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Expression of growth differentiation factor 9, bone morphogenetic protein 15, and anti-Müllerian hormone in cultured mouse primary follicles

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Abstract

Growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), and anti-Müllerian hormone (AMH) play an important role in the primary to secondary follicle transition and follicle activation *in vivo*. In organ culture of neonatal mouse ovaries, it was observed that significantly fewer primary follicles develop to the secondary stage. The objectives of this study were: (1) to compare ovarian follicular populations between organ-cultured neonatal mouse ovaries and freshly isolated age-matched control ovaries; (2) to quantify RNA levels of *Gdf9*, *Bmp15*, and *Amh* in cultured primary follicles; and (3) to immunolocalize GDF9 and AMH in cultured ovaries. Ovaries from 3-day-old (PND 3) mice were cultured for 7 or 10 days in the absence or presence of FSH. Follicular populations were counted in freshly isolated 13-day-old (PND 13) ovaries and organ-cultured ovaries. Transcripts were quantified in isolated primary follicles using real-time RT-PCR, and protein expressions were localized using immunohistochemistry. The number of secondary follicles in organ-cultured ovaries was significantly lower than *in vivo* controls. *Gdf9* and *Bmp15* mRNA expression levels were similar as in controls. *Amh* mRNA levels were significantly ($P<0.05$) lower after day 10 of culture in the absence of FSH. GDF9 and AMH proteins were respectively detected in the oocytes and the granulosa cells (GC) beginning at the primary and primordial stages onward. GDF9 and BMP15 production in cultured primary follicles are not different from *in vivo* controls; hence abnormal early follicular growth was not related to a deficient transcription of these factors.

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Introduction

Primordial follicles are the stock from which all growing follicles are derived. Initiation of ovarian follicular (follicle activation) growth is characterized by morphological changes in primordial follicles, which include: transformation of the flattened granulosa cells (GC) into cuboidal cells, proliferation of GC, and oocyte growth. Only about 0.1% of primordial follicles that initiate growth will ever proceed to ovulation, the vast majority of growing follicles undergoing atresia (Hsueh *et al*. 1994). The primary to secondary follicle transition seems to be a bottleneck in the culture systems of different species (Wandji *et al*. 1996, 1997, Fortune *et al*. 1998, Hovatta *et al*. 1999, Yu & Roy 1999, Devine *et al*. 2002, Hreinsson *et al*. 2002, Otala *et al*. 2002, Schmidt *et al*. 2005, Sadeu *et al*. 2006, Yang & Fortune 2006, Sadeu & Smitz 2008) in the prospect of increasing reproductive efficiency, and hopefully to help infertile women after gonadotoxic treatment.

Over the past years, the growth of rodent (Eppig & O'Brien 1996, Parrott & Skinner 1999, Klinger & de Felici 2002, Obata *et al*. 2002, Kezele & Skinner 2003, O'Brien *et al*. 2003, Lee *et al*. 2004, Nilsson & Skinner 2004, Shen *et al*. 2006*a*, 2006*b*, 2007), cattle (Wandji *et al*. 1996, Braw-Tal & Yossefi 1997, Fortune *et al*. 1998), and primate (Wandji *et al*. 1997), including human (Hovatta *et al*. 1999, Wright *et al*. 1999, Hreinsson *et al*. 2002, Sadeu *et al*. 2006) primordial follicles has been investigated *in vitro*. In these ovarian culture experiments, primordial follicles within newborn or neonatal mouse ovaries, ovarian cortex of fetal or adult bovine ovaries as well as fetal baboon ovaries, or fetal or adult human ovaries were able to initiate growth and to develop to primary or secondary follicles. But generally, only a few growing primary follicles progressed to the secondary stage in cattle, primates, and humans. Meiotically competent oocytes have been mainly obtained after isolation and *in vitro* growth of mouse primary or secondary follicles as single functional units (Spears *et al*. 1994, Cortvrindt *et al*. 1996, Eppig & O'Brien 1996, O'Brien *et al*. 2003, Lenie *et al*. 2004, Kreeger *et al*. 2005, Shen *et al*. 2006*b*, 2007). Regarding the production of mature oocytes *in vitro*, there is scarce proof of concept in the non-rodent species (Hirao *et al*. 1994).

It is known that the primary to secondary transition is induced by an autocrine/paracrine regulatory process that involves growth factors produced by the oocyte and GC. Growth differentiation factor-9 (GDF9) and bone

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morphogenetic protein 15 (BMP15) are oocyte-specific proteins secreted by growing oocytes in rodents, sheep, and humans (McGrath *et al*. 1995, Dube *et al*. 1998, Fitzpatrick *et al*. 1998, Aaltonen *et al*. 1999, Bodensteiner *et al*. 1999, Otsuka *et al*. 2000). They are known to control folliculogenesis by acting on GC in developing follicles. Studies in genetic mutations have elucidated the role of these proteins in regulating the primary to secondary follicle transition. Mutations in GDF9 (Dong *et al*. 1996) and BMP15 (Galloway *et al*. 2000, Hanrahan *et al*. 2004) result in growth arrest at the primary stage. Furthermore, consistent with its role in controlling follicular growth, GDF9 has been shown to enhance the development and growth of human (Hreinsson *et al*. 2002), and rodent (Hayashi *et al*. 1999, Nilsson & Skinner 2002) early-stage follicles when added to culture media.

