (i.e. Gaussian) curve, and analyzed by ANOVA. In order to control for the variation between animals in follicle number and primordial follicle pool, transformed values for each experiment were normalized to the control by dividing the developing follicle values by the experiment's control mean. Therefore, the relative untreated control mean is set to equal one, which allowed the data between experiments to be quite consistent. Bartlet's test for equal variances was performed on the pooled datasets for both the raw percentage data and the transformed and normalized data. In both cases, the sample data exhibited no significant deviation from Gaussian distribution (data not shown), indicating that transformation and normalization likely did not skew the data.

Microarray and bioinformatics analysis

RNA was hybridized to the Affymetrix (Santa Clara, CA, USA) rat 230 2.0 gene chip. The Genomics Core in the Center for Reproductive Biology at Washington State University performed the analysis as previously described (McLean et al. 2002, Shima *et al.* 2004). Briefly, RNA from control and treated cultured ovaries were reverse transcribed into cDNA which was transcribed into biotin-labeled RNA. Biotin-labeled RNA was then hybridized to the Affymetrix rat 230 2.0 gene chips. Biotinylated RNA was then visualized by labeling with phycoerythrin-coupled avidin. The microarray chip was scanned on an Affymetrix Gene Chip Scanner 3000 (Affymetrix). The microarray image data were converted to numerical data with GeneChip Operating Software (GCOS version 1.1; Affymetrix) using a probe set scaling factor of 125. An absolute analysis was performed with GCOS to assess the relative abundance of the transcripts based on signal and detection calls (present, absent, or marginal). This information was imported into Genespring software (Silicon Genetics, Redwood City, CA, USA) and normalized using the recommended defaults. This includes setting signal values below 0.01 to a value of 0.01, total chip normalization to the 50th percentile, and normalization of each chip to the median. Unless otherwise indicated, in order for a transcript to be considered present, it had to be both flagged as present in the GCOS present/absent call, and have an expression level >75. Briefly, the 16 sets of oligonucleotides for a specific gene were used to make comparisons of a signal to statistically determine a present call using a one-sided Wilcoxon's signed rank test. In order for a transcript to be considered changed between treatment groups, it had to exhibit at least a two-fold change between the means of the treatments and have a Student's *t*-test P value of 0.05 between treatments. The raw signal cutoff was between 75 and 200 depending on the specific analyses as outlined in Results. Therefore, the data presented are for genes that were determined to be statistically present and found to be statistically different from control with a given treatment.

Two different experiments were performed involving two different sets of animals, RNA sample preparations, and microarray chips. Therefore, two control and AMH-treated samples were analyzed on two different chips. This allowed a 2×2 factorial comparison with all present/absent calls and changes in expression to be statistically significant for further analysis. The R^2 for the comparison between microarray chips was found to be R^2>0.94,

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which indicated negligible total variability between chips, experiments, and samples. The R^2-value and statistical analysis confirms that the chip number used was appropriate. The number of chips required for specific experiments has previously been reviewed (Chen *et al.* 2004). Previous studies have demonstrated that microarray data are validated with quantitative PCR data (Shima *et al.* 2004, Kezele *et al.* 2005*b*). Due to the presence of 16 different oligonucleotide sets for each specific gene being used on the microarray versus only a single primer set for a gene in a quantitative PCR, the microarray is more effective at eliminating false-positive or -negative data and provides a more robust quantitation of changes in gene expression. However, validation of microarray data was performed with selected genes using a real-time PCR procedure, as previously described (Kezele *et al.* 2005*b*). The genes selected for real-time PCR confirmation in the current study are *BMP4, GDF9*, and *KITL*. As presented in Results, similar data were obtained with the real-time PCR analysis as with the microarray analysis.

Statistical analysis

Comparisons between two groups using percentage data were performed using the Mann–Whitney test (Fig. 1). These same data (Fig. 1) were also arcsine transformed and subjected to an ANOVA, which yielded the same significant differences between treatment groups. Data from further organ culture experiments (Fig. 2) were arcsine transformed and normalized as described in Materials and Methods. Multiple comparison tests using transformed and normalized data were performed using Tukey's multiple comparison test after a significant difference was found with ANOVA. Groups were considered significantly different if P 0.05. All statistics were calculated with the help of GraphPad Prism version3.0a (GraphPad Software, San Diego, CA, USA).

Results

Organ culture experiments were performed to investigate the actions of AMH on primordial to primary follicle transition. Ovaries from 4-day-old rats were placed into an organ culture system and cultured for 10 days in the absence or presence of 50 ng/ml AMH. Following culture, the ovaries were fixed, sectioned, stained with H&E, and subjected to morphological analysis. A dose curve with AMH demonstrated a similar response (i.e. suppression of follicle development) with either 50 or 250 ng/ml AMH, such that 50 ng/ml were selected as the minimum dose needed to obtain an optimal response (data not shown). The cultured organs have previously been shown to maintain viability in culture with no apoptosis or morphological abnormalities (Parrott & Skinner 1999, Kezele *et al.* 2005*b*), and was also observed in the current study (data not shown). Total follicle numbers did not change during culture or after any of the treatments (data not shown), as previously described (Parrott & Skinner 1999, Nilsson *et al.* 2001*a*, 2002, 2006). Therefore, treatment with AMH and other growth factors only influenced follicle development and not follicle numbers or viability. Four-day-old rat ovaries primarily contain (>70%) primordial follicles (Kezele *et al.* 2005*b*). After 10 days of culture, untreated control ovaries contained 57±2.0% (mean±S.E.M.) primordial follicles and 43% developing follicles, demonstrating some endogenous primordial to primary follicle transition in culture (Fig. 1). Ovaries treated with AMH contained 68±1.5% primordial and 32% developing follicles after culture (Fig. 1). The

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proportion of arrested primordial follicles in AMH-treated ovaries was significantly (P=0.0024) higher than the proportion of primordial follicles in control cultures, demonstrating an arrest in endogenous follicle transition. These results confirm that AMH inhibits the primordial to primary follicle transition in this culture system.

