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Secretion profiles from *in vitro* cultured follicles, isolated from fresh prepubertal and adult mouse ovaries or frozen–thawed prepubertal mouse ovaries

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Summary

In vitro folliculogenesis could be a new technology to produce mature oocytes from immature follicles that have been isolated from cryopreserved or fresh ovarian tissue. This technique could also be a tool for evaluation of oocyte quality and/or for determination of follicular parameters during follicular growth. Our objective was to characterize in mice the secretion profiles of follicles that had been isolated mechanically during *in vitro* follicular growth and in relation to the growth curve. Early preantral follicles from fresh prepubertal and adult mouse ovaries or frozen–thawed prepubertal mouse ovaries were cultured individually in microdrops under oil for 12 days. Each day, two perpendicular diameters of the follicles were measured. From day-3 to day-12 of culture, culture medium was collected and preserved for determination of inhibin B, anti-Müllerian hormone (AMH) and estradiol levels. At the end of the culture, after maturation, the status of the oocyte was evaluated. Follicular growth and their individual hormone production did not always correlate. Inhibin B was never secreted from follicles of less than 200 μm diameter, whether the follicles were examined when fresh or after freezing–thawing. Estradiol secretion was never observed in frozen–thawed follicles. AMH was mainly secreted between day-3 and day-9. Despite similar morphological aspects at the start of culture, follicles selected for *in vitro* folliculogenesis were found to be heterogeneous and differed in their ability to grow and to produce hormones, even if they had similar growth curves. Follicles from frozen–thawed ovaries developed slowly and produced fewer hormones than freshly collected follicles.

Keywords: AMH, Estradiol, Follicular secretion, *In vitro* folliculogenesis, Mice

Introduction

In vitro folliculogenesis is a technology designed to produce mature oocytes starting from immature follicles isolated from fresh or from cryopreserved ovarian tissue. Most of the research on early folliculogenesis has been carried out in rodents. Rodent models offer several advantages: the growth phase *in vitro* is completed within 12 days and prepubertal mouse ovaries contain a high density of preantral follicles. Three types of culture models have been developed to grow follicles from mouse ovaries: (i) organ culture (Eppig & O'Brien, 1996); (ii) culture of enzymatically isolated cumulus–oocyte cell complexes (COCs) (Eppig & Schroeder, 1989); or (iii) culture of mechanically dissected intact preantral follicles (Boland *et al.*, 1993; Cortvrindt *et al.*, 1996). The second system was developed by Eppig's group (Eppig & Schroeder, 1989): ovaries of prepubertal mice were digested enzymatically and COCs were cultured in large groups on collagen membranes for 10 days prior to *in vitro* maturation. Eppig and colleagues demonstrated the capacity of their model by successfully obtaining live young. Others studies, with the same culture system, reported growth and development of mechanically intact follicular structures at the late

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preantral stage from older mice and for a short period (Nayudu & Osborn, 1992; Boland & Gosden, 1994; Hartshorne *et al.*, 1994; Spears *et al.*, 1994). A simplified culture system was developed by Cortvrindt *et al.* (1996) for *in vitro* maturation of early preantral mouse ovarian follicles. The follicles were cultured singly in 20 µl droplets under oil in a medium supplemented with recombinant follicle stimulating hormone at 37 °C and 5% CO_2 in air for an optimal period of 12 days. Their report showed that preservation of the three-dimensional structure of the follicle was not required for maturation of oocytes from primary stage to Graafian follicles. This model made it easy to follow and evaluate individual follicle development, without altering environmental parameter. The production of fertilizable mouse oocytes from cultures of small follicles isolated from cryopreserved ovarian cortex has been reported by many authors (Cortvrindt *et al.*, 1996; Newton *et al.*, 2001, Dela Peña *et al.*, 2002). To date, the mouse is the only species in which offspring have been obtained after *in vitro* growth, maturation and fertilization from the primordial stage (Boland *et al.*, 1993; Eppig & O'Brien, 1996).

In vitro folliculogenesis, in mice, has also been developed as a study model in order to evaluate and/or characterize follicular growth and oocyte maturation mechanisms (Pesty *et al.*, 2007). At the present time, little is known in detail about hormone secretion within follicles during *in vitro* follicular growth. Only few studies have reported hormone assays in medium collected during follicle culture. *In vitro* ovulation from individual ovarian follicles was first demonstrated in 1993 (Boland *et al.*, 1993). The same report demonstrated the secretion of estradiol and progesterone during follicle culture. The features of a 12-day *in vitro* follicular culture system in microdrops under oil for early preantral mouse follicles that permits the production of meiotically competent and fertilizable oocytes (including estradiol and progesterone production) were clarified (Cortvrindt *et al.*, 1996). Subsequently, secretion of hormones, essentially estradiol, testosterone and progesterone, produced *in vitro* by mice follicles have been used as a test of functionality (Demeestere *et al.*, 2002; Liu *et al.*, 2002; Wycherley *et al.*, 2004; Calongos *et al.*, 2008). An inhibin B assay has also been carried out in order to assess the physiological function of granulosa cells in culture (Newton *et al.*, 2001). However no study to date has reported anti-Müllerian hormone (AMH) assays during *in vitro* follicular growth. Indeed, little is known on the rate and kinetics of production of estradiol, inhibin B and AMH by follicles in culture.

The present study is designed to characterize the capacity of secretion of follicles isolated from their ovarian environment during *in vitro* folliculogenesis with or without prior freezing, in relation to follicular growth and maturation status of the oocyte. The parameters studied were: (i) the growth curve; (ii) oocyte meiotic maturation; and (iii) estradiol, inhibin B and AMH secretion.

Materials and methods

This study describes 20 follicles that were isolated from different origins: (i) fresh or frozen–thawed; (ii) prepubertal; or (iii) from adult ovaries.

