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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

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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 BMPRIB and BMPRII 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 BMPRIB and BMPRII were also found in oocyte cytoplasm. In conclusion, expression of GDF9, BMP15 and their receptors BMPRIB and BMPRII 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

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.

Aside from recent autograft reports (Donnez et al., 2004; Oktay et al., 2004; Meirow 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),

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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 (TGFβ) 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 BMPRIB and BMPRII 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 mm^2 were prepared and cultivated on filters (0.4 μm 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 μg/L, bovine serum albumin (BSA) 0.5 mg/L, and linoleic acid 4.7 μg/L) (Sigma), BSA 1.25 mg/ml (Sigma), streptomycin 50 μg/ml (Sigma), and penicillin-G 75 μg/ml (Sigma).

The culture inserts were preequilibrated 2 hr before culture with 400 μl of culture medium in a humidified incubator under 5% CO_2, 5% O_2, and 90% N_2, 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 μm slices were cut by microtome (Leitz, Wetzlar, Germany). Twelve slices were cut per hemi-ovary and culture-time, at 60 μm 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 μm 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×.

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 mm^3), using TRIzol® reagent (Sigma), according to the manufacturer's protocol.

mRNA (10 μg) 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 μl (50 ng/μl) random hexamers (Invitrogen), 2 μl (0.1 M) DTT (Invitrogen), 1 μl dNTPs (10 mM each dNTP, Applied Biosystems), 4 μl 5X RT buffer (Applied Biosystems) in a final volume of 20 μl. The reaction was terminated by heating at 70°C for 10 min. cDNA was precipitated and resuspended in 50 μl 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 μl reaction mixture: 1X Taq polymerase reaction buffer (Eurobio, Les Ulis, France) containing 1 μl (0.5 μg/μl) of each primer, 1 μl 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
GFD9 1 (586 pb)	s	GTTCTGTATGATGGGCACGG	1981–2000	AF078545 Ovis aries
GDF9 2	as	AGGGCGCTACGAGAAGAGTC	3672–3691	AF078545 Ovis aries
BMP15 1 (483 pb)	s	CTATTGCCCACCTGCCTGAG	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 μl $MgCl_2$ (50 mM), 1.5 IU Taq DNA polymerase (Eurobio), and 10 μl 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 μl 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, BMPRIB and BMPRII

Slices (4 μm) were cut for each paraffin-embedded hemi-ovary on D0, D7, and D15. GDF9 and BMP15 were located on serial 4 μm 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 peroxydase 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 μg/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 μg/ml. After incubation with primary antibody, slices were washed in PBS and incubated for 30 min with the secondary anti-rabbit IgG conjugated to peroxydase (EnVision DakoCytomation, Carpinteria, CA). The slices were then washed twice and stained with diaminobenzidine complex (DAB+: DakoCytomation) for 1 min.

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

After two washes with deionized water, for GDF9, BMP15, BMPRIB 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, BMPRIB 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 = 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