In order to characterize how AMH interacts with growth factors that stimulate the primordial to primary follicle transition, the ovaries were cultured and treated with AMH in combination with bFGF, KITL, or KGF. After culture, the ovaries were fixed, stained, and subjected to morphological evaluation (Fig. 2). The proportion of developing follicles was normalized to the control for each experimental replicate in order to account for variation in untreated control follicular development between replicates. Ovaries treated with AMH, bFGF, or with the combination of AMH and bFGF demonstrated that the proportion of developing follicles in AMH-treated ovaries was the same as that in the combination AMH- and bFGF-treated ovaries (Fig. 2A). Similarly, organ culture experiments in which ovaries were treated with AMH, KITL, or AMH and KITL demonstrated that the proportion of developing follicles in AMH-treated ovaries was the same as in AMH- and KITL-treated ovaries (Fig. 2B). Experiments in which ovaries were treated with AMH, KGF, or AMH, and KGF demonstrated that the proportion of developing follicles in AMH-treated ovaries was the same as in AMH- and KGF-treated ovaries (Fig. 2C). These observations indicate that AMH is able to suppress the stimulatory effects of bFGF, KITL, and KGF on primordial to primary follicle transition.

The effect of AMH on the ovarian transcriptome was investigated to help elucidate the mechanism of AMH action. Ovaries were dissected from 4-day-old rat pups and placed into an organ culture system. The ovaries were cultured for 48 h with or without 50 ng/ml AMH. Ovaries from the same treatment groups were pooled into duplicate samples for each treatment, and total RNA was isolated and used for microarray gene expression analysis using Affymetrix rat 230 2.0 chips. The microarray analysis demonstrated that 707 transcripts were statistically changed (i.e. greater than twofold) in ovaries with AMH treatment when compared with controls, as per the criteria described in Materials and Methods. A dendrogram demonstrates alterations in the ovarian transcriptome (Fig. 3) with 164 genes increased and 543 genes decreased after AMH treatment. Therefore, the predominant action of AMH is to suppress the expression of a large number of genes. A full list of these changed genes, as well as the raw signal data from the Affymetrix microarrays, is available in the Supplemental Table 1 which can be viewed online at www.reproduction-online.org/supplemental/. These regulated gene transcripts were categorized when possible according to function. The number of genes up- and downregulated in each functional category is shown in Fig. 4. The categories with the highest number of genes with known functions were those involved in transport, signaling, and transcription.

An examination of cellular signaling pathways and associated gene transcripts that change in expression following AMH treatment identified two major pathways that were affected (i.e. transforming growth factor b (TGF-β) and MAP kinase (MAPK) signaling pathways). The TGF-β signaling pathways and associated genes that change in expression after AMH treatment are shown in Fig. 5. Although AMH is a TGF-β family member and utilizes the TGF- signaling pathway, many of the key genes (e.g. *Smads*) are suppressed by AMH,

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suggesting an inhibition of other TGF-β family member actions (e.g. BMP and activin). The MAPK pathway shown in Fig. 6 also demonstrates that a number of key regulatory genes in this pathway are suppressed. The suppression of the MAPK pathway would effect the actions of stimulatory growth factors and inhibit cell proliferation.

Extracellular signaling molecules are thought to play an important role in regulating the primordial to primary follicle transition (Skinner 2005). Candidate signaling molecules that may play a role in follicle transition were identified by examining the list of gene transcripts that change in ovaries treated with AMH. A list of growth factors, cytokines, and other paracrine signaling molecules and their receptors was developed. These candidate signaling factors are presented in Table 1. While the majority of these regulatory factors are decreased in AMH-treated ovaries, six transcripts are upregulated (shown in bold). Secreted growth factors stimulated by AMH include vascular endogenous growth factor (VEGF), growth differentiation factor 1-like (GDF1-like), colony-stimulating factor 1, bone morphogenetic protein antagonist 1 (Cktsf1b1), and angiopoietin 2 (Agpt2). Interestingly, the expression of both the c-kit receptor (KIT) and the FGF receptor (FGFR1) are suppressed by AMH (Table 1), which may correlate with the ability of AMH to suppress KITL and bFGF actions. The expression of KITL and bFGF themselves did not change with AMH treatment (data not shown). AMH also suppressed GDF9 and BMP15 expression (Table 1), which correlates with a decrease in the number of primary follicles. BMP4 has been shown to promote primordial to primary follicle transition (Nilsson & Skinner 2003), and AMH suppressed the expression of BMP4 (Table 1). To help validate the microarray procedure and confirm the effects on KITL, GDF9, and BMP4, a real-time PCR was performed and gave a 0.4-fold suppression in GDF9 expression, 0.5-fold suppression in BMP4 expression, and no change in KITL expression (data not shown), which are similar to the results shown in Table 1 for GDF9 and BMP4. Therefore, the real-time PCR data for these three genes confirmed the microarray data.