BMP15 and GDF9 play a synergistic role in folliculogenesis. Mice lacking both copies of the *Bmp15* gene and one copy of the *Gdf9* gene show increased oocyte loss and decreased late-stage follicles, whereas those lacking both copies of *Gdf9* and *Bmp15* genes show follicle growth arrests at the primary stage similar to the *Gdf9* knockout mice (Yan *et al*. 2001).

Anti-Müllerian hormone (AMH) is expressed postnatally by the GC of developing follicles from the early primary stage (oocyte surrounded by a mixture of flattened and cuboidal GC) to the early antral stage (Durlinger *et al*. 2002*a*, 2002*b*). Increased levels of AMH secreted by growing follicles have been associated with the decreased activation of the pool of primordial follicles (Behringer *et al*. 1990). It is proposed that AMH inhibits the primordial to primary transition. In addition, a homozygous mouse knockout model for AMH shows an early depletion of the primordial follicle pool that is consistent with the role of AMH in regulating the activation of primordial follicles (Durlinger *et al*. 1999).

GDF9, BMP15, and AMH are major regulators for the growth and function of ovarian follicles, and it was questioned whether in the *in vitro* culture situation the actions of these factors might be altered in comparison with their observed roles *in vivo*, where they decrease the number of secondary follicles. Therefore, to investigate whether the decrease in the number of growing follicles was related to any abnormal level of expression of mRNAs for GDF9, BMP15, and AMH, primary follicles were isolated from cultured intact neonatal mouse ovaries and used to address the question. The aims of the study were: (1) to compare the ovarian follicular population between cultured neonatal mouse ovaries and freshly isolated age-matched ovaries; (2) to quantify mRNA of key growth factors in activated follicles in relation to the stage they have reached *in vitro*; and (3) to immunolocalize GDF9 and AMH in cultured ovaries.

Results

Follicular development in vivo *and* in vitro

Gross morphological examination of ovaries cultured in the presence of follicle-stimulating hormone (FSH) showed similar stages of follicle development compared with those cultured without FSH. In that respect, the ovaries cultured for 10 days in the absence of FSH were used to quantify ovarian follicular populations. Follicular development *in vitro* was evaluated by comparing both the developmental stages of the follicles and the number of different stages of follicles between PND 3 ovaries cultured for 10 days and freshly isolated ovaries of age-matched PND 13 mice (Table 1).

Before culture, the ovaries of PND 3 mice mainly contained primordial follicles (Fig. 1A and B). But after 10 days of culture, there was also the appearance of early and small secondary follicles (Fig. 1C and D) besides primordial and primary follicles. Ovarian and follicular morphologies in the histological sections demonstrated the viability of the organ after culture (Fig. 1D). The growing follicles were mostly concentrated in the central part of the cultured ovary. In *in vivo* ovaries, they were uniformly distributed (Fig. 1F). The mean number of healthy primordial, primary, and early secondary follicles was not statistically ($P>0.05$) different between PND 3 ovaries cultured for 10 days and control PND 13 ovaries (*in vivo*). However, there were significantly fewer small secondary follicles in cultured ovaries, compared with *in vivo* ovaries (Table 1). By normalizing to percentages, the number of small secondary follicles in the cultured ovary was about 4.8±1.5% of that of the *in vivo* ovary. Furthermore, in contrast to the cultured ovary, a more advanced stage of follicle development (medium secondary follicle) was found in the *in vivo* ovary. (Fig. 1E and F). There was no statistical difference in the number of atretic follicles between *in vivo* and *in vitro* cultured ovaries (Table 1).

Table 1 Early follicular development in PND 3 mouse ovaries after 10 days of culture, compared with 13 days *in vivo* (PND 13 ovaries).

	Follicle stage (mean±S.E.M.)					
Ovaries (*n*)	Primordial	Primary	Early secondary	Small secondary	Medium secondary	Atretic
In vivo (5)	5082.0±887.8	103.0±19.3	28.4±3.8	73.0±5.6[a]	35.8±6.6[a]	65.2±10.7
Culture (13)	4227.7±607.7	169.2±22.5	21.0±3.3	3.5±1.1[b]	0.0±0.0[b]	86.2±18.7

Values with different superscript letters within the same column are significantly different ($P<0.0001$).