Animals and collection of ovaries

Animals were obtained from Charles River Laboratories. F1 hybrid (C57Bl/6 × CBA) 12-day-old and 12-week-old female mice were used. At 12 days, murine ovaries contain mostly primordial and early preantral follicles, with one or two layers of granulosa cells and some theca cells (Cortvrindt *et al.*, 1996). Mice were sacrificed by cervical dislocation. The ovaries were removed and immediately transferred to a dissection medium, consisting of Brahma I (Cryo BioSystem®) supplemented with 10% fetal calf serum (FCS) (SVF, Gibco-BRL®), 100 IU/ml penicillin (Sigma-Aldrich®) and 100 µg/ml streptomycin (Sigma-Aldrich®). All animal experimentation described was conducted in accordance with accepted standards of humane animal care.

Cryopreservation and thawing of ovaries from prepubertal mice

Each ovary was transferred into a cryovial containing 1 ml of Brahma I, supplemented with 1.5 M dimethylsulfoxyde (DMSO, Sigma–Aldrich®), 10% FCS (Gibco-BRL®) and 10% sucrose (Sigma–Aldrich®) and was equilibrated for 30 min in the cryoprotective solution at 4 °C. The cryovials were then loaded into an automated freezer (Planer Kryo 360, CryoBioSystem®) and the temperature was lowered from 4 °C to –9 °C at a rate of 2 °C/min. After automatic seeding, the vials were cooled to –40 °C at a rate of 0.3 °C/min. Finally, the vials were cooled to –140 °C at 10 °C/min before being transferred to liquid nitrogen for storage. For thawing, cryovials were placed in the air for 30 s at room temperature, then for 5 to 10 min at 37 °C, then rinsed in four washing steps (DMSO at 1.5 M, 1 M, 0.5 M and 0 M) (Oktay *et al.*, 2000) and placed in the dissection medium.

Isolation and selection of follicles

Follicles were isolated from fresh or frozen–thawed ovaries by mechanical dissection under a stereomicroscope, using 27-gauge sterile needles to ensure that the follicular structure remained intact. Isolated follicles

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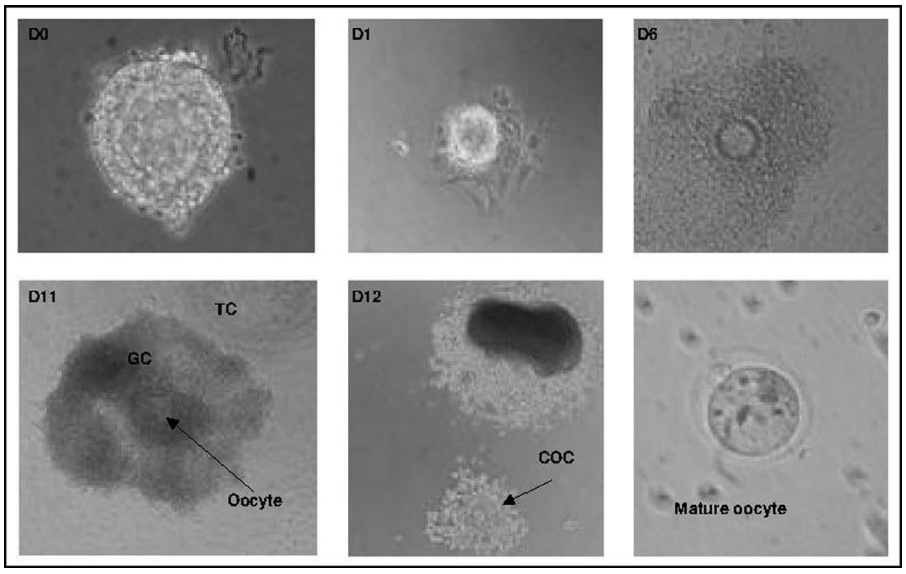

Figure 1 Different aspects of follicles during culture. D = day of culture. COC = cumulus–oocyte complexes; GC = granulosa cells; TC = theca cells.

were selected based on the following criteria: intact follicle with two or three layers of granulosa cells and some adhering theca cells, with a clearly visible, round, central oocyte and with a follicle diameter between 100 and 130 μm.

Follicle culture

Once isolated, the follicles were rinsed three times in the dissection medium and put in culture using the open model developed by Cortvrindt *et al.* (1996). Each follicle was cultured individually in 20 μl microdrops, under detoxified mineral oil (Cryo BioSystem®), at 37 °C, in a medium equilibrated overnight before the start of culture, under an atmosphere containing 5% CO_2 in air. The culture medium consisted of α-MEM GlutaMAX (Gibco-BRL) supplemented with 5% FCS, 10 μg/ml transferrin (Boehringer Mannheim®), 5 g/ml insulin (Boehringer Mannheim®) and 100 mIU/ml recombinant follicle stimulating hormone (FSH) (Schering-Plough). Petri dishes were identified with a letter (A–Z). Ten follicles were cultured per Petri dish. The follicles were cultured for 12 days: day-1 was defined as the first day of the culture.

One hundred and ninety-eight fresh follicles and 98 frozen–thawed follicles from prepubertal ovaries and 54 fresh follicles from adults' ovaries were cultured.

Collection of culture medium

Every day, from day-3 to day-12 of follicle culture onwards, the culture medium was refreshed by collecting and then adding 10 μl of medium. The 10-μl samples of collected medium from surviving follicles were diluted to 1 in 16 in α-MEM GlutaMAX supplemented with 40 mg/l BSA (Sigma-Aldrich®), and frozen at –20 °C, follicle per follicle, for later assay of inhibin B, AMH and estradiol. This dilution used was the minimum one necessary to carry out assays.

Measurement of follicle and oocyte diameters

Each day of the experiment, follicles were observed under an inverted microscope (Nikon). Only those follicles with all components completely individual, that is, with a central oocyte surrounded by a granulosa cell mass and a peripheral theca cell monolayer, were examined. For each follicle, two perpendicular diameters were measured using a calibrated ocular micrometer, at magnifications of ×100 or ×200. Follicle diameter measurements considered the granulosa cell mass, but not the theca cells (Figure 1). Follicles with developmental irregularities such as a non-spherical or non-central oocyte, or with evidence of atresia, or a dark granulosa layer, were disregarded.