Discussion

The objective of the current study was to characterize the mechanisms of AMH action to inhibit the primordial to primary follicle transition. Initially, AMH interactions with growth factors that stimulate follicle transition were investigated. AMH treatment was found to inhibit endogenous and growth factor stimulated primordial to primary follicle transition in rat ovaries (Fig. 1). Observations support the inhibitory actions of AMH on primordial follicles, as previously reported (Durlinger *et al.* 1999, 2002*b*, Ikeda *et al.* 2002, Gigli *et al.* 2005, Carlsson *et al.* 2006). In contrast, one study with cultured human ovarian cortex demonstrated that AMH stimulated primordial follicle transition (Schmidt *et al.* 2005). This study is distinct from others due to conflict with the findings of Carlsson *et al.* (2006), who found that primordial follicle transition was inhibited in human ovarian cortex treated with AMH. When organ cultures have a dramatic change in follicle numbers during culture, the actions of agents such as AMH may influence follicle viability, independent of developmental effects. Therefore, culture systems where follicle number does not change, such as that used in the current study, are more suited for developmental studies to assess the actions of follicle development. The majority of studies designed in this manner have demonstrated that AMH suppresses primordial follicle development, including the current

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observations. The specific AMH site of action on the primordial follicle remains to be elucidated. The source of AMH will be the granulosa cells of larger developing follicles, such that AMH mediates interactions between follicles and provides a mechanism by which larger follicles may influence (i.e. suppress) primordial follicle development.

The AMHR is localized to granulosa cells of secondary and antral follicles, but localization in early primordial stage follicles is uncertain. Although the AMHR is expressed in ovaries containing only early developmental stage follicles (Kezele *et al.* 2005*b*), the localization is unknown (Durlinger *et al.* 2002*a*). Therefore, the specific AMH site of action on the primordial follicle remains to be elucidated. This is a limitation of the current study with regards to making conclusions about specific cell–cell interactions and sites of action. In contrast, the source of AMH is known to be the granulosa cell of larger developing follicles. Therefore, AMH mediates interactions between follicles and provides a mechanism for larger follicles to influence (i.e. suppress) primordial follicle development.

How AMH interacts functionally with stimulatory growth factors was characterized using organ culture experiments in which ovaries were treated with AMH in combination with bFGF, KITL, or KGF. It was found that AMH inhibits the stimulatory actions of all these growth factors. Observations suggest that there are multiple sites of action that AMH must utilize to inhibit primordial to primary follicle transition. AMH needs to influence the oocyte to inhibit KITL actions and influence the granulosa cell to inhibit bFGF actions. AMH acts on the precursor theca cells to inhibit KGF action. The ability of AMH to suppress KITL, GDF9, and BMP15 expression suggests a potential action on the oocyte.

The ability of AMH to suppress FGFR1 expression suggests a potential action on the granulosa cells. The ability of AMH to suppress BMP4 expression suggests a potential action on precursor theca cells. Further studies are needed to determine the direct and indirect actions of AMH on the various cells of the developing primordial follicle. However, all the cell types appear to either directly or indirectly respond to AMH.

The mechanisms by which AMH inhibits primordial to primary follicle transition were examined with a microarray analysis of the mRNA transcripts expressed in AMH-treated ovaries compared with untreated control ovaries. Ovaries from 4-day-old rats were cultured for 2 days in the absence or presence of AMH treatment and then RNA was isolated. Forty-eight hours of AMH treatment was judged to be long enough for some downstream effector genes to alter expression. After 48 h of treatment, negligible morphological differences are observed between treated and untreated ovaries (Nilsson *et al.* 2006), so the composition of the follicles and the proportion of the various cell types in the ovary remain constant between treatment groups. Therefore, the changes in mRNA transcript levels seen in treated ovaries are due to changes in gene expression rather than changes in cell proliferation and altered populations of the different cell types that occur in the 10-day cultures.

An examination of the list of genes that are regulated after AMH treatment identified a number of extracellular growth and regulatory factors (Table 1). Some of these factors are known to be involved in follicle development. GDF9 and BMP15 are oocyte-derived growth factors that are known to stimulate early follicle development after the primary stage

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(McGrath *et al.* 1995, Dong *et al.* 1996, Dube *et al.* 1998, Laitinen *et al.* 1998, Hayashi *et al.* 1999, Otsuka *et al.* 2000, Vitt *et al.* 2000, Vitt & Hsueh 2001, Findlay *et al.* 2002, Monget *et al.* 2002, Nilsson & Skinner 2002, Otsuka & Shimasaki 2002, Wu & Matzuk 2002). The activin receptor (ACTR1) has been shown to be present in early stage oocytes and influence later stages of follicle development (Drummond *et al.* 2002). BMP4 is known to stimulate primordial to primary follicle transition (Nilsson & Skinner 2003). The c-kit receptor tyrosine kinase has KITL as a ligand, and KITL stimulates primordial to primary follicle transition (Huang *et al.* 1993, Packer *et al.* 1994, Parrott & Skinner 1999, Nilsson & Skinner 2004, Hutt *et al.* 2006). Interestingly, KITL mRNA levels were themselves not regulated by AMH. Both microarray and real-time PCR data confirmed the reduction in BMP4 and GDF9 expression, and a lack of effect on KITL expression. For each of the above regulated growth factors or receptors, treatment with AMH decreased mRNA transcript expression, consistent with the action of AMH to inhibit primordial to primary follicle transition.

