

Follicular Growth In Vitro: Detection of Growth Differentiation Factor 9 (GDF9) and Bone Morphogenetic Protein 15 (BMP15) During In Vitro Culture of Ovine Cortical Slices

LIONEL MERY,^{1,2*} ANNICK LEFEVRE,^{1,2} MEHDI BENCHAI, ^{1,2} BANU DEMIRCI,^{1,3}
BRUNO SALLE,^{1,2} JEAN-FRANCOIS GUERIN,^{1,2} AND JACQUELINE LORNAGE^{1,2}

¹Département de Médecine et Biologie de la Reproduction, Hôpital Edouard Herriot/CECOS, Lyon, France

²Unité INSERM U 418, Hôpital Debrousse, Lyon, France

³Laboratoire de Zootechnie, Ecole Nationale Vétérinaire de Lyon, Marcy l'Etoile, France

ABSTRACT Primordial follicles from different mammal species can survive and enter the growth phase in vitro but do not develop beyond the primary stage. The hypothesis was that, in sheep, in vitro follicular growth is arrested because of a lack of secretion of GDF9 and/or BMP15. Cortical slices of 0.3–0.5 mm thickness issued from 5- to 6-month-old lambs were cultured for 15 days. The pieces were fixed on days 0, 2, 4, 7, 10, and 15 of culture. Follicle morphology, RT-PCR exploration of GDF9 and BMP15 mRNA, immunohistochemical location of their proteins and their receptor BMPRII and BMPRI were assessed at different time of culture. The mean percentage of primordial follicles decreased from 58.6% (day 0) to 13.4% (day 15) ($P < 0.01$), whereas that of primary follicles increased from 3.2% (day 0) to 31.5% on day 4 ($P < 0.01$), then remained stable until day 15 (35.6%). The percentage of atretic follicles increased from 14.7% (day 0) to 27.1% (day 15) ($P < 0.05$). A few secondary follicles were observed on days 4 and 10, representing 1.0%, and 2.1% of the total number of follicles. GDF9 and BMP15 mRNAs were detected from harvesting (day 0) up to day 15 following culture. At the same time, positive immunoreactions for GDF9, BMP15 and for BMPRII and BMPRI were also found in oocyte cytoplasm. In conclusion, expression of GDF9, BMP15 and their receptors BMPRII and BMPRI are detected during in vitro culture of ovine cortical slices. *Mol. Reprod. Dev.* 74: 767–774, 2007. © 2006 Wiley-Liss, Inc.

Key Words: GDF9; BMP15; ovary; in vitro follicle growth; sheep

Aside from recent autograft reports (Donnez et al., 2004; Oktay et al., 2004; Meirou et al., 2005), the other follicle growth procedures, in vivo xenograft, or in vitro culture are proving limited. Ovarian tissue xenografting has given some encouraging results in immunodeficient (SCID, NUDE) mice (Gook et al., 2001), but raises ethical issues with regard to its application. In vitro culture is an interesting alternative, since it rules out the risk of cancer cells being transmitted in the graft (Shaw et al., 1996). A few studies have been performed on in vitro culture of cryopreserved ovarian tissue, but results have been unsatisfactory, with a poor follicle survival rate (Hovatta et al., 1997; Isachenko et al., 2003).

In vitro fresh ovarian tissue follicle growth was achieved in mice as long ago as 1965 (Blandau et al., 1965). Eppig & O'Brien achieved complete in vitro follicle growth, with several life offspring (Eppig and O'Brien, 1996; O'Brien et al., 2003). In vitro culture in large mammals (cows, ewes, humans) has enabled primordial follicle growth to be initiated, but early growth arrest intervenes at the primary follicle stage (Wandji et al., 1996; Cecconi et al., 1999; Hovatta et al., 1999).

The mechanisms of in vivo or in vitro follicle growth remain unclear. The kit ligand/c-kit interaction seems to be involved in growth initiation (Parrott and Skinner, 1999). Kit ligand (stem cell factor [SCF]) secreted by granulosa cells acts on the oocyte via the c-kit receptor on the oocyte membrane. SCF secretion in response to a rise in intracellular AMPc (Packer et al., 1994) seems to be regulated by various inhibitory or stimulatory paracrine factors. Various molecules, such as Anti-Müllerian Hormone (AMH) (Durlinger et al., 2002),

INTRODUCTION

Ovarian tissue banks represent a real hope for all women obliged to undergo cancer treatment and who wish to conserve fertility. Reserve follicles, however, have to go through a long growth stage in vivo in case of grafting or of several months in vitro following thawing before reaching the preovulatory follicle stage.

*Correspondence to: Lionel Mery, Département de Médecine et Biologie de la Reproduction, Hôpital Edouard Herriot/CECOS, Lyon, France. E-mail: lionel.mery@chu-lyon.fr

Received 4 August 2006; Accepted 11 September 2006

Published online 8 December 2006 in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/mrd.20661

retinoblastoma protein (pRb) (Picton, 2001), the Wilms tumor suppressor gene protein (WT1) (Hsu et al., 1995), or somatostatin (SST) (Goddard et al., 2001), appear to play inhibitory roles. Conversely, follicle growth initiation could be due to a rise in local stimulatory factors such as epidermal growth factor (EGF) (Picton, 2001), bone morphogenic protein 7 (BMP7) (Lee et al., 2001), basic fibroblast growth factor (bFGF) (Nilsson et al., 2001), leukemia inhibiting factor (LIF) (Nilsson et al., 2002), or bone morphogenic protein 4 (BMP4) (Nilsson and Skinner, 2003). For the stages following follicle growth initiation, 2 of the 40 members of the transforming growth factor β (TGF β) superfamily (Chang et al., 2002), growth differentiation factor 9 (GDF9) and bone morphogenic protein 15 (BMP15), appear to be essential for primary follicles to reach the secondary follicle stage. Several studies have supported these hypotheses: GDF9-deficient (Knock Out) mice (Dong et al., 1996) and homozygous ewes deficient in GDF9 and BMP15 (Galloway et al., 2000; Hanrahan et al., 2004) are sterile, with follicles arrested at the primary stage. Identical results were obtained in ewes immunized against GDF9 and/or BMP15 (Juengel et al., 2004). In humans, ovarian insufficiency has been associated with a BMP15 gene mutation (Di Pasquale et al., 2004), and Hreinsson et al. (2002) obtained an increased number of secondary follicles by adding GDF9 to the ovarian tissue culture medium.