In vitro oocyte maturation

At day-12 of culture, the intact follicles were transferred to 20 μl microdrops of maturation medium under oil. Oocyte maturation was induced by the addition to the culture medium of 1.5 IU/ml human chorionic gonadotrophin (hCG) (Sigma–Aldrich®) and 5 ng/ml epidermal growth factor (EGF) (Boehringer Mannheim®). Sixteen to 20 h later, the released oocytes were classified according to their maturation stage as

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Table 1 Follicles used in the study

Follicle origin (*n*)	Follicular growth	Oocyte status after *in vitro* maturation (follicle no.)
Prepubertal mice, fresh (9)	Good	MII (D7, E10, E1)
	Medium	MII (D4, F1); MI (C1 H8)
	Absence of	GV (A7, A2)
Prepubertal mice, frozen–thawed ovaries (7)	Good	MII (H5, G1)
	Medium	MII (H10); GV (G2, G9)
	Absence of	GV (I6, H2)
Adult mice, fresh (4)	Good	MII (R8)
	Medium	MII (Q4); MI (R5)
	Absence of	GV (R7)

Twenty follicles were selected *a posteriori* for study according to their origin (prepubertal or adults ovaries), their treatment (fresh or frozen–thawed follicles), their growth curve (good, medium or absence of follicular growth) and the oocyte status after *in vitro* maturation (germinal vesicle [GV], metaphase I [MI] or metaphase II [MII]). According to their place in each Petri dish, follicles were designated by the letter given to the petri dish (A to Z) and the number of the microdrop containing the follicle (1 to 10).

germinal vesicle (GV), as oocyte blocked in metaphase I (MI), or as in metaphase II (MII).

Follicles selected for the present study

Follicles were chosen *a posteriori* at random within each group, based on three criteria: (i) their origin (fresh vs. frozen–thawed and prepubertal vs. adult ovaries); (ii) their size at day-12 of culture: good follicular growth (GFG) if size was $\geq$ 500 μm, medium follicular growth (MFG) if the size was between 200 and 500 μm or absence of follicular growth (AFG) if the size was <200 μm at day-12; and (iii) oocyte nuclear status at day-12 after *in vitro* maturation (GV, MI or MII).

The follicles were identified by the letter given to the Petri dish (A–Z) in which they were located and by the number of the microdrop in which they were cultured (1–10). In total, 20 follicles were chosen (Table 1). (1) Nine fresh follicles isolated from prepubertal mouse ovaries: (a) three follicles with a GFG (D7, E10, E1): a MII oocyte was obtained in every instance after *in vitro* maturation; (b) four follicles with a MFG split into two with a MII oocyte (D4, F1) and two with a MI oocyte (C1, H8); and (c) two follicles with AFG (A7, A2), whose oocyte was GV. (2) Seven follicles isolated from frozen–thawed prepubertal mice ovaries: (a) two follicles with a GFG and a MII oocyte each time (H5, G1); (b) three follicles with a MFG: one with a MII oocyte (H10), and two with a GV oocyte (G2, G9); and (c) two follicles with AFG (I6, H2) and a GV oocyte. And (3) four fresh follicles isolated from adult mouse ovaries: (a) one follicle with GFG (R8) and a MII oocyte; (b) two follicles with a MFG : one with a MII oocyte (Q4), and one with a MI oocyte (R5); and (c) one follicle with AFG (R7) and a GV oocyte.

Hormone assays from individually cultured follicles

Hormone assays were carried out on culture medium samples collected daily, for each follicle, from day-3 to day-12. Out of the 200 culture medium samples expected, 177 were able to be analysed, however 23 samples were 'missing'. Three series of assays were organized, each series was composed of samples that had various types of follicular growth (GFG, MFG or AFG) and that had different origins or had different features (fresh vs. frozen–thawed; prepubertal vs. adult; mature vs. immature oocyte). The hormone assays were performed in the following order: first 100 μl were used for inhibin B assay, then 20 μl for AMH and finally estradiol from the remaining volume adjusted to 50 μl with the dilution medium.

Estradiol assay

Estradiol was measured from 50 μl of diluted culture medium with an RIA from Clinical Assay (Estradiol-2; DiaSorin). The analytical sensitivity and inter-assay coefficient of variation were 5 pg/ml and <10% respectively for all measurement ranges. The radioimmunoassay (RIA) technique was chosen for its very high sensitivity. Under the various dilutions, the smallest detectable concentration of estradiol was 135 pg/ml. Below that concentration, the E2 secretion was characterized as 'absent'.

Inhibin B assay

The amount of inhibin B was measured by ELISA starting from 100 μl of culture medium using the OBI-DSL ultra-sensitive kit (Reference: MCA 1312KZZ). Within a measurement range of 5–1000 pg/ml, the intra-assay and inter-assay variation coefficients were both <10%. For values between 1000 and 2000 pg/ml, the variation coefficient was between 10 and 20%. The analytical sensitivity was 5 pg/ml.

Anti-Müllerian hormone assay

AMH was determined starting from 20 μl of culture medium using Diagnostic Systems Laboratories Active® MIS/AMH (Müllerian inhibiting substance/anti-Müllerian hormone) (Reference DSL-10–14400). For AMH determination, the intra-assay and inter-assay variation coefficient were <8% for all measurement ranges. The detection limit was 0.10 ng/ml.

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Results

The results presented refer to the final concentration, taking into account the required dilution rate. In order to present all the parameters in the same figure, the estradiol curve was represented using estradiol results divided by 10.

Follicular growth

For fresh follicles from prepubertal ovaries (n = 198) (Figure 2)

For follicles with a GFG, follicular growth had two phases: (i) slow growth until day-6 (E1, E10) or day-8 (D7); and (ii) subsequent acceleration; which coincided with a loss of spherical structure. Antrum formation could be observed on day-11. For follicles with a MFG, even if they reached a similar final size, follicular growth curve appeared to be different. F1 follicle reached its final size at day-10, contrary to D4, H8 and C1. For follicles with AFG, follicular growth was quasi non-existent.