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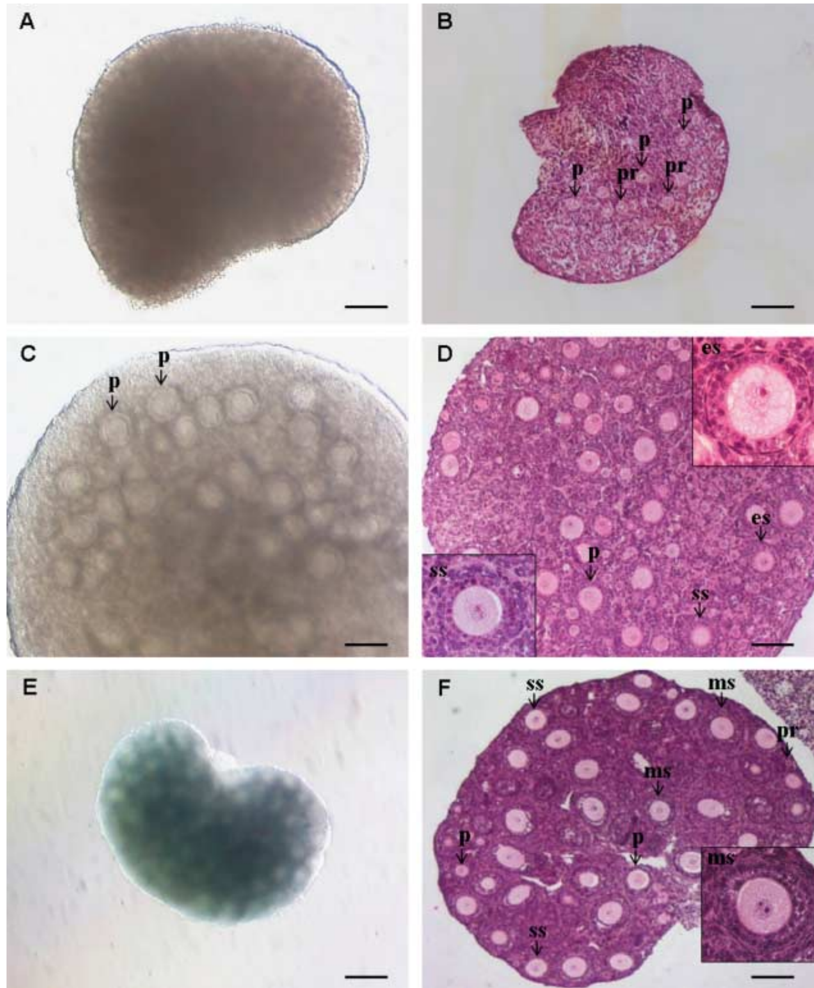

Figure 1 Photographs of freshly isolated as well as cultured mouse ovaries and the corresponding representative ovarian sections. (A and B) Freshly isolated PND 3 mouse ovary with many primordial (pr) and a few primary (p) follicles. (C and D) PND 3 mouse ovary after culture *in vitro* for 10 days with many primary (p), early secondary (es + right inset), and a few small secondary (ss + inset) follicles. (D) Growing follicles are mostly found in the central part of the ovary. (E and F) Freshly isolated PND 13 mouse ovary with many primary (p), early secondary (es), small secondary (ss), and medium secondary (ms + inset) follicles. (F) Most small secondary follicles are found at the periphery of the ovary, and the medium secondary follicles at the center. Scale bars represent: (A, B, E, and F) 100 µm and (C and D) 40 µm.

Expressions of Gdf9*,* Bmp15*, and* Amh *mRNAs in mouse follicles*

To investigate whether the decrease in the number of growing follicles was reflected in the expression levels of growth-related factors, oocyte-specific markers (GDF9 and BMP15) and GC marker (AMH) were examined using real-time PCR. The gene expression analysis was performed on RNA from 13 groups of primary follicles (88–100 µm; $n=46$) from freshly isolated PND 13 ovaries (controls) and 38 groups of primary follicles (50–95 µm; $n=16$ (D7); $n=14$ (FSH D7); $n=59$ (D10); $n=50$ (FSH D10)) (Fig. 2) from cultured PND 3 ovaries. *Gdf9* mRNA expression was present in all samples analyzed. In the cultured follicles, a trend towards an increase ($P=0.09$) in the mRNA expression level from D7 to D10 was found for *Gdf9* and *Bmp15* (Fig. 3). There was no difference in mRNA expression level of both oocyte-specific markers (*Gdf9* and *Bmp15*) at D10 for follicles cultured in the absence or presence of FSH, compared with the *in vivo* controls (Fig. 3).

Quantitative analysis revealed that *Amh* mRNA expression was present in all samples analyzed. The mRNA expression level increased from D7 to D10 but did not change between the different culture conditions within D7 or D10 (Fig. 3). In the follicles cultured for 7 and 10 days in the absence of FSH, a five- and two-fold significantly ($P<0.05$) lower *Amh* mRNA expression was found respectively, compared with the *in vivo* controls.

Figure 2 Morphological characteristics of *in vivo* and *in vitro* grown mouse primary follicles. (A) Primary follicles freshly isolated from PND 13 ovaries. (B) Primary follicles isolated from PND 3 ovaries cultured *in vitro* for 10 days. Bars = 20 µm.