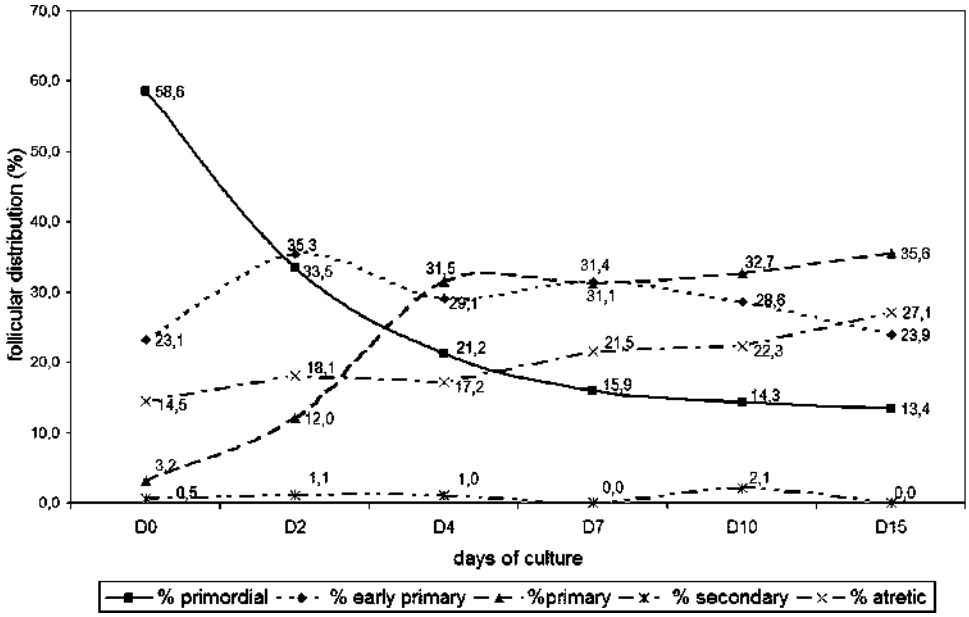

Fig. 1. Distribution of the atretic, primordial, early primary, primary, and secondary follicles in ewes during 15 days of culture.

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 BMPRIB and BMPRII

On D0, the various follicles types showed positive BMPRIB 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 BMPRIB 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; Meirow 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)

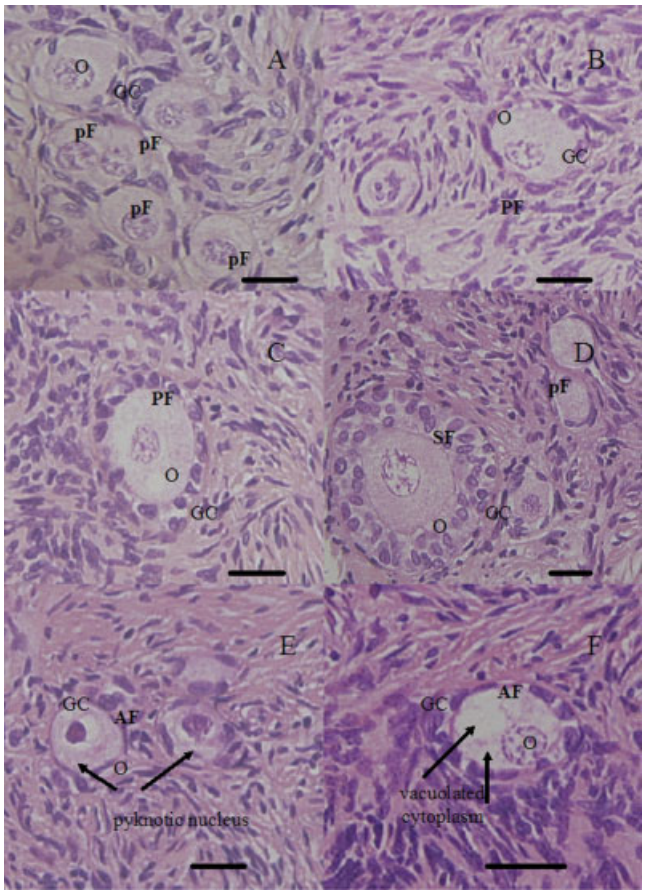

Fig. 2. morphological aspect of ovarian follicle, (HPS coloration). primordial follicles (**A**), primary follicle (**B**,**C**), secondary follicle (**D**), apoptotic follicles (**E**,**F**), pycnotic nucleus (E), and cytoplasmic vacuole (F). pF, primordial follicle; PF, primary follicle; SF, secondary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

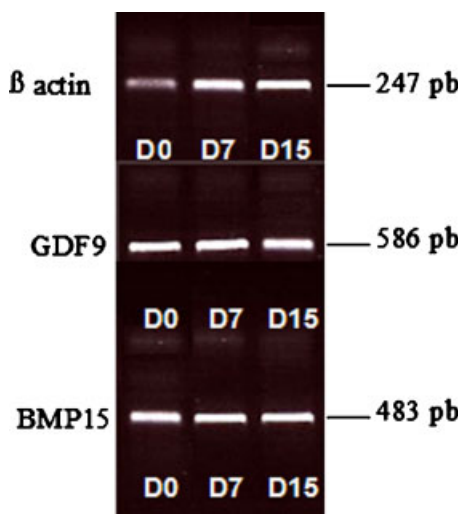

Fig. 3. Expression of GDF9, BMP15, and positive control β actin during culture on a representative gel. BMP15 and GDF9 are expressed at D0, D7, and D15 of culture.