Other extracellular signaling factors that changed with AMH treatment have previously been identified in ovaries, but have not been shown to be involved in follicle transition. These include Bambi (Loveland *et al.* 2003), Fstl (Herrera *et al.* 2005), Mif (Suzuki *et al.* 1996, Wada *et al.* 1997, 1999, Bove *et al.* 2000, Ostrer 2000, Matsuura *et al.* 2002, Saitoh 2003), TGFb2 (Nilsson *et al.* 2001*b*, Gueripel *et al.* 2004), Itga6 (Terpe *et al.* 1994, Frojdman & Pelliniemi 1995, Fujiwara *et al.* 1995, 1996, Giebel *et al.* 1996, Zuccotti *et al.* 1998, Burns *et al.* 2002, Le Bellego *et al.* 2005), and VEGF (Lam & Haines 2005, Fraser 2006). These are now interesting candidate genes and experiments will be needed to determine whether these factors have any role in primordial to primary follicle transition.

From previous information on the candidate regulatory factors described earlier, it is possible to speculate about their potential roles in primordial follicle transition. Bambi is an inhibitor of BMPs (Loveland *et al.* 2003), and BMP4 and BMP7 have been implicated in regulating primordial to primary follicle transition (Shimasaki *et al.* 1999, Lee *et al.* 2001, Nilsson & Skinner 2003). Agpt2 is a negative regulator of Agpt1 (Lindell *et al.* 2001), and angiogenesis is important for follicular development. Similarly, VEGF is a modulator of angiogenesis (Lam & Haines 2005, Fraser 2006). The traditional view of VEGF as a stimulator of vascular development would seem to be at odds with VEGF increasing when follicle development is inhibited. However, the *VEGF* gene undergoes alternative splicing and the VEGF 165b isoform has been shown to inhibit angiogenesis (Bates *et al.* 2002, Woolard *et al.* 2004). It is possible that the increased VEGF message expression seen after AMH treatment may be an increase in this inhibitory isoform. Alternatively, VEGF is known to have signaling roles beyond that of promoting vascular development (Lam & Haines 2005). Of interest are those regulatory factors that have increased mRNA expression in the presence of AMH, which include Cktsf1b1, VEGF, Agpt2, and Itga6. These gene products may act with AMH to inhibit follicle transition. Since AMH treatment alone does not completely inhibit primordial to primary follicle transition (Fig. 1), other factors or signals such as Sdf1 (Holt *et al.* 2006) are also likely to be necessary to maintain the arrested primordial follicle pool.

The expression microarray study was performed with mRNA extracted from whole ovaries. Therefore, it is not possible to know what cell type (oocyte, granulosa cell, stromal/

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interstitial cell) is showing a change in expression for any particular gene after AMH treatment. Further studies are necessary to determine which genes are expressed in which cell types. A mixture of arrested and developing follicles exists in the cultured ovaries of these experiments. The magnitude of the AMH-induced gene expression changes will be due to the actions of a combination of these follicle stages. A limitation of the current study is that multiple cell types and follicle stages exist in the analysis.

Several gene transcripts that are part of the TGF-β signaling pathways were found to change mRNA expression levels in AMH-treated ovaries (Fig. 5). These included a decrease in TGF-β expression and an increase in decorin expression, an inhibitor of TGF-β actions (Droguett *et al.* 2006). This combined decrease in TGF-β expression and increase in decorin expression would result in a negative regulation of TGF-β signaling. BMP levels are also decreased in response to AMH treatment. Therefore, two different ligand inputs into TGF-β signaling pathways are decreased. This is coupled with a decrease in some Smad proteins, which are intra-cellular transducers and regulators of TGF-β signaling (Massague *et al.* 2005). While some transcripts are increased, most notably the receptors BMPR2, Acvr1, Acvr2, and NodalR2, the overall effect of AMH exposure appears to decrease TGF-β pathway signaling into processes such as cell differentiation, angiogenesis, and cell cycle regulation. This is a mechanism that AMH may utilize to suppress the primordial to primary follicle transition. Since AMH is a TGF-β family member, the actions of AMH either bypasses this decrease in TGF-β pathway signaling or this may be part of the negative feedback responses to AMH.

Similarly, several gene transcripts that are part of the MAPK signaling pathways were found to change mRNA expression in AMH-treated ovaries (Fig. 6). These include several important mediators of MAPK signaling such as AKT, MEKKI, ERK, p53, CREB, and c-fos. Transcripts are both up- and downregulated in this pathway. Tissue- or cell type-specific effects on the processes of cell proliferation or differentiation would result in the regulation of MAPK signaling. These observations are consistent with AMH regulating the primordial to primary follicle transition through alterations in the MAPK pathway.

In summary, the current study has described some potential mechanisms by which AMH inhibits the primordial to primary follicle transition. AMH was found to interact with different growth factors that stimulate follicle transition, suggesting that AMH acts on the signaling pathways which promote follicle transition. Analysis of mRNA expression revealed that TGF-β signaling pathways were downregulated with AMH treatment, which could lead to decreased cell differentiation and decreased angiogenesis, as well as an influence on the cell cycle. The microarray analysis also revealed some previously unrecognized extracellular signaling factors that may regulate primordial to primary follicle transition. Further investigation of these candidate regulatory factors will result in a better understanding of the mechanisms that regulate primordial to primary follicle transition. Observations may lead to new therapeutic targets to slow the loss of reproductive function in women, control menopausal transition, or treat some forms of infertility, such as premature ovarian failure.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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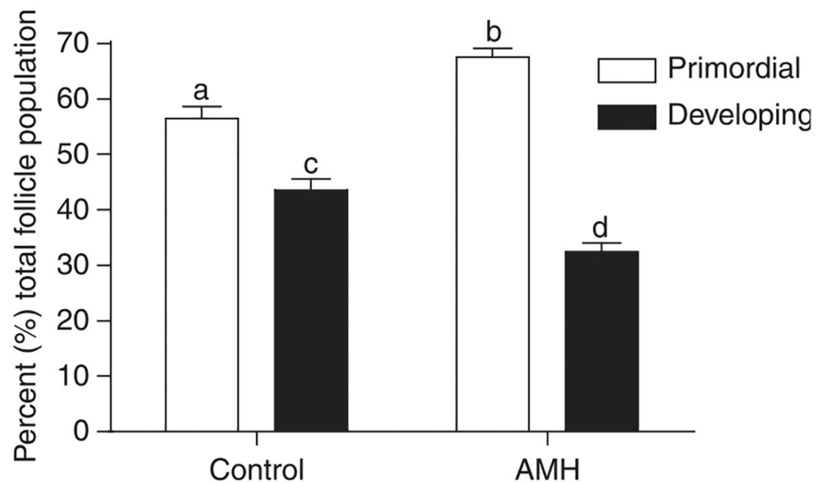