The primary follicle stage growth arrest found *in vitro* suggests a possible lack of growth factors such as GDF9 and/or BMP15. To test this hypothesis, we used RT-PCR and immunohistochemistry to analyze GDF9 and BMP15 expression at harvesting (day 0) and at various times during culture of ewe ovarian tissue. In the same time, we used immunohistochemistry to analyze BMPRII and BMPRI expression, receptors of GDF9 and BMP15.

MATERIALS AND METHODS

Sheep Ovary Collection

Five ovaries from 5- to 6-month-old lambs were collected at the slaughterhouse (1 ovary per lamb). The ovaries were placed in X-vivo, a serum-free organ transport medium, (Bio Whittaker, Walkersville, ML) and transported to our laboratory at 10°C.

Culture Conditions and Methods

Cortical slices (0.3–0.5 mm) with an area of 3 mm² were prepared and cultivated on filters (0.4 μ m Millicell-CM; Millipore Co., Bedford, MA) in DMEM-Ham F-12 medium (Sigma, St Louis, MO) supplemented with ITS + 1 (i.e., insulin 10 mg/L, transferrin 5.5 mg/L, selenium 5 μ g/L, bovine serum albumin (BSA) 0.5 mg/L, and linoleic acid 4.7 μ g/L) (Sigma), BSA 1.25 mg/ml (Sigma), streptomycin 50 μ g/ml (Sigma), and penicillin-G 75 μ g/ml (Sigma).

The culture inserts were preequilibrated 2 hr before culture with 400 μ l of culture medium in a humidified incubator under 5% CO₂, 5% O₂, and 90% N₂, at 37°C.

Three slices were placed in each well. The volume of culture medium was adjusted so that the pieces of ovarian cortex were covered by a very thin film generated by surface tension. Culture medium was changed every 2 days.

The slices were cultured for 15 days. On the day before (D0) and on days 2, 4, 7, 10, and 15 of culture, they were fixed in Bouin liquid (Chimie-Plus, Denicé, France) for morphological examination. On D0, D7, and D15, they were either fixed in 4% formalin in PBS for immunohistochemistry, or put in –196°C liquid nitrogen (to avoid any deterioration in mRNA) for RT-PCR.

Morphological Study of Follicles

After inclusion in paraffin, semi-serial 4 μ m slices were cut by microtome (Leitz, Wetzlar, Germany). Twelve slices were cut per hemi-ovary and culture-time, at 60 μ m intervals to prevent any follicles being counted twice, and stained in HPS Hematoxylin (Speci, Varennes-sur-Allier, France), Phloxine B (Réactifs RAL, Bordeaux, France), and Saffron (Speci). Follicles were categorized as: primordial (20–30 μ m oocyte arrested in diploten of prophase I (oocyte I), surrounded by <15 flat cells), early primary (oocyte I surrounded by cubic and flat cells); primary (oocyte I surrounded by a complete layer of cubic cells), secondary (oocyte I surrounded by at least 2 layers of cubic cells), or atretic (pyknotic oocyte nucleus and or vacuolated cytoplasm). The slides were examined using a magnification 400 \times .

RT-PCR Analysis of GDF9 and BMP15 Expression

Extraction of mRNA and Reverse Transcription.

mRNAs was extracted from cortex slices (100 mg of tissue) cut in small pieces (1 mm³), using TRIzol[®] reagent (Sigma), according to the manufacturer's protocol.

mRNA (10 μ g) was reverse-transcribed by incubating at 37°C for 1 hr with 200 IU M-MLV reverse transcriptase (Invitrogen, Life Technologies, Carlsbad, CA), 2 IU RNase inhibitor (Applied Biosystems, Foster city, CA), 2 μ l (50 ng/ μ l) random hexamers (Invitrogen), 2 μ l (0.1 M) DTT (Invitrogen), 1 μ l dNTPs (10 mM each dNTP, Applied Biosystems), 4 μ l 5X RT buffer (Applied Biosystems) in a final volume of 20 μ l. The reaction was terminated by heating at 70°C for 10 min. cDNA was precipitated and resuspended in 50 μ l deionized water.

PCR amplification. PCR primers and the sizes of PCR products are listed in Table 1. Primers were used to span an exon-intron-exon region, when possible, allowing discrimination between amplicons derived from cDNA templates or from genomic DNA templates. For all PCR amplifications, negative (water only) and positive (primers for β actin) controls were included. Amplification reactions were carried out in a 50 μ l reaction mixture: 1X Taq polymerase reaction buffer (Eurobio, Les Ulis, France) containing 1 μ l (0.5 μ g/ μ l) of each primer, 1 μ l dNTPs (10 mM each dNTP: Applied

TABLE 1. Oligonucleotides Primers Used for PCR Analysis of Sheep Tissue

Target gene/length	Sense	Primer sequence (5' 3')	Position	GenBank accession number
GDF9 1 (586 pb)	s	GTTCTGTATGATGGGCACGG	1981–2000	AF078545 Ovis aries
GDF9 2	as	AGGGCGCTACGAGAAGAGTC	3672–3691	AF078545 Ovis aries
BMP15 1 (483 pb)	s	CTATGCCACCTGCCTGAG	326–345	AF236078 Ovis aries
BMP15 2	as	TTTGCCCAACATGTTCCATG	620–639	AF236078S2 Ovis aries
β actin 1 (247 pb)	s	CACGGCATTGTCACCAACTGG	303–323	V39357 Ovis aries
β actin 2	as	AGTCCATCAGGATGCCAGTGG	528–550	V39357 Ovis aries

Biosystems), 1.5 μ l MgCl₂ (50 mM), 1.5 IU Taq DNA polymerase (Eurobio), and 10 μ l of cDNA. The program comprised an initial denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 62°C for 30 sec, and extension at 72°C for 30 sec. Final extension was done at 72°C for 10 min.