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

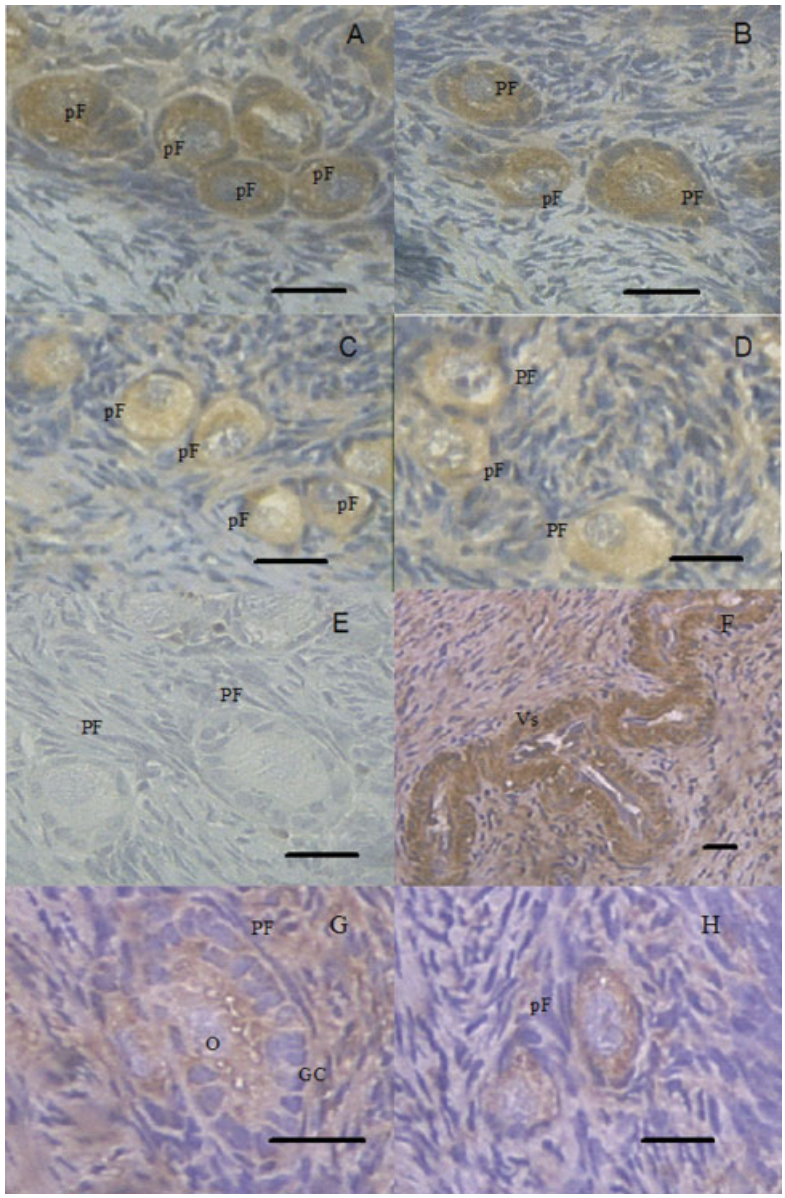

Fig. 4. BMP15, GDF9, BMPRIB and BMPRII immunoreactivity in sheep ovarian tissue. Positive immunoreaction for BMP15 at D0 (**A**) and D7 (**B**). Positive immunoreaction for GDF9 at D0 (**C**), D15 (**D**), and negative control (**E**). Positive immunoreaction for BMPRIB at D0 (**F**) and D7 (**G**).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

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 TGFβ 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. TGFβ 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). TGFβ superfamily members act via membrane-bound serine threonine kinase receptors. BMPRII and ALK5 (TGFβRI) have recently been identified as GDF9 receptors (Vitt et al., 2002; Mazerbourg et al., 2004), and BMPRII and ALK6 (BMPRIB) 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 BMPRIB 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 theirs receptors BMPRIB 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.

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