Figure 1.

Effect of AMH treatment on primordial to primary follicle transition in cultured ovaries. Ovaries from 4-day-old rats were placed into culture for 10 days. Cultured ovaries were treated with 50 ng/ml AMH or were left untreated as controls. After culture, all ovaries were fixed, stained, and subjected to morphological analysis. The follicles per ovary cross-section were categorized as being either primordial or developing (which includes all follicles having undergone the primordial to primary transition). Data are presented as the mean± S.E.M. with data pooled from five separate experiments, n=9–15 per group. Different superscript letters indicate a significant (P<0.01) difference between AMH treated and control by Mann–Whitney test.

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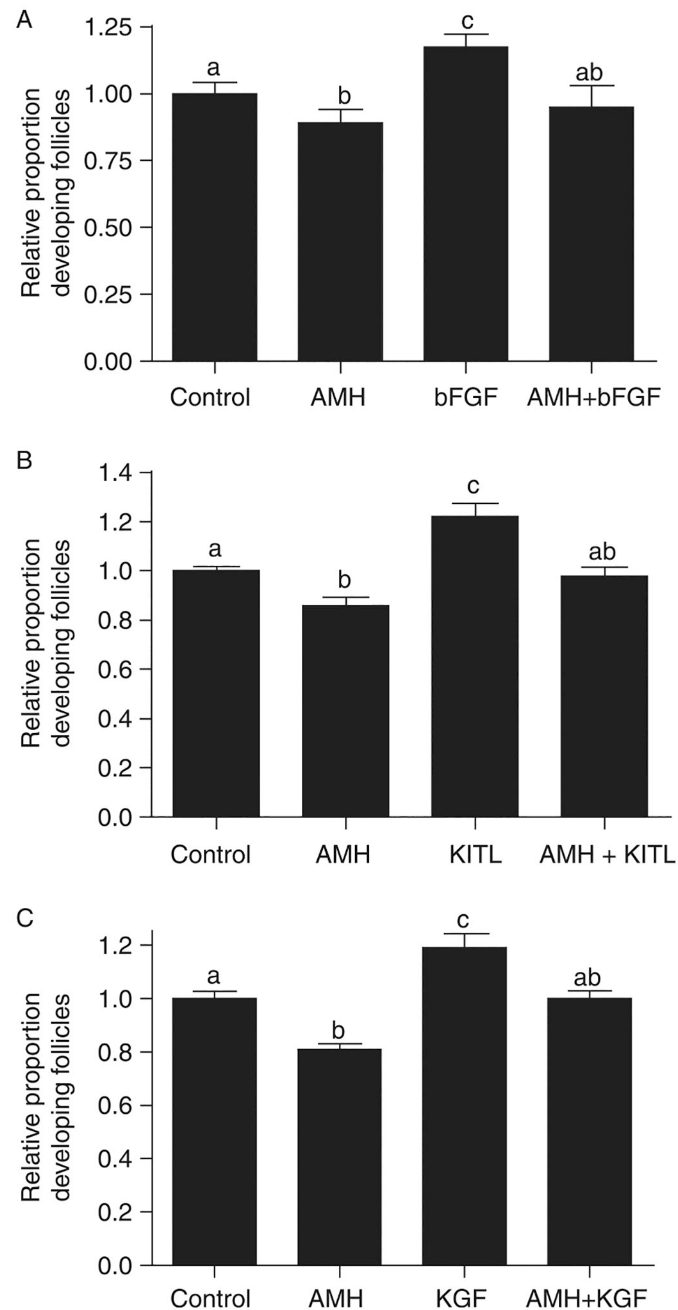

Figure 2.

Effect of AMH treatment in combination with stimulatory growth factors on primordial to primary follicle transition in cultured ovaries. Cultured ovaries were treated for 10 days with AMH alone or in combination with bFGF, KITL, or KGF, or were left untreated as controls (control). After culture, all ovaries were fixed, stained, and subjected to morphological analysis. The follicles per ovary cross-section were categorized as being either primordial or developing. Data are presented as the mean (± S.E.M.) proportion of developing follicles normalized to each experimental control mean. Data were pooled from three or more separate experiments. One-way ANOVA showed a significant (P<0.01) difference in treated

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ovaries. Different superscript letters indicate a significant ($P<0.05$) difference by *post hoc* Tukey's test. (A) AMH treatment in combination with bFGF, n=5–19 per group.(B) AMH treatment in combination with KITL, n=6–19 per group.(C) AMH treatment in combination with KGF, n=8–19 per group.