The PCR products (5 μ l each) were analyzed on 1.5% agarose gels stained with 1 mg/ml of ethidium bromide (Invitrogen), and molecular sizes were determined with the 1 kb molecular weight marker (Invitrogen).

Immunohistochemical Location of GDF9, BMP15, BMPRII and BMPRII

Slices (4 μ m) were cut for each paraffin-embedded hemi-ovary on D0, D7, and D15. GDF9 and BMP15 were located on serial 4 μ m slices cut from the ovaries of five different ewes. These slices were mounted on Starfrost[®] (Knittel Glaser, Braunschweig, Germany) slides, dried for 24 hr at 37°C, deparaffinized in three successive washes of methyl cyclohexane (Merck, Darmstadt, Germany), rehydrated in a graded ethanol series (100, 95, 70%, Hexalab, Saint-Priest, France) and then placed in PBS buffer (Sigma). Heat treatment antigen unmasking was performed by microwaving in 10 mM sodium citrate buffer (pH = 6, Sigma) for 15 min. After two washes in deionized water, endogenous peroxidase blocking was performed by incubating the deparaffinized slices in 3% hydrogen peroxide (Santa Cruz Biotechnology, Inc., CA) for 10 min. The slices were washed twice in PBS. All subsequent incubations were performed at room temperature in a dark wet chamber.

For GDF9, the processed tissue slices were incubated for 2 hr with the primary antibody, goat anti-human GDF9 at concentration of 2 μ g/ml (Santa Cruz Biotechnology, Inc.). After incubation with primary antibody, slices were washed in PBS and incubated for 30 min with biotinylated secondary anti-goat antibodies (Santa Cruz Biotechnology, Inc.). Next, the slices were washed twice and incubated for 30 min with an HRP-streptavidin reagent (Santa Cruz Biotechnology, Inc.). The slices were then washed twice and stained with diaminobenzidine complex (Santa Cruz Biotechnology, Inc.) for 10 min.

For BMP15, the processed tissue slices were incubated for 2 hr with the primary antibody, rabbit anti-human BMP15 provided by A. Gougeon at concentration of 4 μ g/ml. After incubation with primary antibody, slices were washed in PBS and incubated for 30 min with

the secondary anti-rabbit IgG conjugated to peroxidase (EnVision DakoCytomation, Carpinteria, CA). The slices were then washed twice and stained with diaminobenzidine complex (DAB+: DakoCytomation) for 1 min.

For BMPRII and BMPRII, we performed the protocols used for GDF9 with the primary antibody (Santa Cruz Biotechnology, Inc.), goat anti-human BMPRII at concentration of 4 μ g/ml and goat anti-human BMPRII at concentration of 4 μ g/ml.

After two washes with deionized water, for GDF9, BMP15, BMPRII and BMPRII, counter-staining was performed with Mayer's hematoxylin diluted to 1:10 for 2 min. Finally, the slices were washed twice and mounted with aqueous mounting medium (DakoCytomation).

For nonspecific staining, negative controls were performed: first incubation with primary antibody dilutant or with normal goat IgG (Santa Cruz Biotechnology, Inc.) for GDF9, BMPRII and BMPRII, and nonimmune rabbit serum for BMP15.

Statistical Analysis

The percentages of healthy primordial, early primary, primary, secondary, and atretic follicles were calculated for 12 cortical slices per ovary (N = 5). The chi-square test was used in order to compare the distribution of follicles percentages. The Mann-Whitney test was used to determine whether a given day of culture differed from the others. Unistat 4.6 software was used for statistical analysis (Unistat, London, UK). Differences were considered significant at $P \leq 0.05$.

RESULTS

In Vitro Culture

The total number of follicles examined overall in the five ewes was 2,348. During culturing, the mean percentage of primordial follicles fell from 58.6% (D0) to 13.4% (D15) ($P < 0.01$); that of primary follicles rose from 3.2% (D0) to 31.5% by D4 ($P < 0.01$), remaining stable thereafter until D15 (35.6%) (Fig. 1 and Table 2). The mean percentage of intermediate follicles rose from 23.1% (D0) to 31.4% by D7 ($P = \text{NS}$), then fell back to 23.4% by D15. The mean percentage of atretic follicles kept rising, from 14.7% (D0) to 27.1% (D15) ($P < 0.05$). Only a few secondary follicles were found: none on D7 or D15, and only 0.5, 1.1, 1.0, and 2.1% on D0, D2, D4, and

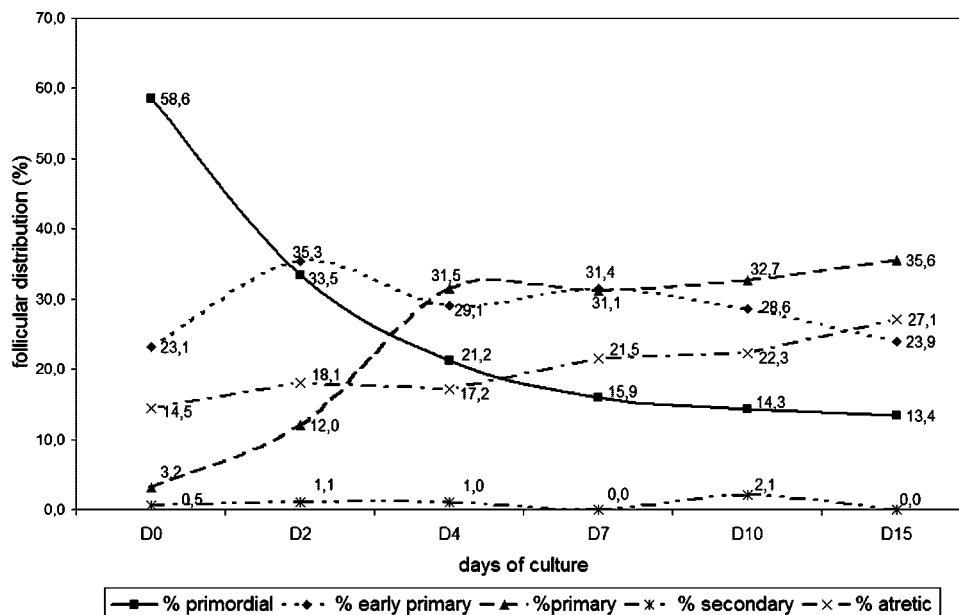


Fig. 1. Distribution of the atretic, primordial, early primary, primary, and secondary follicles in ewes during 15 days of culture.