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Localization of GDF9 protein in cultured mouse ovaries

The ovaries cultured in the absence of FSH for 10 days were further used for immunohistochemical analysis. A weak to strong immunostaining for GDF9 was detected in the oocytes. The immunostaining was weak in primary follicles (Fig. 4A and C) and absent in primordial (pr) follicles (Fig. 4E) and some primary follicles (Fig. 4C; Table 2). No staining was found in the negative control sections (Fig. 4G).

Localization of AMH protein in cultured mouse ovaries

AMH protein expression was detected in cuboidal GC. The immunoreactivity was absent in primordial follicles surrounded with flattened GC (Fig. 4F; Table 2) but present in cuboidal GC of primordial follicles

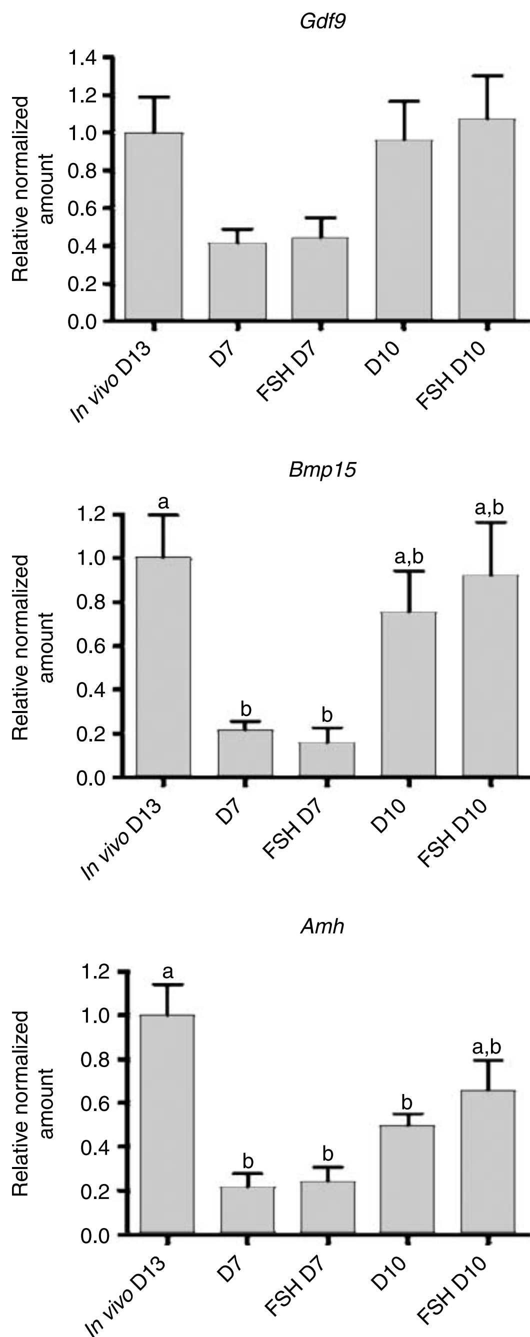

Figure 3 Expression levels of *Gdf9*, *Bmp15*, and *Amh* mRNAs during mouse primary follicle growth *in vivo* and *in vitro*. *Gdf9*, *Bmp15*, and *Amh* mRNAs were measured in freshly isolated primary follicles (*in vivo* D13) and in primary follicles isolated after 7 (D7) and 10 days (D10) of ovary organ culture in the absence (D7 and D10) or presence (FSH D7 and FSH D10) of FSH. Normalized values are represented with the average expression *in vivo* (*In vivo* D13) as a calibrator. Values are means ± s.e.m. $n=13$ (*in vivo*), 6 (D7), 5 (FSH D7), 13 (D10), and 14 (FSH D10). Bars with different letters are significantly different ($P<0.05$).