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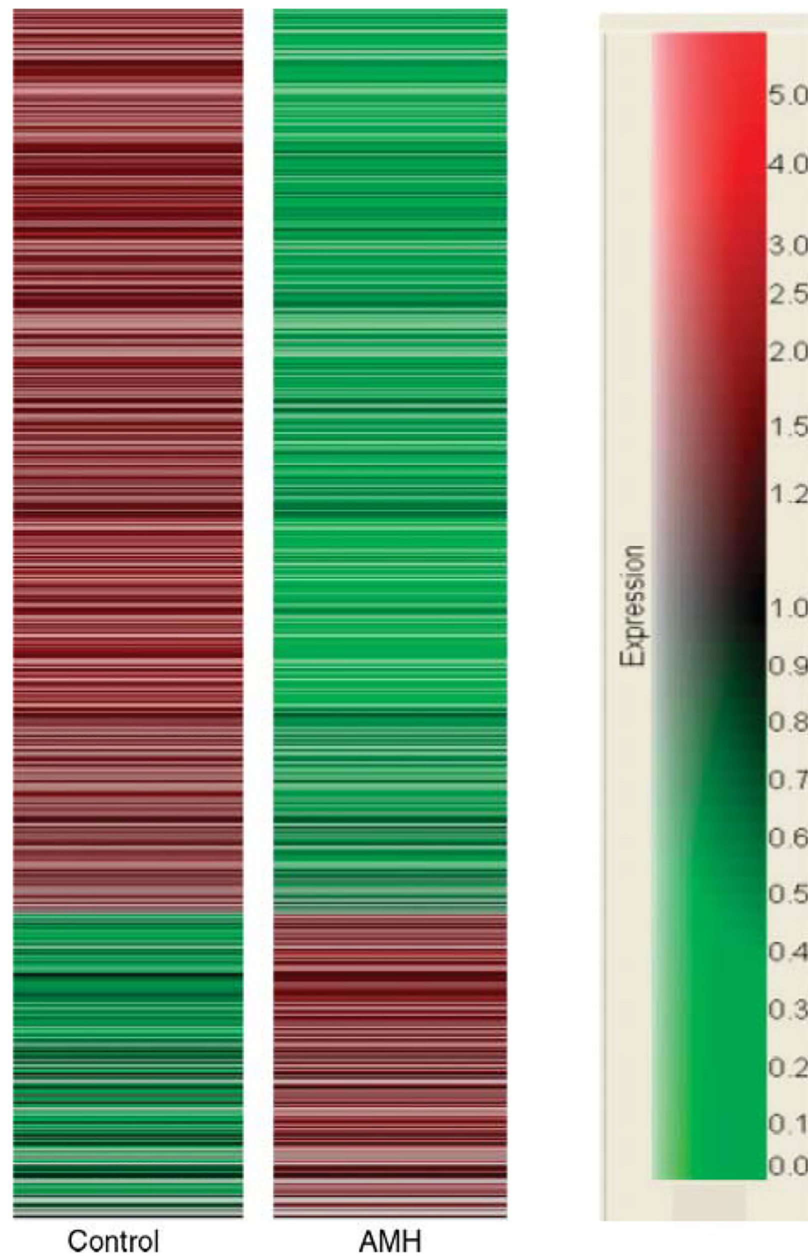

Figure 3.

Dendrogram of microarray analysis results showing transcripts that change expression level in AMH-treated versus control ovaries. Seven hundred and seven genes show an expression change as per the criteria described in Materials and Methods between AMH-treated and control ovaries. Compared with controls, 164 genes are increased and 543 genes are decreased in AMH-treated ovaries. Red, increase in expression levels; green, decrease in expression levels. Relative expression changes are according to the scale at right.

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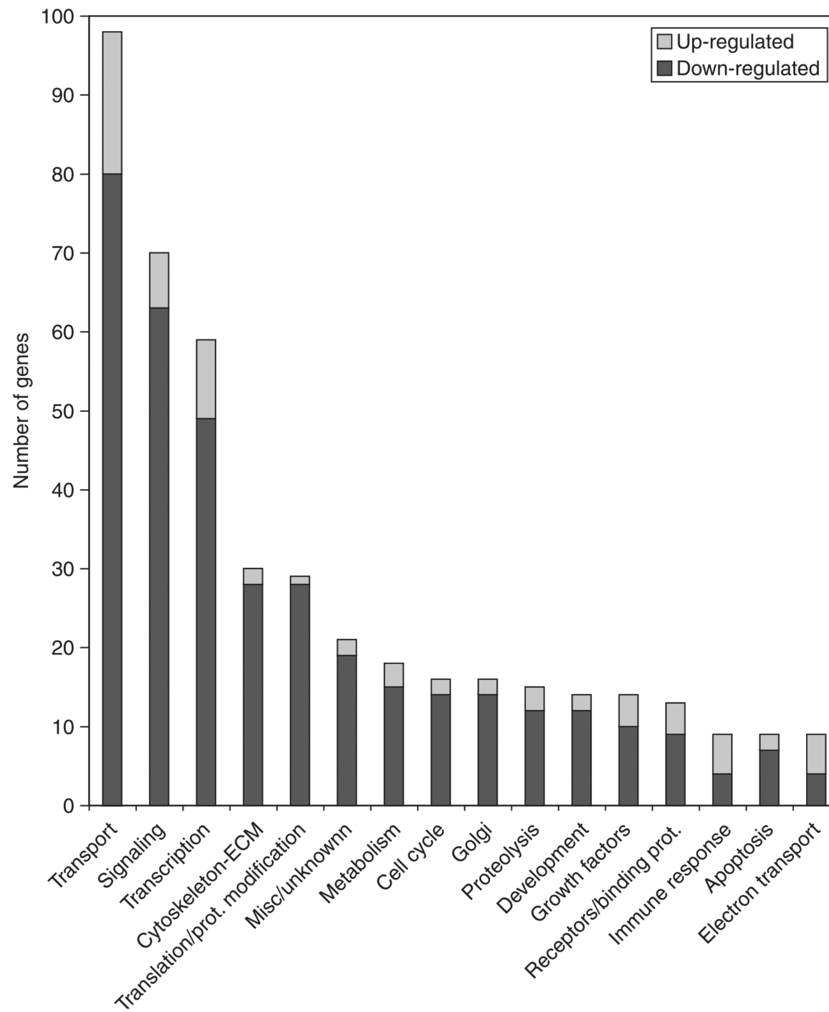

Figure 4.