D10, respectively. Figure 2 shows the morphological aspect of follicles during culture.

Expression of GDF9 and BMP15 by RT-PCR

GDF9 and BMP15 mRNA was found on D0 and D7 through D15. All five ewes showed the same electrophoresis gel profile for all three stages of culture, attested by the presence of a band at 586 pb for GDF9 and 483 pb for BMP15 (Fig. 3).

Immunolocation of GDF9 and BMP15

Tissue in the various slices taken from the five ewes contained primordial, early primary, primary, and a very few secondary follicles. On D0, these various types—mainly primordial—showed positive GDF9 and BMP15 immunoreactions in the oocyte cytoplasm (Fig. 4). Follicle type distribution on D7 and D15 differed from D0 (Fig. 1). Positive GDF9 and BMP15 immunoreactions, identical to those of D0, were observed in the oocyte cytoplasm of the various follicle types (Fig. 4). No such labeling was to be found in the various negative controls.

Immunolocation of BMPRII and BMPRII

On D0, the various follicles types showed positive BMPRII and BMPRII immunoreactions in the oocyte cytoplasm, we also found positive immunoreactions in the endothelial cells of the vassals (capillary). For D7 and D15, positive BMPRII and BMPRII immunoreactions, identical to those of D0, were observed in the oocyte cytoplasm of the various follicle types and in endothelial cells. No such labeling was to be found in the various negative controls.

DISCUSSION

Ovarian cortex oocyte cryopreservation is the method that presently seems to offer the best guarantee of fertility preservation. Our team has already obtained several pregnancies in ewes via autograft of cryopreserved tissue, up to 2 years postgrafting (Salle et al., 2003). Pregnancies and births are now being reported in humans (Donnez et al., 2004; Meirou et al., 2005). When, however, the risk of transmitting malignant cells in the graft seems excessive (Shaw et al., 1996), in vitro primordial follicle culture would be a useful alternative.

TABLE 2. Proportions in Mean Percentage (Standard Error of Mean Into Brackets) of Atretic, Primordial, Early Primary, Primary, and Secondary Follicles Originating From Five Different Ewes During Culture

Day of culture	Primordial (%)	Early primary (%)	Primary (%)	Secondary (%)	Atretic (%)
D0	58.6 (± 3.1)	23.1 (± 4.0)	3.2 (± 1.7)	0.5 (± 0.3)	14.5 (± 1.8)
D2	33.5 (± 4.5)	35.3 (± 5.8)	12.0 (± 3.1)	1.1 (± 0.4)	18.1 (± 3.1)
D4	21.2 (± 3.1)	29.1 (± 6.0)	31.5 (± 5.8)	1.0 (± 0.6)	17.2 (± 1.3)
D7	15.9 (± 1.9)	31.4 (± 1.9)	31.1 (± 2.0)	0.0 (± 0.0)	21.5 (± 2.6)
D10	14.3 (± 1.2)	28.6 (± 2.3)	32.7 (± 1.6)	2.1 (± 0.9)	22.3 (± 2.3)
D15	13.4 (± 0.7)	23.9 (± 1.7)	35.6 (± 2.2)	0.0 (± 0.0)	27.1 (± 2.5)

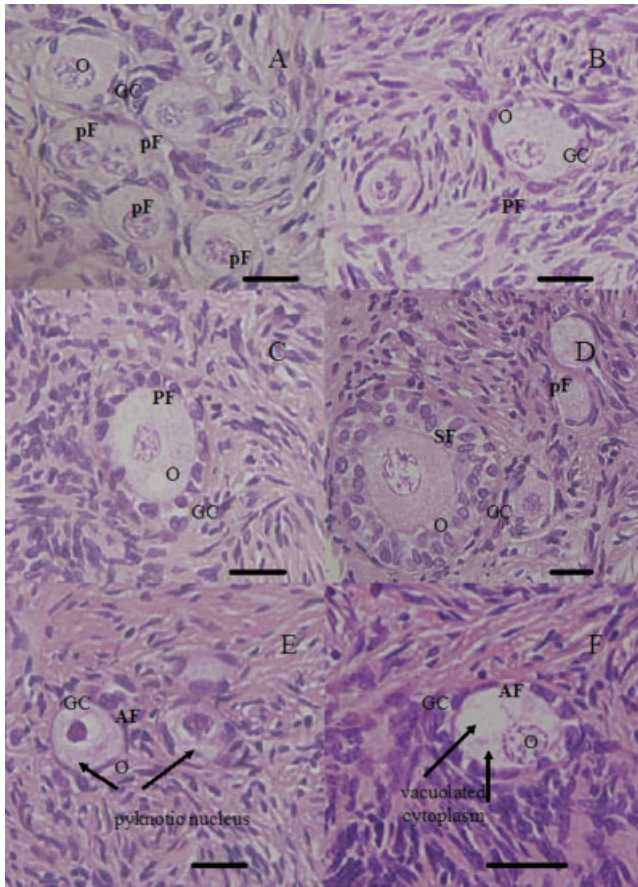


Fig. 2. morphological aspect of ovarian follicle, (HPS coloration). primordial follicles (A), primary follicle (B,C), secondary follicle (D), apoptotic follicles (E,F), pyknotic nucleus (E), and cytoplasmic vacuole (F). pF, primordial follicle; PF, primary follicle; SF, secondary follicle; AF, apoptotic follicle; O, oocyte; GC, granulosa cells; scale bars represent 20 μ m.