For frozen–thawed follicles from prepubertal ovaries (n = 98) (Figure 3)

For follicles with a GFG (H5, G1), follicular development had two phases: (i) slow growth until day-8; and (ii) subsequent acceleration. Among follicles with a MFG, the H10 follicle with a MII oocyte after *in vitro* maturation did not show growth acceleration until day-10, contrary to G9 and G2 follicles with a MI oocyte after *in vitro* maturation whose growth curve showed acceleration at day-8. For follicles with AFG, follicular growth was quasi non-existent.

For fresh follicle from adults ovaries (n = 54) (Figure 4)

In a follicle with a GFG (R8), follicular growth acceleration started on day-5. In follicles with a MFG, follicular growth acceleration was observed starting on day-8 for R5 with a MII oocyte after *in vitro* maturation and starting on day-7 for Q4 with a MI oocyte after *in vitro* maturation. For follicles with AFG, follicular growth was quasi non-existent.

Secretion profiles of follicles

Fresh follicles from prepubertal ovaries (Figure 2)

For follicles with a good follicular growth (GFG) (E1, E10 and D7). Estradiol was undetectable (<135 pg/ml) until day-6 for E1 and E10 and until day-9 for D7, and each time until the follicle size had reached 200 μm. Increasing amounts of estradiol were then released, with a sharp rise from day-6 to day-10 with a final level ranging from 1159 to 2490 pg/ml. A major peak of estradiol (5767 pg/ml) was observed, on day-9, in E1 only. A similar curve was recorded for inhibin B secretion, with a sharp rise at day-6 for E1 and E10 and at day-9 for D7. However, final inhibin B concentrations were very similar for E1, E10 and D7, even though their follicular growth curves were different. Inhibin B production always started when the follicle size reached 200 μm. AMH was mainly produced from day-3 (2 ng/ml) to day-9 (14, 6 ng/ml for D7) with a peak at day-6/day-7. A decrease in AMH secretion was observed when the follicle size exceeded 600 μm. All these follicles with a GFG (E1, E10 and D7) presented an oocyte in metaphase II after *in vitro* maturation.

In follicles with a medium follicular growth (MFG) (F1, D4, H8, C1). F1 and D4 follicles with a metaphase II oocyte after *in vitro* maturation did not produce estradiol. A small rate of estradiol production was observed in the C1 follicle at day-12 and in H8, starting on day-9 and each time the follicle diameter reached 200 μm. These two follicles presented a MI oocyte after *in vitro* maturation. Regarding inhibin B production, F1 with a metaphase II oocyte after *in vitro* maturation and H8 with a MI oocyte after *in vitro* maturation secreted in the same way inhibin B when their follicle size reached 200 μm. In H8, inhibin B production coincided with estradiol secretion. In C1 a small rate of inhibin B was recorded before the follicle size had reached 200 μm. AMH production was poor in all the MFG follicles and never exceeded 4.3 ng/ml.

In follicles with absence of follicular growth (AFG) (A7, A2). Secretion profiles of follicles with no follicular growth and with a GV oocyte after maturation *in vitro*: A7, A2 did not show secretion of either estradiol or inhibin B, but small quantities of AMH (<6 ng/ml) were secreted between day-7 and day-10.

Frozen–thawed follicles from prepubertal ovaries (Figure 3)

In follicles with a good follicular growth (GFG) (H5, G1). No estradiol secretion was recorded during follicular culture, even though a metaphase II oocyte was obtained after *in vitro* maturation. A curve similar to that obtained with fresh follicles with GFG was determined for inhibin B secretion but with a delay: the production was undetectable until day-9. Inhibin B secretion started when the follicle size exceeded 200 μm. A lower and later production of AMH was recorded for GFG follicles compared to fresh follicles. This delay was correlated with the follicular growth curve. A lower secretion of AMH was observed in fresh GFG follicles of 350–400 μm diameters, compared to those of 600 μm diameters. These two follicles with a GFG presented a metaphase II oocyte after *in vitro* maturation.

In follicles with a medium follicular growth (MFG) (H10, G9, G2). No estradiol production was found. Inhibin B production in the H10 follicle, with a MII oocyte after *in vitro* maturation, was quasi non-existent contrary