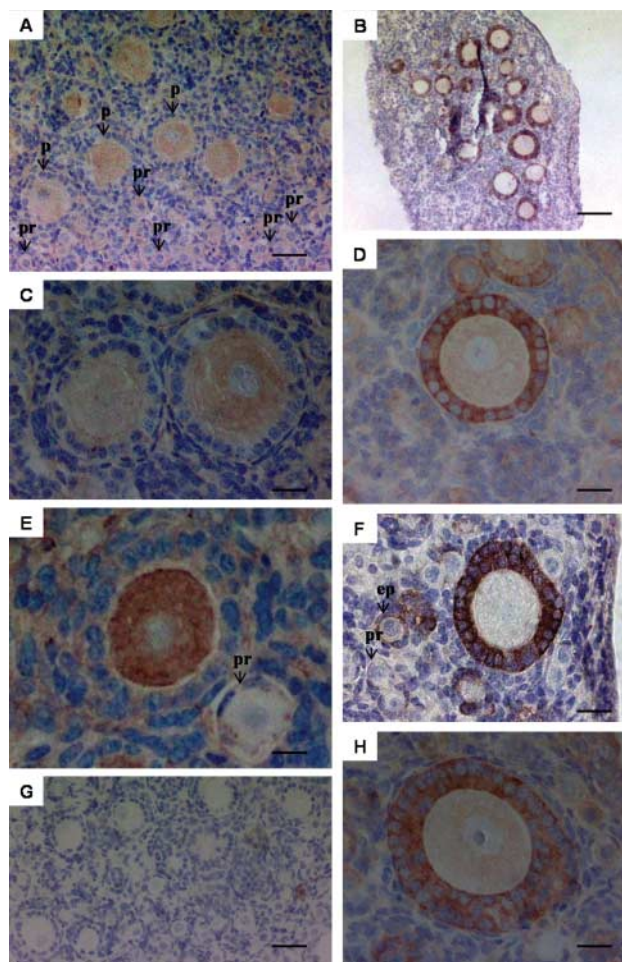

Figure 4 Representative tissue sections of cultured mouse ovaries showing an absence of GDF9 expression in (A and E) primordial (pr) follicles and positive expression in (A and C) primary (p) and (E) early secondary follicles. Note the (C) weak and (E) strong intensity of the staining in oocyte. AMH expression is absent in (F) primordial (pr) follicles with one layer of flattened GC, while present in (F) primordial (ep) follicles with a mixture of flattened and cuboidal GC, (B and D) primary, (F) early secondary, and (H) small secondary follicles. (G) Negative control. Scale bars represent: (A and G) 40 μm, (B) 100 μm, and (C–F, and H) 10 μm.

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Table 2 Expression of growth differentiation factor 9 (GDF9) and anti-Müllerian hormone (AMH) proteins in PND 3 mouse ovary after 10 days of culture.

	GDF9 immunopositive follicles/counted follicles		AMH immunopositive follicles/counted follicles	
Follicle stage	*n*	(%)	*n*	(%)
Primordial (with flattened GC)	0/351	0.0	0/1173	0.0
Primary	27/32	84.4	64/64	100
Early secondary	4/4	100	26/26	100
Secondary	1/1	100	7/7	100

N=5 ovaries, one from each animal; 4–5 (AMH) and 2 (GDF9) sections for each ovary were analyzed.

surrounded with a mixture of flattened and cuboidal GC (Fig. 4F). AMH immunostaining was present in GC in all the primary, early secondary, and secondary follicles examined (Fig. 4B, D, F, and H; Table 2).

Discussion

The present study showed that the transition of the first wave of developing follicles from the primary to secondary stage was interrupted in mouse ovary organ cultures. It also showed that *Gdf9* and *Bmp15*, but not always *Amh*, mRNA levels in primary follicles after culture were similar to control levels in age-matched follicles from *in vivo* ovaries. In addition, it was found that GDF9 protein was restricted to the oocyte from primary follicle onward and AMH to the cuboidal GC from primordial follicles onward as previously described (McGrath *et al*. 1995, Durlinger *et al*. 2002*a*, 2002*b*, Weenen *et al*. 2004, Modi *et al*. 2006). Taken together, the present findings indicate that the block in primary follicle growth into secondary follicles *in vitro* might not result from a deficient transcription of GDF9 and/or BMP15.

Because communications between oocytes and follicular cells all contribute to follicular development, any alterations in growth-supporting factors may disrupt these processes. In this study, the primordial to primary transition *in vitro* proceeded similar to the *in vivo* situation. However, the deficiency in secondary follicle development *in vitro* might suggest an altered hormone processing, a lack of some stage-specific growth factors in the *in vitro* environment, or an alteration of response of GC to oocyte-secreted factors. Given that the null mutation of the *Gdf9* and *Bmp15* genes (Dong *et al*. 1996, Galloway *et al*. 2000, Hanrahan *et al*. 2004) results in growth arrest at the primary stage *in vivo*, it can be postulated that low expression of *Gdf9* and/or *Bmp15* transcripts *in vitro* may contribute to the decrease in the number of small and medium secondary stage follicles observed in ovarian culture experiments. However, there is no evidence for or against this idea in the literature.

Freshly isolated ovaries at PND 3 contained mostly primordial follicles. A low number of these follicles activated *in vitro* during whole-ovary organ culture, similar to previously described observations (Eppig & O'Brien 1996). No significant change in the rate of development of primary and early secondary follicles was found between *in vivo* control and cultured ovaries although there was a trend towards increased primary follicle development *in vitro*. Follicle development *in vivo* was different from *in vitro* conditions in that the number of small secondary follicles was significantly increased *in vivo*. In addition, the development of medium secondary follicles did not occur *in vitro*. These findings show that most of the primordial follicles that initiated growth *in vitro* underwent developmental arrest at the primary stage. The number of atretic follicles was comparable between the *in vivo* and *in vitro* conditions, suggesting that atresia was not affected by the conditions of culture. Similar to the results with rodents, human, bovine, and baboon ovarian cortical tissue cultures showed that only a few growing primary follicles progress to the secondary stage (Wandji *et al*. 1996, 1997, Braw-Tal & Yossefi 1997, Fortune *et al*. 1998, Hovatta *et al*. 1999, Wright *et al*. 1999, Hreinsson *et al*. 2002, Sadeu *et al*. 2006).