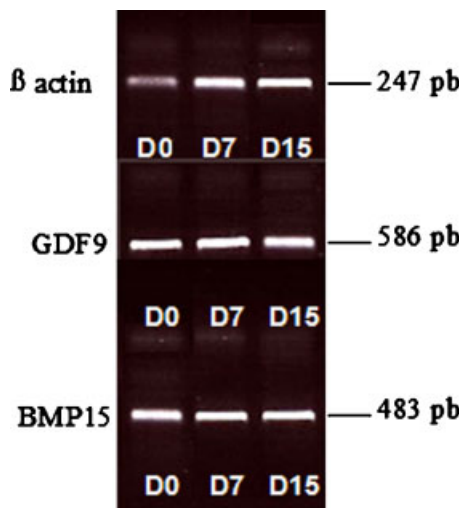


Fig. 3. Expression of GDF9, BMP15, and positive control β actin during culture on a representative gel. BMP15 and GDF9 are expressed at D0, D7, and D15 of culture.

Our results show that, in ewes, primordial follicles cultivated in serum-free medium can enter the growth phase in vitro. The morphological study found a significant fall in primordial follicles, from 58.6% (D0) to 21.2% (D15) ($P < 0.01$) and a proportionally significant rise in the percentage of primary follicles, from 3.2% (D0) to 31.5% as of D4 ($P < 0.01$), thereafter stabilizing through to D15 (36.5%). This early growth initiation, with 31.5% primary follicles by D4, agrees with previous findings in humans (Cecconi et al., 1999), baboons (Wandji et al., 1997), and cows (Wandji et al., 1996; Braw-Tal and Yossefi, 1997).

Various hypotheses have been entertained to account for such early growth initiation. Inhibitory factors present in the ovary and blocking initiation were suggested by Peters et al. (1973). Several molecules, SST (Goddard et al., 2001), AMH (Durlinger et al., 2002), retinoblastoma protein (pRb) (Picton, 2001), and Wilms tumor suppressor gene protein (WT1) (Hsu et al., 1995) have indeed shown such a potential. In the cultures used in the present study, however, these growth-inhibiting factors were absent or too weakly represented, due to the removal of the medulla, as envisaged by Wandji et al. (1996, 1997). Another hypothesis is that the culture medium used may contain growth-activating factors, such as EGF (Picton, 2001), BMP7 (Lee et al., 2001), bFGF (basic fibroblast growth factor) (Nilsson et al., 2001), LIF (Nilsson et al., 2002), or BMP4 (Nilsson and Skinner, 2003).

The culture conditions used in the present study promoted the initiation of follicle growth, but failed to enable the secondary and preantral follicle stages. Despite the initiation of follicle growth, we observed early arrest, with no more than 0.1% secondary follicles on D0, 1.1% on D2, 1.0% on D4, 0% on D7, 2.1% on D10, and 0% on D15 (Fig. 1)—this small number doubtless being follicles already at this stage in the harvested tissue, and remaining during culture. The GDF9 and BMP15 genes code for proteins that turn out to be essential to the early stages of follicle growth, and in particular to the shift from primary to secondary (Fig. 3). On D0, a positive immunoreaction to GDF9 and BMP15 was found in the oocyte cytoplasm of various categories of follicle, testifying to the presence of the corresponding proteins, in agreement with literature reports (Fig. 4). mRNA of GDF9 and BMP15 proteins are expressed by the oocyte in humans (Aaltonen et al., 1999), rodents (Laitinen et al., 1998; Jaatinen et al., 1999), ruminants (Bodensteiner et al., 1999, 2000; Galloway et al., 2000), and marsupials (Eckery et al., 2002), although in some primates (Duffy, 2003), or in goats (Silva et al., 2005), their expression was located in granulosa cells. We detected a positive immunoreaction to GDF9 as of the primordial stage, as reported in ewes, cows, possums, and hamsters (Bodensteiner et al., 1999; Eckery et al., 2002; Wang and Roy, 2004); in humans, rats, and mice, however, GDF9 is expressed only at the primary follicle stage (Fitzpatrick et al., 1998; Laitinen et al., 1998; Aaltonen et al., 1999; Jaatinen et al., 1999). We detected a positive