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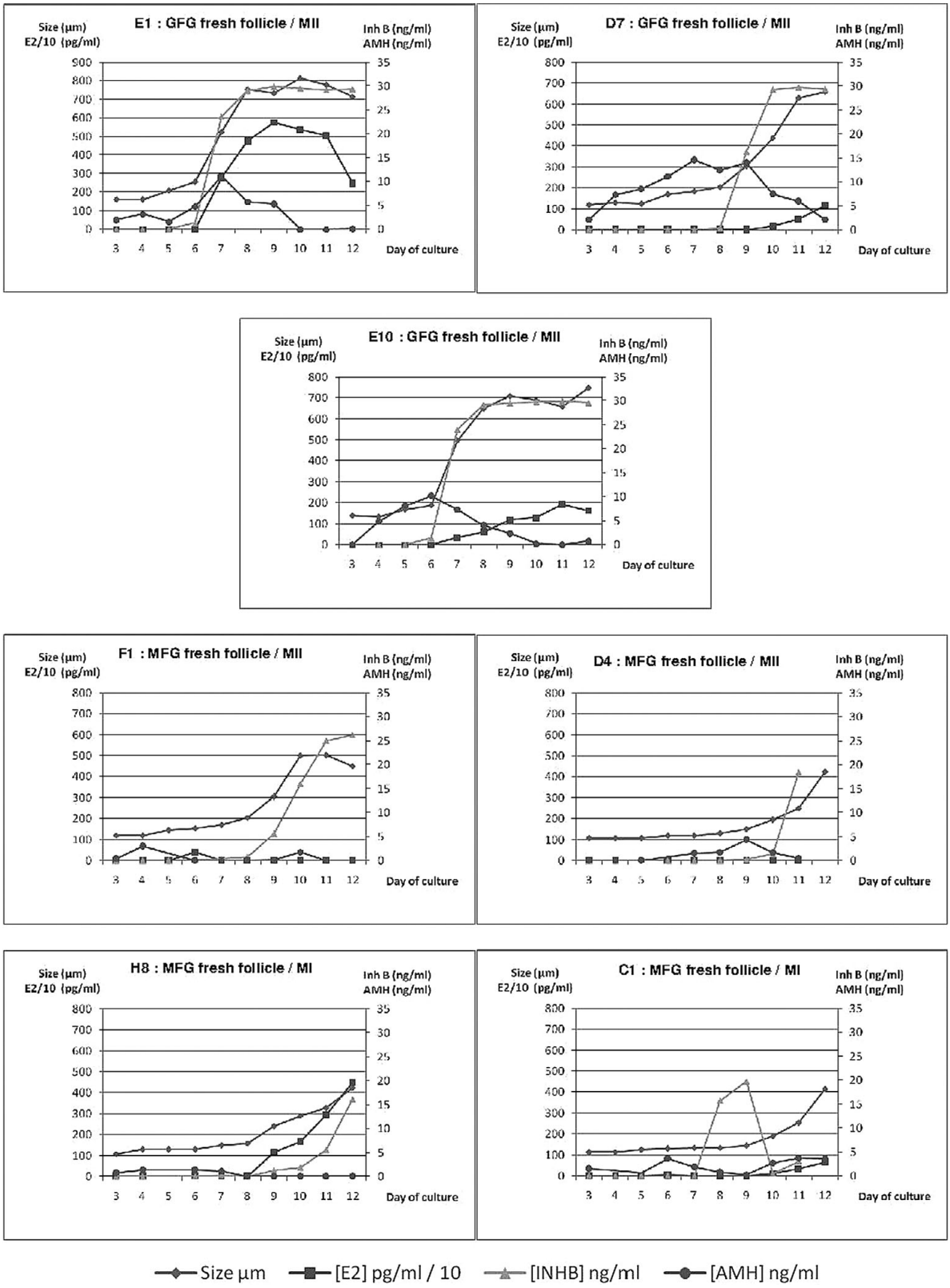

Figure 2 Secretion profiles from studied fresh follicles from prepubertal ovaries with a good (GFG) and medium (MFG) follicular growth. The estradiol graph corresponds to estradiol concentration(E2)/10. On each graph, the identification of the follicle is given. AMH, anti-Müllerian hormone; Inh B, inhibin B.

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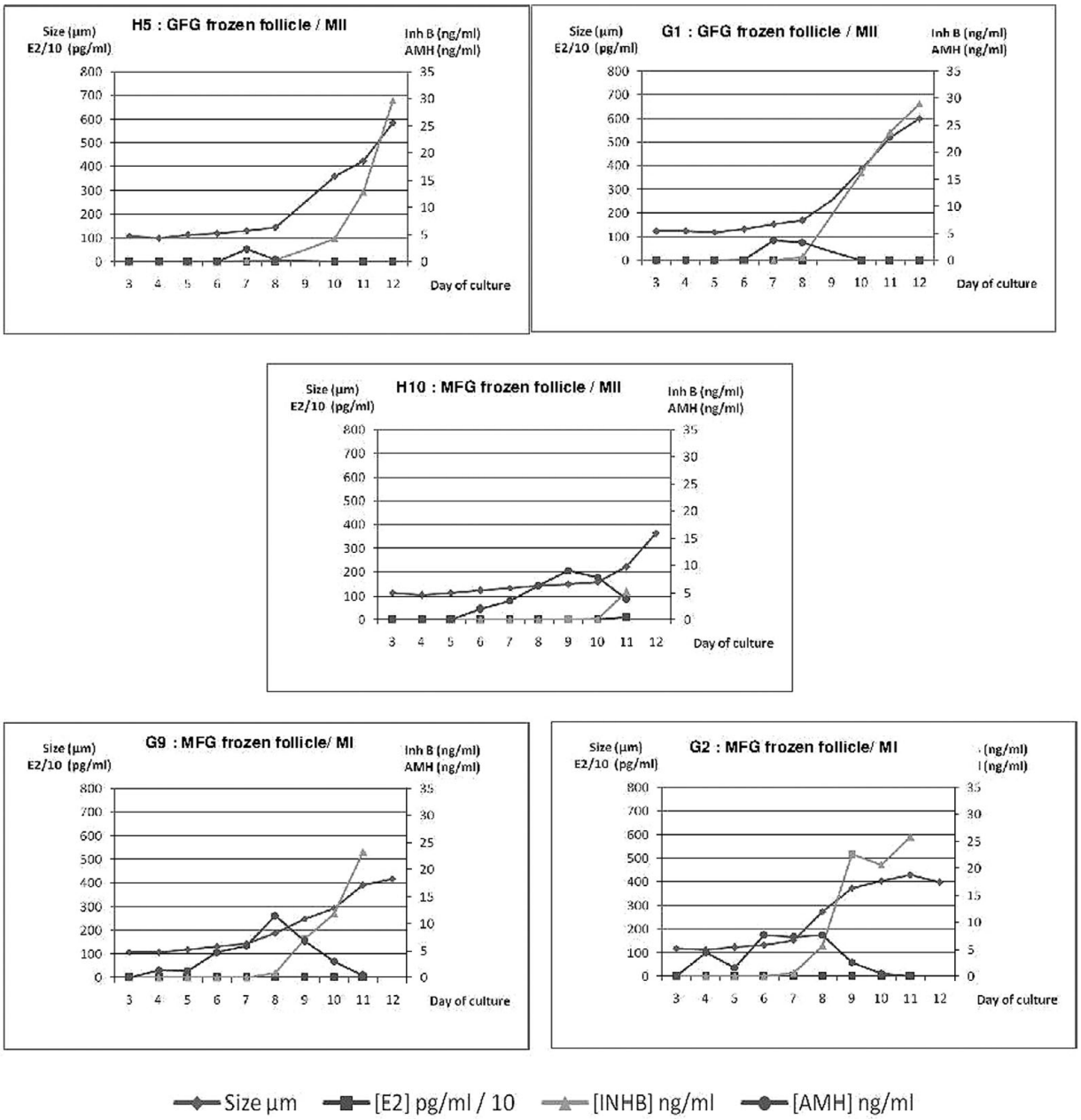