Gene transcripts that change expression level in AMH-treated versus control ovaries categorized according to the physiological function. Lighter portion of bar represents a number of transcripts upregulated in AMH-treated ovaries, while darker portion of bar represents transcripts downregulated.

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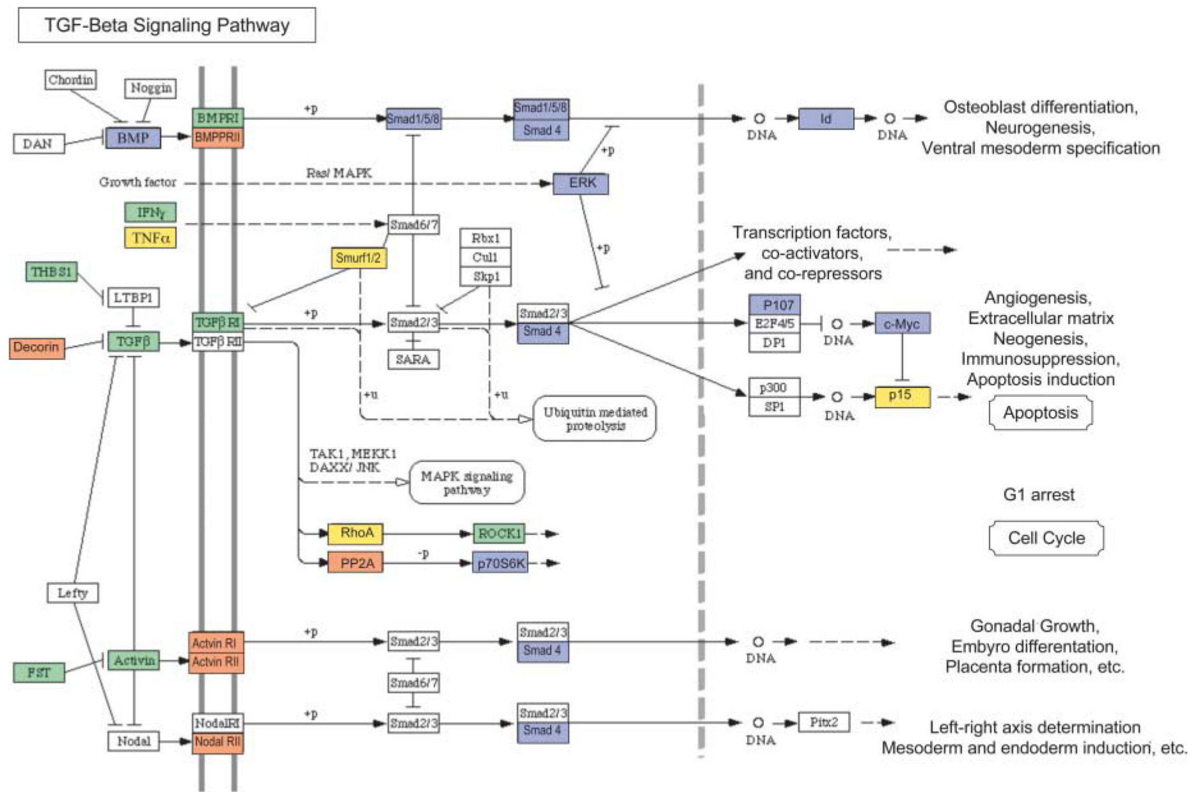

Figure 5.

Illustration of TGF-β signaling pathways proposed to be involved in regulating follicle transition using microarray data. Genes that are upregulated by AMH treatment (1.5-fold change) are shown in orange. Genes that are downregulated (1.5-fold change) are shown in blue. Genes that are not changing in expression level are shown in green. Genes that are not expressed at levels above a raw score of 75 are given in yellow. Genes that are not represented on the Affymetrix RAE 230 2.0 chip are shown in white. Pathways are adapted from KEGG as accessed through Genespring GX 7.3 Expression analysis (Agilent Technologies, Palo Alto, CA, USA).