Gene expression in ovarian organ culture experiments is regularly performed with mRNA extracted from whole ovaries. The limitation in such a methodological approach is that different follicle stages exist in the cultured ovaries and therefore it is impossible to indicate which follicle stage is showing a change in expression of any particular gene. In that respect, in contrast to other studies, the present analysis on mRNA expression in cultured ovaries was performed with RNA from a well-defined follicle class. After 10 days of culture of PND 3 mouse ovaries, the *Gdf9* and *Bmp15* mRNA levels in primary follicles were similar to the control levels in *in vivo* follicles. This observation indicates a normal expression of growth-supporting factors *in vitro*. On the basis of the data from *in vivo* studies, one would expect that normal *Gdf9* and *Bmp15* mRNA expression *in vitro* would correlate with normal primary follicle progression into the secondary stage. This was not the case in our study. Nevertheless, the decreased progression *in vitro* might still depend on GDF9 and BMP15 since these growth factors act as homo- or heterodimers. Their prohormones must first undergo proteolytic cleavage before they can form the bioactive dimers or it is possible that expressions of GDF9 and BMP15 receptors did not occur in present ovarian organ culture experiment.

Early-stage follicles contain growing oocytes and this may explain the increased *Gdf9* and *Bmp15* mRNA levels from day 7 to day 10 of culture. Interestingly, both oocyte-secreted factors showed similar mRNA expression patterns. The observation that *Gdf9* and *Bmp15* mRNA levels are slightly higher in FSH conditions might suggest that FSH could positively regulate *Gdf9* and *Bmp15*. Using

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total RNA from immature mouse ovaries, Guéripel *et al.* (2006) have observed that *Bmp15* but not *Gdf9* mRNA levels increased in the gonadotropin-treated immature mice. This was an *in vivo* study and RNA was extracted from whole ovaries.

A negative regulator of early follicular development is AMH, which has been shown to inhibit the transition from primordial to primary follicles. An increased number of developing follicles was found in mice with null mutation of the *Amh* gene (Durlinger *et al.* 1999). The *Amh* mRNA level was significantly lower after 10 days of culture without FSH. However, this decrease was not reflected in the number of growing follicles, which may suggest that AMH expression *in vitro* could be high enough to keep control over follicle recruitment. By contrast, the *Amh* mRNA level of follicles cultured in the FSH conditions was similar with the control level *in vivo*, suggesting a possible direct or indirect relationship between FSH and AMH. It was shown that FSH-stimulated preantral follicle growth *in vitro* was attenuated in the presence of AMH and, in addition, that more follicles start to grow *in vivo* under the influence of exogenous FSH in AMH-deficient mice compared with the wild types (Durlinger *et al.* 2001). Given that *Amh* mRNA levels were not significantly different between the follicles cultured in the FSH and those cultured in the no FSH conditions, direct evidence to support the role of FSH in regulating AMH expression cannot be provided.

Consistent with *Gdf9* and *Amh* mRNA expressions detected by real-time PCR, we showed by immunohistochemical analysis that GDF9 protein was first detected in oocytes of primary follicles, and AMH, in cuboidal GC of primordial (oocyte surrounded by a mixture of flattened and cuboidal GC) follicles, suggesting that these proteins were present at these stages of follicle development as well as in the more advanced stages where positive immunoreactions were further detected. This provided evidence for the maintenance of the stage-specific specialized GC and oocyte functions during follicle growth *in vitro*. These findings are in agreement with previously described observations from *in vivo* studies where *Gdf9* mRNA expression and protein were demonstrated in oocytes of primary and advanced follicle stages in mice, rats, and humans (McGrath *et al.* 1995, Aaltonen *et al.* 1999, Elvin *et al.* 1999, Hayashi *et al.* 1999, Jaatinen *et al.* 1999), and AMH in cuboidal GC of primordial and advanced follicle stages in mice and humans (Durlinger *et al.* 2002*a*, 2002*b*, Weenen *et al.* 2004).

In summary, primary follicles isolated from cultured PND 3 mouse ovaries were used to determine whether any alteration in the intrinsic expressions of GDF9, BMP15, and AMH could explain the block in follicle transition from the primary to secondary stage in ovary organ culture. It was found that no significant change in expression levels of *Gdf9* and *Bmp15* occurred in primary follicles grown *in vitro* compared with control age-matched follicles grown *in vivo*, whereas significantly lower *Amh* expression was found after *in vitro* growth in the absence of FSH. In addition, GDF9 and AMH protein expressions were detected at similar stages of follicle development as described *in vivo*. The present observations indicated that the disruption of the primary to secondary transition *in vitro* might not result from defective *Gdf9*, *Bmp15*, and/or AMH transcription. Additional investigations of other genes playing a significant role in the oocyte, GC, and theca cell interactions would provide more insight into the abnormal process of follicle development beyond the primary stage *in vitro*.