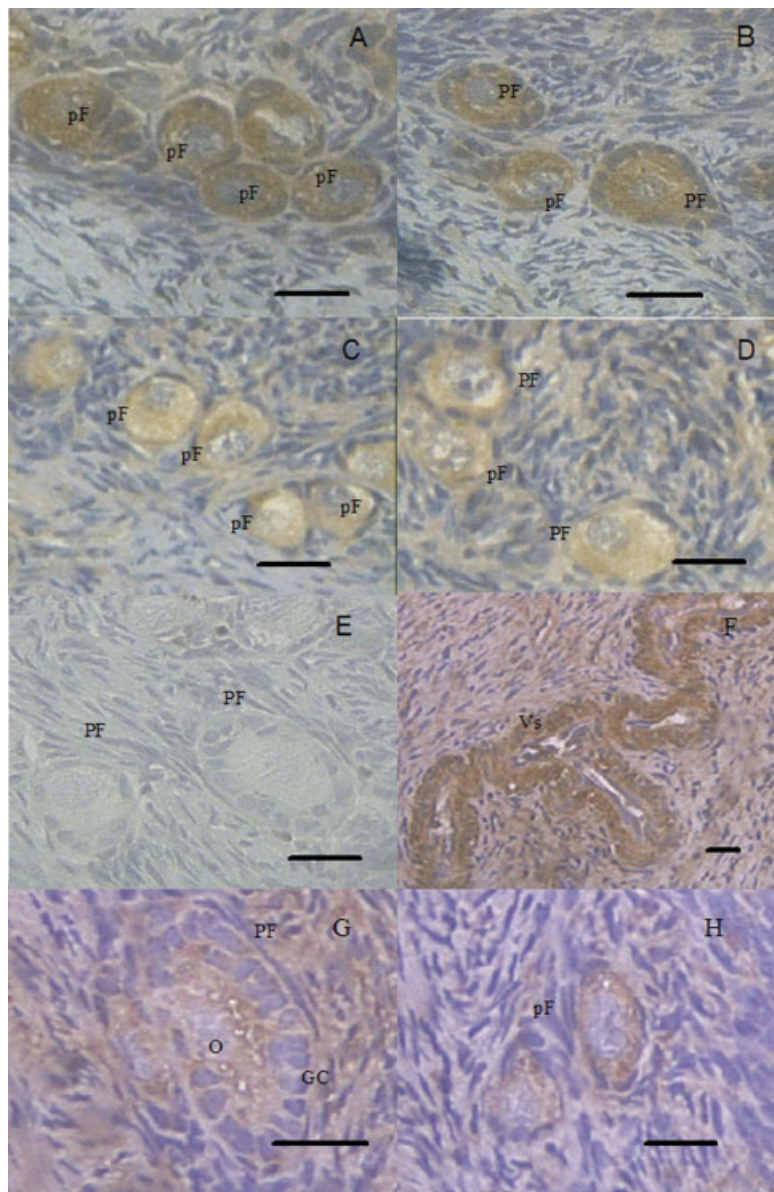


Fig. 4. BMP15, GDF9, BMPRI and BMPRII immunoreactivity in sheep ovarian tissue. Positive immunoreaction for BMP15 at D0 (A) and D7 (B). Positive immunoreaction for GDF9 at D0 (C), D15 (D), and negative control (E). Positive immunoreaction for BMPRI at D0 (F) and D7 (G). Positive immunoreaction for BMPRII at D15 (H). PF, primary follicle; pF, primordial follicle; O, oocyte; GC, granulosa cells; Vs, vassals; scale bars represent 20 μ m.

immunoreaction to BMP15 at the primordial stage, as reported in goats (Silva et al., 2005) and brushtail possums (Eckery et al., 2002); in humans, rats, mice, and sheep, however, BMP15 is expressed only at the primary stage (Laitinen et al., 1998; Aaltonen et al., 1999; Jaatinen et al., 1999; Galloway et al., 2000). These divergent findings may relate to interspecies variations, or to methodological variations (in test sensitivity and specificity) which would account for certain contradictory reports on the same species. In ewes, we detected BMP15 at the primordial follicle stage by immunohistochemical means; whereas Galloway et al.

(2000) detected BMP15 at the primary stage using an *in situ* hybridation technique.

We detected mRNA by RT-PCR (Fig. 3) and the BMP15 and GDF9 proteins in the various follicle types throughout culture (Fig. 4). Our hypothesis that growth arrest at the primary follicle stage might be due to a complete lack of GDF9 and/or BMP15 thus failed to be confirmed. And yet the arrest found here during culture agrees with a number of reports. In GDF9-deficient (Knock Out) mice, sterility and primary-stage arrest have been found (Dong et al., 1996). BMP15-deficient (Knock Out) mice remain fertile, with apparently

normal follicular growth, even if ovulation and fertility are impaired (Yan et al., 2001). GDF9 and BMP15 deficient homozygous ewes (Galloway et al., 2000; Hanrahan et al., 2004) are sterile, with follicles arrested at the primary stage. Identical observations have been made for ewes immunized against GDF9 and/or BMP15 (Juengel et al., 2004). Human ovarian insufficiency has been attributed to a mutation on the BMP15 gene (Di Pasquale et al., 2004), and Hreinsson et al. (2002) obtained an increased number of secondary follicles by adding GDF9 to the ovarian tissue culture medium.

Like others, members of the TGF β super family, GDF9 and BMP15 are produced as preproprotein that must undergo processing and proteolytic cleavage before the secretion of the mature form of the molecules (Laitinen et al., 1998). The mature proteins act via association mechanisms and complex phosphorylation cascades. TGF β superfamily members classically act in the form of homo- or hetero-dimers with a double covalent disulfide bond. BMP15 and GDF9 have no cystein residues, but can form homo- or hetero-dimer complexes by noncovalent bonding (Liao et al., 2003; Shimasaki et al., 2004). TGF β superfamily members act via membrane-bound serine threonine kinase receptors. BMPRII and ALK5 (TGF β RI) have recently been identified as GDF9 receptors (Vitt et al., 2002; Mazerbourg et al., 2004), and BMPRII and ALK6 (BMPRIIB) as BMP15 receptors (Moore et al., 2003). A lack of these receptors could explain the growth arrest, but in our study, the presence of the BMPRIIB and BMPRII proteins were detected by immunohistochemistry during the culture. Once activated, these receptors induce phosphorylation of the intracellular signaling molecules known as Smads. GDF9 can activate the Smads 2 and 3 pathways (Kaivo-Oja et al., 2003), and BMP15 those of Smads 1, 5, and 8 (Moore et al., 2003). A default production of these factors may play a role in growth arrest.

Our present results demonstrate that primary follicle stage growth arrest during in vitro culture seems not be due to complete defective production of GDF9, BMP15 or their receptors BMPRIIB and BMPRII. Nevertheless, it is possible that these factors expression is decreased in cultured ovaries compared to in vivo. This decreased level of expression may be still detectable by techniques employed in our experiment, but may be insufficient to stimulate primary to secondary follicle growth, if such stimulation were to be necessary. Many questions presently remain as to the intimate mechanisms governing early follicle growth, and as yet unknown pathways may be involved in the arrest observed. Progress in vivo could have a knock-on effect in vitro, and vice-versa. Applications will concern both in vitro culture of cryopreserved gamete-bank ovarian tissue and the development of therapy for early ovarian insufficiency.

ACKNOWLEDGMENTS

We would like to thank A. Gougeon (unité INSERM 407, Lyon Sud, France) for providing the anti-BMP15

antibodies; the technicians of our laboratory and from the laboratory d'anatomie-pathologique (Hôpital Edouard Herriot, Lyon, France, Pr Scoazec) for their technical assistance.