Figure 3 Secretion profiles from frozen–thawed follicles from prepubertal ovaries with good (GFG) or medium (MFG) follicular growth. The estradiol (E2) graph corresponds to estradiol concentration/10. On each graph, the identification of the follicle is given. AMH, anti-Müllerian hormone; Inh B, inhibin B.

to that in the G9 and G2 follicles with a MI oocyte after *in vitro* maturation, with a sharp rise of secretion from day-8 or day-9 when the follicle size exceeded 200 µm. H10 showed AMH secretion between day-6 and day-11. G2, G9 follicles secreted AMH, from day-4 to day-10. No AMH was detectable once the follicle size exceeded 400 µm.

In follicles with absence of follicular growth (AFG) (I6, H2). In frozen–thawed follicles with no follicular growth no secretion of either estradiol, inhibin B, or AMH was recorded. Those follicles with AFG presented with a GV oocyte after *in vitro* maturation.

Fresh follicles from adult ovaries (R5, R8, Q4, R7) (Figure 4)

Estradiol concentrations remained under 135 pg/ml in all fresh follicles from adult ovaries, whatever their follicular growth curve was. Inhibin B secretion profiles were similar to those in fresh follicles from prepubertal ovaries. Inhibin B production started once the follicle size reached 200 µm. AMH production was

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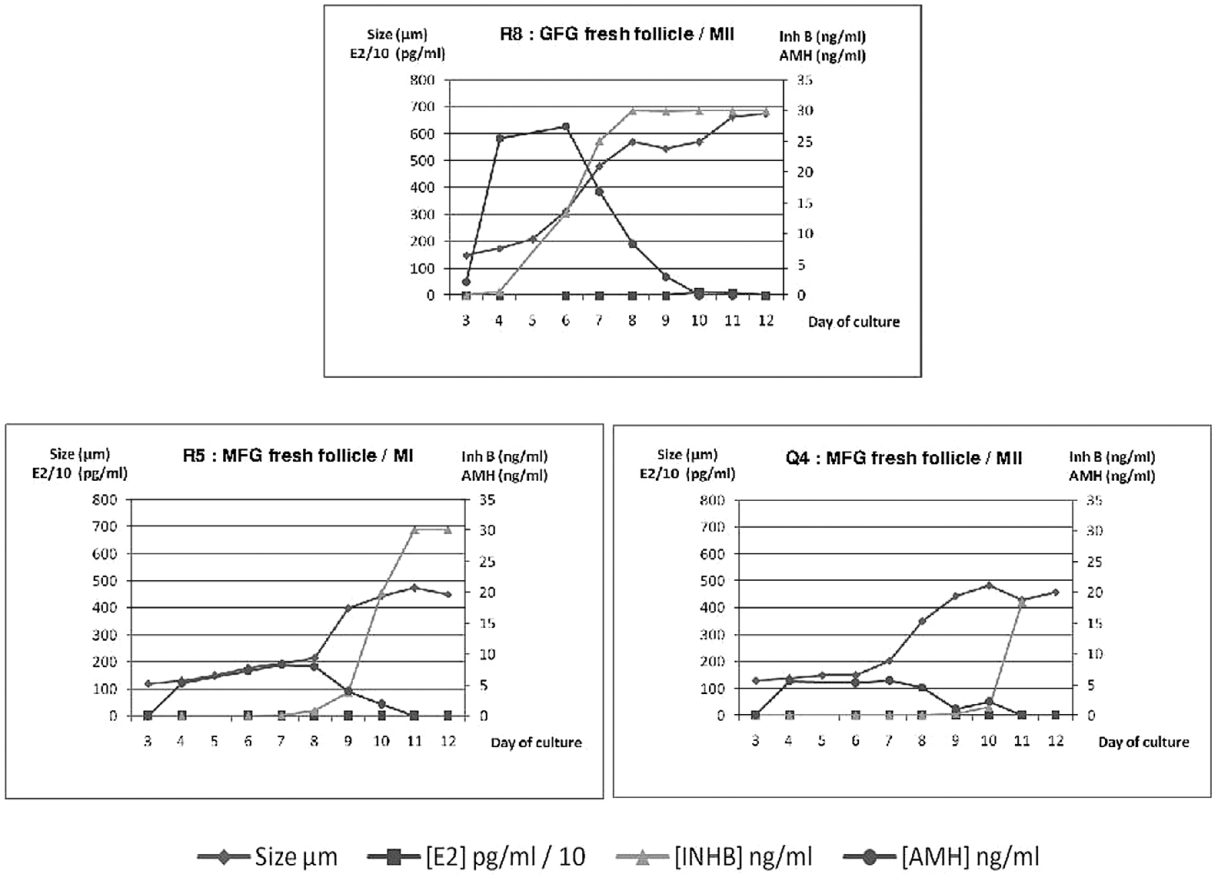

Figure 4 Secretion profiles from fresh follicles from adults' ovaries with good (GFG) or medium (MFG) follicular growth. The estradiol (E2) graph corresponds to estradiol concentration/10. On each graph, the identification of the follicle is given. AMH, anti-Müllerian hormone; Inh B, inhibin B.

three times higher in GFG adult follicles (25 ng/ml at day-4) than in GFG fresh prepubertal follicles. However, we observed a decrease in AMH secretion once the follicle size reached 500 µm in diameter.