Materials and Methods

Animals

Female F1 hybrid (C57BL/6j×CBA/Ca) mice housed and bred according to national standards were used throughout the study. The Ethics Committee for laboratory animal experiments of Vrije Universiteit Brussel approved all procedures.

Postnatal 3-day-old (PND 3) mice were killed by decapitation. Ovaries were aseptically removed and collected in 1.5 ml Leibovitz-L15 medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 IU/ml penicillin. Under a light microscope, the bursa and surrounding tissues were carefully dissected away from the ovaries. The procedure was carried out at 37 °C. Unless indicated, otherwise all chemicals used were purchased from Invitrogen.

Ovary organ culture

Ovaries were cultured as described in Parrot & Skinner (1999) with some modifications. Each well of 4-well culture plates (Nunc, VWR, Leuven, Belgium) was filled with 500 μl Dulbecco's modified Eagle's medium–Ham's F-12 (1:1, v/v) supplemented with 10% FBS, 10 μg/ml insulin, 10 μg/ml transferrin, and without or with 50 mIU/ml FSH. A floating filter (0.4 μm isopore membrane filter, Millipore, Brussels, Belgium) was laid over the medium and the plates were pre-equilibrated for ∼4 h. Ovaries (2–4/well) collected at PND 3 were placed onto the floating filters, covered with drops of medium, and cultured for 7 (D7) or 10 (D10) days at 37 °C in a humidified incubator with 5% CO_2 in air. Three-day-old ovaries cultured for 10 days should be comparable with ovaries of similar age (i.e., ovaries of 13-day-old mice). Consequently, a normal number of small secondary follicles (similar to the development after 13 days *in vivo*) should be obtained. Half the volume of culture medium was replaced with fresh pre-equilibrated medium every 3 days. At the end of cultures, the ovaries were fixed in 4% formaldehyde (Sigma) for morphological or immunohistochemical analysis. Alternatively, they were used for an isolation of primary follicles. Each experiment was performed at least three times. One group of ovaries ($n=5$) was collected from age-matched 13-day-old (PND 13) mice for *in vivo* comparisons of follicular populations and another group ($n=6$) for isolation of primary follicles for *in vivo* comparisons of expressions of *Gdf9*, *Bmp15*, and *Amh* mRNAs.

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Tissue processing

The ovaries were fixed for ∼4 h at room temperature. Subsequently, they were transferred into 70% ethanol at 4 °C until transported to the histology laboratory for processing and embedding into paraffin blocks. The ovaries were completely serial sectioned at 5 μm thickness on a sliding microtome (Bromma, Stockholm, Sweden). Every other set of five consecutive sections was mounted onto a microscope slide.

Quantification of primordial and primary follicles

Thirteen cultured ovaries and five ovaries of PND 13 immature animals were used for the analysis. The sections were deparaffinized, hydrated, and stained with hematoxylin, eosin, and safran. Every tenth section was examined under a light microscope for the presence of follicles. Primordial and primary follicles were counted in the entire sections. To avoid counting the same follicle more than once, only follicles with visible oocyte nucleolus were counted.

Early-stage follicles were classified as: (a) primordial follicle, oocyte surrounded by flattened or a mixture of flattened and cuboidal GC; (b) primary follicle, oocyte surrounded by one layer of cuboidal GC; (c) early secondary follicle, oocyte surrounded by one and a half layer of GC; (d) small secondary follicle, oocyte surrounded by two layers of GC; and (e) medium secondary follicle, oocyte surrounded by two and a half to three layers of GC. Follicles were considered atretic based on the following criteria: pyknotic GC or theca cells, eosinophilia of the ooplasm, a contracted chromatin material, or loss of basement membrane integrity. The size of the ovaries did not change significantly between treatments, and an estimation of the total follicle numbers per ovary was obtained by multiplying the number of primordial or primary follicles present in every tenth section analyzed by 10 to account for the ovarian sections not included in the analysis (Flaws *et al.* 2001).

Quantification of early, small, and medium secondary follicles

The number of healthy early, small, and medium secondary follicles per ovary was determined by exact counts from an examination of each of the entire consecutive 5 μm sections throughout the whole ovary. Attention was paid to avoid double counting in adjacent sections.

Isolation of primary follicles

Primary follicles were isolated from ovaries after 7 or 10 days of culture and from *in vivo* D13 ovaries (of immature mice) by mechanical isolation with fine 25½ gauge needles (Becton Dickinson, Erembodegem, Belgium). The diameter of the follicles was measured with a caliper in the eyepiece of an inverted microscope. A total of 185 primary follicles were isolated and immediately frozen (2–12 follicles/cryovial) in liquid nitrogen and stored until analyzed for gene expression as described below.