REFERENCES

- Aaltonen J, Laitinen MP, Vuojolainen K, Jaatinen R, Horelli-Kuitunen N, Seppa L, Louhio H, Tuuri T, Sjoberg J, Butzow R, Hovata O, Dale L, Ritvos O. 1999. Human growth differentiation factor 9 (GDF-9) and its novel homolog GDF-9B are expressed in oocytes during early folliculogenesis. *J Clin Endocrinol Metab* 84:2744–2750.
- Blandau R, Warrick E, Rumery RE. 1965. In vitro cultivation of fetal mouse ovaries. *Fertil Steril* 16:705–715.
- Bodensteiner KJ, Clay CM, Moeller CL, Sawyer HR. 1999. Molecular cloning of the ovine growth/differentiation factor-9 gene and expression of growth/differentiation factor-9 in ovine and bovine ovaries. *Biol Reprod* 60:381–386.
- Bodensteiner KJ, McNatty KP, Clay CM, Moeller CL, Sawyer HR. 2000. Expression of growth and differentiation factor-9 in the ovaries of fetal sheep homozygous or heterozygous for the inverdale prolificacy gene (FecX(I)). *Biol Reprod* 62:1479–1485.
- Braw-Tal R, Yossefi S. 1997. Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary. *J Reprod Fertil* 109:165–171.
- Cecconi S, Barboni B, Coccia M, Mattioli M. 1999. In vitro development of sheep preantral follicles. *Biol Reprod* 60:594–601.
- Chang H, Brown CW, Matzuk MM. 2002. Genetic analysis of the mammalian transforming growth factor-beta superfamily. *Endocr Rev* 23:787–823.
- Di Pasquale E, Beck-Peccoz P, Persani L. 2004. Hypergonadotropic ovarian failure associated with an inherited mutation of human bone morphogenetic protein-15 (BMP15) gene. *Am J Hum Genet* 75:106–111.
- Dong J, Albertini DF, Nishimori K, Kumar TR, Lu N, Matzuk MM. 1996. Growth differentiation factor-9 is required during early ovarian folliculogenesis. *Nature* 383:531–535.
- Donnez J, Dolmans MM, Demylle D, Jadoul P, Pirard C, Squifflet J, Martinez-Madrid B, van Langendonck A. 2004. Livebirth after orthotopic transplantation of cryopreserved ovarian tissue. *Lancet* 364:1405–1410.
- Duffy DM. 2003. Growth differentiation factor-9 is expressed by the primate follicle throughout the periovulatory interval. *Biol Reprod* 69:725–732.
- Durlinger AL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA, Themmen AP. 2002. Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. *Endocrinology* 143:1076–1084.
- Eckery DC, Whale LJ, Lawrence SB, Wylde KA, McNatty KP, Juengel JL. 2002. Expression of mRNA encoding growth differentiation factor 9 and bone morphogenetic protein 15 during follicular formation and growth in a marsupial, the brushtail possum (*Trichosurus vulpecula*). *Mol Cell Endocrinol* 192:115–126.
- Eppig JJ, O'Brien MJ. 1996. Development in vitro of mouse oocytes from primordial follicles. *Biol Reprod* 54:197–207.
- Fitzpatrick SL, Sindoni DM, Shughrue PJ, Lane MV, Merchenthaler IJ, Frail DE. 1998. Expression of growth differentiation factor-9 messenger ribonucleic acid in ovarian and nonovarian rodent and human tissues. *Endocrinology* 139:2571–2578.
- Galloway SM, McNatty KP, Cambridge LM, Laitinen MP, Juengel JL, Jokiranta TS, McLaren RJ, Luiro K, Dodds KG, Montgomery GW, Beattie AE, Davis GH, Ritvos O. 2000. Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. *Nat Genet* 25:279–283.
- Goddard I, Bauer S, Gougeon A, Lopez F, Giannetti N, Susini C, Benahmed M, Krantic S. 2001. Somatostatin inhibits stem cell factor messenger RNA expression by Sertoli cells and stem cell factor-induced DNA synthesis in isolated seminiferous tubules. *Biol Reprod* 65:1732–1742.