Discussion

This study was designed to characterize in relation to follicular growth the secretion capacity of fresh or frozen-thawed follicles that had been isolated from their ovarian environment during *in vitro* folliculogenesis. The aim of this report was to describe isolated follicles secretion and not elaborate some correlation, it is why this uses 20 follicles, corresponding to a large number of assays. To our knowledge, this is the first report to describe secretion from a single isolated follicle under these experimental conditions i.e. follicles cultured individually for 12 days in microdrops of culture medium under oil. This paper also describes for the first time assay of AMH in the culture medium during *in vitro* folliculogenesis.

Our results on follicular growth are in keeping with those in previously described in the literature (Cortvrindt *et al.*, 1996; Mousset-Simeon *et al.*, 2005). Follicular development of GFG fresh follicles (E1, E10 and D7) showed two phases: (i) a slow growth until day-5; followed by (ii) acceleration in follicles freshly isolated from ovaries. At day-11, a small cavity that corresponded to reaching the antral stage was observed. Follicles isolated from frozen–thawed prepubertal mouse ovarian tissue developed slowly compared with freshly collected follicles. Contrary to Newton's observations, in our experiment, at the end of the culture period, the diameter of the frozen–thawed follicles was similar to that of fresh ones, based on our follicle selection (Newton *et al.*, 2001). Moreover, such frozen–thawed follicles were found to produce mature oocytes (H5, G1 and H10). Newton and colleagues observed in their report that, after 8 days of individual culture in 96-well plates, fresh follicles had a larger diameter than frozen–thawed follicles (Newton *et al.*, 2001). They indicated that fresh follicles had a larger granulosa cell population at the end of culture; which may due to delayed proliferation or to early cell death of the granulosa cells as a result of the freezing/thawing process.

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Very few papers have evaluated hormone production during *in vitro* folliculogenesis. The first study reporting hormone production by ovarian follicles in a culture medium was that of Boland and colleagues (in 1993). Follicles were cultured individually in 20 µl droplets under oil for 6 days on a 96V-well microtitre plate. Estradiol production was found to increase at day-3 and decrease at day-6, whether or not LH was added to the culture medium. In further studies, hormone production was used as a marker for follicular growth, i.e. to evaluate a novel follicle culture system (Cortvrindt *et al.*, 1996; Liu *et al.*, 2002; Wycherley *et al.*, 2004; Xu *et al.*, 2006), to assess the effect of the isolation procedure of mouse preantral follicles on their subsequent *in vitro* development (Demeestere *et al.*, 2002), or the effects of supplements such as IGF1 (Demeestere *et al.*, 2004), aromatase inhibitor (Hu *et al.*, 2002) or melatonin (Adriaens *et al.*, 2006) on *in vitro* follicular development. Recently, follicular secretion was measured to test if follicular somatic cell steroidogenesis could be used as an indicator for quality of the enclosed oocytes (Liu *et al.*, 2006). Estradiol production was measured in these various studies, but inhibin B secretion was only evaluated in two studies (Cortvrindt *et al.*, 1996; Newton *et al.*, 2001), and AMH production in none. *In vitro* steroidogenesis was measured for a shorter time culture period (Boland *et al.*, 1993) compared with other studies or was measured in a different experimental model, such as culture on 96-well plates (Boland *et al.*, 1993; Wycherley *et al.*, 2004). Even in studies involving singly cultured follicles, hormone production was evaluated on a pool of collected medium, from follicles that were morphologically and functionally quite different (Cortvrindt *et al.*, 1996; Liu *et al.*, 2002; Segino *et al.*, 2005). One report evaluated hormone production follicle by follicle, but the results were expressed as means (Segino *et al.*, 2005). Liu and colleagues did measure and report hormone production follicle by follicle (Liu *et al.*, 2006) using the same system as described here but for only 10 days of culture. Our study, therefore, for the first time describes the analysis of single isolated follicles rather than examination of a pool.

In this study, estradiol production by follicles in culture and their kinetics were variable dependent upon the type of follicle and varied even among follicles with similar growth curves. Therefore, we describe here values for individual follicles rather than means of several follicles.

Our results regarding estradiol production in follicles isolated from fresh prepubertal mouse ovaries are in keeping with those reported in earlier studies in terms of values (Cortvrindt *et al.*, 1996; Liu *et al.*, 2002; Segino *et al.*, 2005). Estradiol secretion in our study ranged from 1159 and 5767 pg/ml at day-10 for GFG fresh follicles, compared with Cortvrindt *et al.* who had found an average of 3000 ng/l per 48 h at day-10. Segino *et al.* evaluated estradiol secretion at day-12, day-14 and day-16 for fresh and frozen–thawed mouse follicles. They found a significantly lower estradiol production in follicles from frozen–thawed ovaries (1339 pg/ml) than in follicles from fresh ovaries (3091 pg/ml; $p < 0.001$) at day-12. These amounts of estradiol indicated that, despite the loss of three-dimensional structure, the culture system retained the integrity of follicular structure: granulosa, theca and oocyte remained functional, as described previously by Cortvrindt *et al.* (1996). Liu *et al.* confirmed that estradiol concentrations in the samples of medium collected could be used to monitor follicular estradiol production *in vitro* even when follicles are cultured in microdrops under oil (Liu *et al.*, 2002). Liu *et al.* collected and pooled samples of follicle culture medium from the same day of culture. As estradiol is lipid soluble, it can easily diffuse into the covering oil. When comparing estradiol concentrations in the culture medium with that in adjacent oil throughout the 12-day culture, they found that only 1% of the follicular secretory products had diffused into adjacent oil. Conversely, they found a correlation between estradiol concentrations from day-4 to day-10 and granulosa cell proliferation. Boland *et al.* also measured estradiol and progesterone concentrations in culture medium samples collected every 24 h from individual primary follicles cultured in 96-well plates for 6 days (Boland *et al.*, 1993). He reported that estradiol production was higher in cultures supplemented with FSH, but that progesterone was undetectable, suggesting that premature luteinisation did not occur during culture. *In vivo*, FSH is essential for aromatase enzyme activity (P450 aromatase) stimulating steroidogenesis, for granulosa cell differentiation by stimulating the expression of LH receptors and for follicular antrum formation. In the culture medium FSH stimulates estradiol production and may also stimulate follicles to grow in size or granulosa cell density to increase: estradiol was detected in mouse follicles *in vitro* starting at the time of antrum formation, when the diameter reached a minimum of 300 µm (Boland *et al.*, 1993). Another study involving estradiol assays during *in vitro* folliculogenesis showed an increase in production at day-6 (Wycherley *et al.*, 2004).