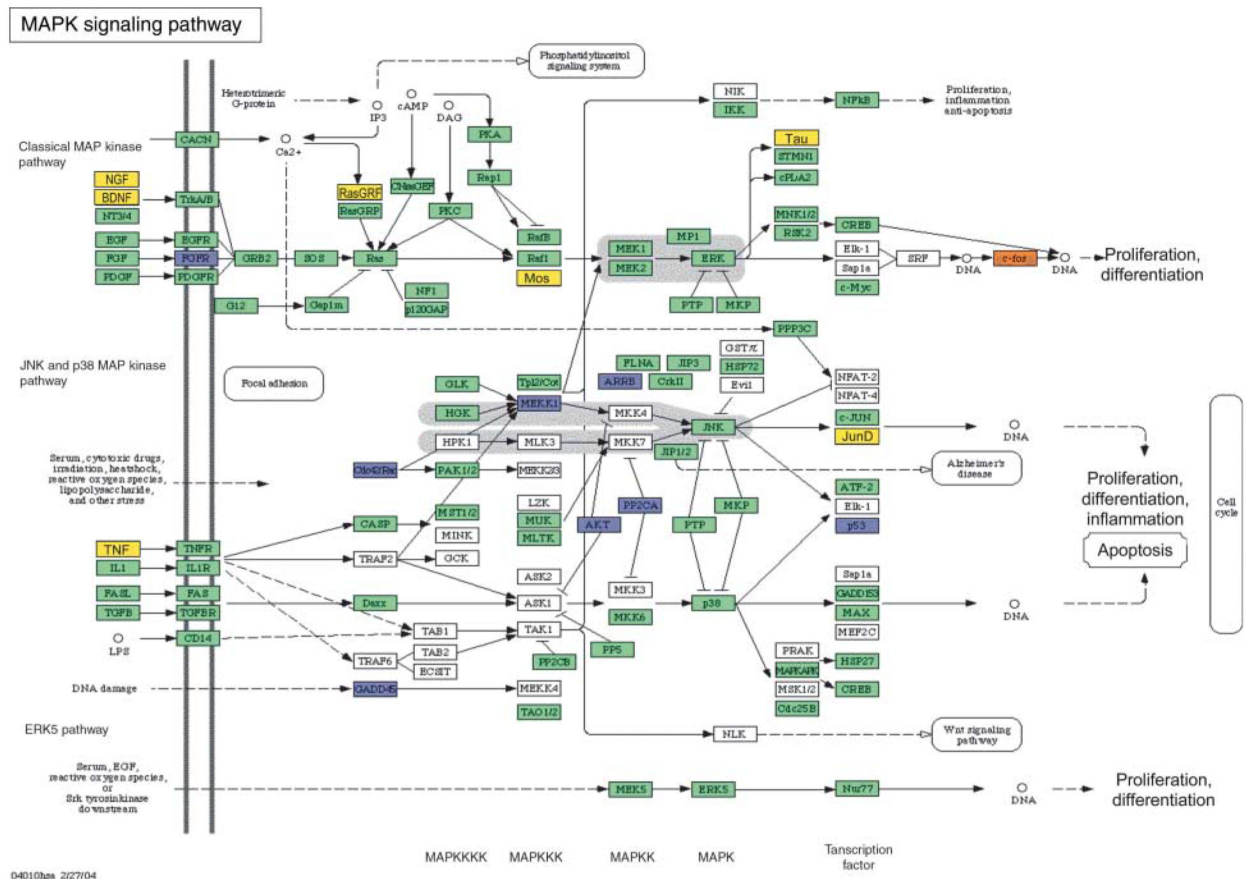

Figure 6.
Illustration of MAPK signaling pathways proposed to be involved in regulating follicle transition using microarray data. Genes that are upregulated by AMH treatment (1.5-fold change) are shown in orange. Genes that are downregulated (1.5-fold change) are shown in blue. Genes that are not changing in expression level are shown in green. Genes that are not expressed at levels above a raw score of 75 are given in yellow. Genes that are not represented on the Affymetrix RAE 230 2.0 chip are shown in white. Pathways are adapted from KEGG as accessed through Genespring GX 7.3 Expression analysis (Agilent Technologies, Palo Alto, CA, USA).

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Table 1

Candidate regulatory factors for primordial follicle development.

Control	MIS	Common name	Genbank no.	Description
Microarray signal				
144 ± 15	68 ± 18	Acvr1	BM389711	Activin type I receptor
64 ± 19	**181 ± 23**	**Agpt2**	**BI275292**	**Angiopoietin 2**
972 ± 110	356 ± 129	Bambi	AF387513	BMP and activin membrane-bound inhibitor
125 ± 17	51 ± 0.1	Bmp4	NM_012827	Bone morphogenetic protein 4
131 ± 16	22 ± 14	Bmp15	NM_021670	Bone morphogenetic protein 15
250 ± 56	100 ± 35	Ccl7-like	BF419899	Similar to small inducible cytokine A7 precursor
201 ± 45	41 ± 21	Ccl21b-like	BI282920	Similar to chemokine ligand 21b
7 ± 7	**48 ± 13**	**Cktsf1b1/Grem1**	**NM_019282**	**Cysteine knot superfamily 1, BMP antagonist 1, Grem 1**
241 ± 19	68 ± 23	Fgfr1	S54008	Fibroblast growth factor receptor 1
370 ± 38	76 ± 35	Fstl	NM_024369	Follistatin-like
46 ± 12	**119 ± 14**	**GDF1-like**	**BI289525**	**Similar to GDF1 embryonic growth factor**
1729 ± 144	386 ± 181	Gdf9	NM_021672	Growth differentiation factor 9
50 ± 11	**115 ± 20**	**Itga6**	**AI137931**	**Integrin, α6**
718 ± 2	556 ± 30	Kit	AI454052	c-Kit receptor tyrosine kinase
1630 ± 122	153 ± 153	Mif	NM_031051	Macrophage migration inhibitory factor
103 ± 11	31 ± 13	Obrgrp/Leprot	NM_020099	OB receptor gene-related protein/leptin receptor homolog
235 ± 24	42 ± 29	Scye1	AI454911	Endothelial monocyte-activating polypeptide 2
99 ± 12	48 ± 12	Sdfr2-like	AI179412	Similar to stromal cell-derived factor receptor 2
83 ± 12	15 ± 10	Tgfb2	AF135598	Transforming growth factor, β2
347 ± 86	**705 ± 99**	**Vegf**	**AI175732**	**Vascular endothelial growth factor**

Bold, upregulated transcripts.