- Gook DA, McCully BA, Edgar DH, McBain JC. 2001. Development of antral follicles in human cryopreserved ovarian tissue following xenografting. *Hum Reprod* 16:417–422.
- Hanrahan JP, Gregan SM, Mulsant P, Mullen M, Davis GH, Powell R, Galloway SM. 2004. Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased ovulation rate and sterility in Cambridge and Belclare sheep (*Ovis aries*). *Biol Reprod* 70:900–909.
- Hovatta O, Silye R, Abir R, Krausz T, Winston RM. 1997. Extracellular matrix improves survival of both stored and fresh human primordial and primary ovarian follicles in long-term culture. *Hum Reprod* 12:1032–1036.
- Hovatta O, Wright C, Krausz T, Hardy K, Winston RM. 1999. Human primordial, primary and secondary ovarian follicles in long-term culture: Effect of partial isolation. *Hum Reprod* 14:2519–2524.
- Hreinnsson JG, Scott JE, Rasmussen C, Swahn ML, Hsueh AJ, Hovatta O. 2002. Growth differentiation factor-9 promotes the growth, development, and survival of human ovarian follicles in organ culture. *J Clin Endocrinol Metab* 87:316–321.
- Hsu SY, Kubo M, Chun SY, Haluska FG, Housman DE, Hsueh AJ. 1995. Wilms' tumor protein WT1 as an ovarian transcription factor: Decreases in expression during follicle development and repression of inhibin- α gene promoter. *Mol Endocrinol* 9:1356–1366.
- Isachenko E, Isachenko V, Rahimi G, Nawroth F. 2003. Cryopreservation of human ovarian tissue by direct plunging into liquid nitrogen. *Eur J Obstet Gynecol Reprod Biol* 108:186–193.
- Jaatinen R, Laitinen MP, Vuojolainen K, Aaltonen J, Louhio H, Heikinheimo K, Lehtonen E, Ritvos O. 1999. Localization of growth differentiation factor-9 (GDF-9) mRNA and protein in rat ovaries and cDNA cloning of rat GDF-9 and its novel homologue GDF-9B. *Mol Cell Endocrinol* 156:189–193.
- Juengel JL, Hudson NL, Whiting L, McNatty KP. 2004. Effects of immunization against bone morphogenetic protein 15 and growth differentiation factor 9 on ovulation rate, fertilization, and pregnancy in ewes. *Biol Reprod* 70:557–561.
- Kaivo-Oja N, Bondestam J, Kamarainen M, Koskimies J, Vitt U, Cranfield M, Vuojolainen K, Kallio JP, Olkkonen VM, Hayashi M, Moustakas A, Groome NP, ten Dijke P, Hsueh AJ, 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.
- Laitinen M, Vuojolainen K, Jaatinen R, Ketola I, Aaltonen J, Lehtonen E, Heikinheimo M, Ritvos O. 1998. A novel growth differentiation factor-9 (GDF-9) related factor is co-expressed with GDF-9 in mouse oocytes during folliculogenesis. *Mech Dev* 78:135–140.
- Lee WS, Otsuka F, Moore RK, Shimasaki S. 2001. Effect of bone morphogenetic protein-7 on folliculogenesis and ovulation in the rat. *Biol Reprod* 65:994–999.
- Liao WX, Moore RK, Otsuka F, Shimasaki S. 2003. Effect of intracellular interactions on the processing and secretion of bone morphogenetic protein-15 (BMP-15) and growth and differentiation factor-9. Implication of the aberrant ovarian phenotype of BMP-15 mutant sheep. *J Biol Chem* 278:3713–3719.
- Mazerbourg S, Klein C, Roh J, Kaivo-Oja N, Mottershead DG, Korchynskiy O, Ritvos O, Hsueh AJ. 2004. Growth differentiation factor-9 signaling is mediated by the type I receptor, activin receptor-like kinase 5. *Mol Endocrinol* 18:653–665.
- Meirow D, Levron J, Eldar-Geva T, Hardan I, Fridman E, Zalel Y, Schiff E, Dor J. 2005. Pregnancy after transplantation of cryopreserved ovarian tissue in a patient with ovarian failure after chemotherapy. *N Engl J Med* 353:318–321.
- Moore RK, Otsuka F, Shimasaki S. 2003. Molecular basis of bone morphogenetic protein-15 signaling in granulosa cells. *J Biol Chem* 278:304–310.
- Nilsson EE, Skinner MK. 2003. Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. *Biol Reprod* 69:1265–1272.
- Nilsson E, Parrott JA, Skinner MK. 2001. Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. *Mol Cell Endocrinol* 175:123–130.
- Nilsson EE, Kezele P, Skinner MK. 2002. Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries. *Mol Cell Endocrinol* 188:65–73.
- O'Brien MJ, Pendola JK, Eppig JJ. 2003. A revised protocol for in vitro development from primordial follicles dramatically improves their developmental competence. *Biol Reprod* 68:1682–1686.
- Oktay K, Buyuk E, Veeck L, Zaninovic N, Xu K, Takeuchi T, Opsahl M, Rosenwaks Z. 2004. Embryo development after heterotopic transplantation of cryopreserved ovarian tissue. *Lancet* 363:837–840.
- Packer AI, Hsu YC, Besmer P, Bachvarova RF. 1994. The ligand of the c-kit receptor promotes oocyte growth. *Dev Biol* 161:194–205.
- Parrott JA, Skinner MK. 1999. Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. *Endocrinology* 140:4262–4271.
- Peters H, Byskov AG, Lintern-Moore S, Faber M. 1973. Proceedings: Follicle growth initiation in the immature mouse ovary: Extraovarian or intraovarian control? *J Reprod Fertil* 35:619–620.
- Picton HM. 2001. Activation of follicle development: The primordial follicle. *Theriogenology* 55:1193–1210.
- Salle B, Demirci B, Franck M, Berthollet C, Lornage J. 2003. Long-term follow-up of cryopreserved hemi-ovary autografts in ewes: Pregnancies, births, and histologic assessment. *Fertil Steril* 80:172–177.
- Shaw JM, Bowles J, Koopman P, Wood EC, Trounson AO. 1996. Fresh and cryopreserved ovarian tissue samples from donors with lymphoma transmit the cancer to graft recipients. *Hum Reprod* 11:1668–1673.
- Shimasaki S, Moore RK, Otsuka F, Erickson GF. 2004. The bone morphogenetic protein system in mammalian reproduction. *Endocr Rev* 25:72–101.
- Silva JR, van den Hurk R, van Tol HT, Roelen BA, Figueiredo JR. 2005. Expression of growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15), and BMP receptors in the ovaries of goats. *Mol Reprod Dev* 70:11–19.
- Vitt UA, Mazerbourg S, Klein C, Hsueh AJ. 2002. Bone morphogenetic protein receptor type II is a receptor for growth differentiation factor-9. *Biol Reprod* 67:473–480.
- Wandji SA, Srsen V, Voss AK, Eppig JJ, Fortune JE. 1996. Initiation in vitro of growth of bovine primordial follicles. *Biol Reprod* 55:942–948.
- Wandji SA, Srsen V, Nathanielsz PW, Eppig JJ, Fortune JE. 1997. Initiation of growth of baboon primordial follicles in vitro. *Hum Reprod* 12:1993–2001.
- Wang J, Roy SK. 2004. Growth differentiation factor-9 and stem cell factor promote primordial follicle formation in the hamster: Modulation by follicle-stimulating hormone. *Biol Reprod* 70:577–585.
- Yan C, Wang P, DeMayo J, DeMayo FJ, Elvin JA, Carino C, Prasad SV, Skinner SS, Dunbar BS, Dube JL, Celeste AJ, Matzuk MM. 2001. Synergistic roles of bone morphogenetic protein 15 and growth differentiation factor 9 in ovarian function. *Mol Endocrinol* 15:854–866.