Gdf9, Bmp15, *and* Amh *mRNA expressions in primary follicles*

RNA extraction and RT

Total RNA was extracted from pooled follicles using the RNeasy Micro kit (Qiagen) according to the manufacturer's instructions. An exogenous control 10 pg of luciferase mRNA (Promega) was added and samples were eluted in 14 μl RNase-free water and stored at −80 °C. Subsequently, RT of 10 μl total RNA was carried out using the iScript cDNA Synthesis Kit (Bio-Rad laboratories) according to the manufacturer's instructions using the blend of oligo(dT) and random hexamers in a total volume of 20 μl. Negative controls were generated by omitting the RNA or the RT enzyme. Reverse transcribed cDNA was diluted 2:5 with DEPC-treated water and stored at −80 °C until real-time PCR was performed.

Real-time RT-PCR

Quantitative PCR was performed on the LightCycler 480 (Roche Diagnostics). PCR primer sequences are indicated in Table 3. Amplification reactions were carried out in a total volume of 15 μl containing 2 μl cDNAs, 7.5 μl SYBR Green PCR Master Mix 2× (Roche Diagnostics), and 0.6 μM forward and reverse gene-specific primers. Cycle amplification protocol was as follows: 95 °C for 10 min followed by 55 cycles of 95 °C for 10 s. and 60 °C for 30 s. For the quantification, standard curves were generated by amplifying serial dilutions of each amplicon. The specificity of the PCR products was checked by the melting curve analysis performed after the amplification cycles, and was further confirmed by sequencing the amplicons. The amount of gene of interest was normalized with the amount of luciferase in the same sample and with the number of follicles in the sample. Luciferase has been used as an appropriate exogenous control gene for quantitative PCR

Table 3 Primers pairs used for real-time PCR.

Gene	Sequence (5′–3′)	Amplicon size (bp)	GenBank accession no.
Gdf9	F: TACCGTCCGGCTCTTCAGT R: TTAAACAGCAGGTCCACCATC	93	NM_008110.1
Bmp15	F: CAGTAAGGCCTCCCAGAGGT R: AAGTTGATGGCGGTAAACCA	113	NM_009757.3
Amh	F: GGGGAGACTGGAGAACAGC R: AGAGCTCGGGCTCCCATA	67	NM_007445.2

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studies (Pennetier *et al.* 2006). Each sample was tested in triplicate and the normalized expression level of each gene was then related to the average amount as found in the *in vivo* control samples, PND 13.

Immunohistochemistry

The sections were incubated overnight at 37 °C and then for 2 h at 60 °C, at which point they were deparaffinized, hydrated, and incubated with 3% H_2O_2–methanol solution for 30 min. Then they were rinsed with distilled water and treated in citrated buffer (pH 6) for 20 min at 95 °C. After cooling the sections to room temperature, they were washed with three changes of PBS for 5 min each and preincubated with 10% normal rabbit serum for 20 min at room temperature, and incubated with primary antibodies overnight at 4 °C in a humidified chamber. The primary antibodies included: goat polyclonal IgG (Santa Cruz, Heidelberg, Germany) diluted to 1:50 (GDF9, sc-12244), and 1:200 (AMH, sc-6886) in PBS with 5% BSA. After that, the sections were washed with three changes of PBS with 0.1% Triton for 5 min each. All other incubations and washes were done at room temperature. The sections were further incubated for 30 min with biotinylated polyclonal rabbit anti-goat IgG (Dako, Heverlee, Belgium) diluted to 1:200 (GDF9) and 1:400 (AMH) in PBS with 5% BSA. Next, the sections were washed with three changes of PBS with 0.1% Triton for 5 min each, before being incubated for 30 min with extravidin diluted to 1:200 in PBS. Afterwards, the sections were then washed with three changes of PBS for 5 min each. Binding of the primary antibody was detected with diaminobenzidine (DAB; Sigma) solution (0.7 mg/ml) according to the manufacturer's instructions. The sections were washed with running and distilled water and then incubated with $CuSO_4$ for 5 min before being washed again with distilled and running water. The nuclei were counterstained with hematoxylin. Labeled cells were distinguished from unlabeled ones based upon the brown DAB precipitate. The primary antibodies were replaced by the antibody diluents in negative control sections. Negative controls were included in each run. Five cultured ovaries from five animals were used and two to five sections for each ovary were analyzed. Sections immunostained for GDF9 and AMH proteins were examined for the percentage of labeled follicles relative to the total number of follicles to assess growth factor expression.

Statistical analysis

All data are expressed as mean±s.e.m. Statistical analysis of follicle number was performed by Student's *t*-test and the differences in *Gdf9*, *Bmp15*, and *Amh* mRNA levels between control and cultured groups were determined by one-way ANOVA followed by Tukey's *post hoc* test. The GraphPad Prism 4.01 statistical analysis software (GraphPad Software Inc., San Diego, CA, USA) was used for all analysis. $P<0.05$ was considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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