In our study, estradiol was undetectable in follicles isolated from fresh ovaries until their diameter reached 240 µm. Then a subsequent rise occurred when follicular growth started, at least, in E1, E10, D7 and H8 follicles. But our data demonstrate that a good follicular growth and a metaphase II oocyte can be obtained even in the absence of estradiol secretion. Indeed, follicles with an average follicular growth may or may not produce estradiol and may or may not

produce a metaphase II oocyte (F1, D4 and H8). This was demonstrated earlier by addition of an aromatase inhibitor to the culture medium (Hu *et al.*, 2002).

Whereas we observed an absence of estradiol production during follicular culture of follicles from frozen–thawed ovaries, Segino *et al.* in 2005 reported that on the 16th day of culture, the diameter and estradiol production of follicles isolated from frozen–thawed ovaries reached the same level as that in fresh follicles on day-12 (Segino *et al.*, 2005). Possibly, the duration of culture in our experiment was too short, and we might have observed estradiol production if we had extended the culture of follicles to 16 days as Segino *et al.* did. A methodological problem can be ruled out as we found estradiol in each series of assays on samples from follicles with good or medium follicular growth and in the absence of follicle growth. Yet, the necessary dilution of the samples analysed may have been such that the concentration of estradiol was below the sensitivity threshold of our assay technique. But undetectable estradiol results may reveal a very weak concentration. Alternatively, this situation could result from the freezing–thawing process on granulosa cell proliferation (Cortvrindt *et al.*, 1996; Newton *et al.*, 2001). Conversely, in our study, estradiol was undetectable in the medium from follicles isolated from frozen ovaries, even if their oocyte reached metaphase II stage after maturation *in vitro* (H5, G1 and H10). Our hypothesis is that the freezing–thawing process may also delay aromatase mechanisms, or alter the communication pathway between theca and granulosa cells. It may be interesting to assay Δ4-androstedione in the culture medium in forthcoming studies.

An absence of estradiol was found during culture of follicles from adult mice even when follicular growth was normal and when a metaphase oocyte was obtained under *in vitro* maturation. An earlier study had demonstrated that mature oocytes can be obtained by culture of preantral follicles retrieved from adult mice but no estradiol assay in the culture medium was made (Kim *et al.*, 2009). Thus, our study may be the first to demonstrate the surprising absence of estradiol by preantral adult follicles grown *in vitro*.

Our study confirmed that inhibin B is secreted by growing follicles, and it reflects specifically the growth of granulosa cells (Cortvrindt *et al.*, 1996). Indeed, no inhibin B secretion was found in non-growing follicles. In estradiol-secreting follicles, inhibin and estradiol production started at the same time. From then on, inhibin B production in MFG fresh or frozen–thawed follicles kept increasing (F1, D4, H8, H10, G9 and G2). In GFG fresh follicles (E1, D7, E10 and R8), the values reached the plateau which may reflect the limits of the assay technique i.e. reaching the higher limit of measurement range. Moreover, we never observed, in our culture system inhibin B secretion until the follicle size reached 250 μm, whatever the follicular growth curve, the origin or the completion of a metaphase II oocyte at day-12 with one exception (C1). Our results are not in keeping with those of Newton *et al.* who found that fresh follicles produced more inhibin B than follicles isolated from frozen–thawed tissue (Newton *et al.*, 2001).

As far as we are aware from the current literature, this study is the first one to demonstrate wide differences in AMH concentration in the culture medium of individual follicles. AMH was found to be actively produced between day-3 and day-9 of *in vitro* folliculogenesis in mice. AMH decreased when estradiol and inhibin B secretion increased significantly, that is when follicular growth started, that is to say when follicle size exceed 600 μm. This study confirms that AMH is mainly secreted by small preantral follicles, from secondary to early tertiary stages, whereas no more AMH is produced once a pre-antrum cavity has formed.

AMH production was lower and delayed in all fresh or frozen–thawed follicles, with MFG and never exceeded 4.3 ng/ml, lower than in GFG fresh or frozen–thawed follicles. AMH secretion was quasi non-existent for AFG follicles whatever their origin.

Interestingly, we found AMH level in the medium used for culture of adult ovaries to be three times higher than in that of prepubertal ovaries. AMH inhibition of aromatase activity could explain the lake of estradiol secretion by granulosa cells as shown by Josso and colleagues (2001). It may be that adult follicles acquire gradually the capacity to remove an intracrine inhibitor present in prepubertal ovaries.

In our study, non-growing follicles with a GV oocyte never produced any hormones after *in vitro* maturation.

Our study suggests that the normal nuclear/cytoplasm oocyte maturation probably requires a specific follicular size. But critical details of oocyte maturation, including both nuclear and cytoplasmic elements, are still poorly understood (Eppig *et al.*, 1994). In the culture system described by Cortvrindt *et al.* (1996) and in our own study, the normal three dimensional follicular structure was lost, but all components: theca cells, granulosa cells and oocytes, remained functional, as demonstrated by estradiol, inhibin and AMH secretion. The acquisition of meiotic competence by mouse oocytes coincides with the time of antrum formation. The proportion of oocytes that achieved nuclear maturation was not correlated with hormonal profile secretion. Follicles that exhibited medium growth produced estradiol, inhibin B and AMH during culture or not, irrespective of the oocyte final maturation stage. The capacity to obtain a metaphase II oocyte cannot be predicted from the

hormonal secretion profile. As all follicles with good growth gave mature oocytes, we were not able to demonstrate a positive correlation between the level of estradiol production and the percentage of mature oocytes obtained. Non-growing follicles produced only GV stage oocytes after *in vitro* maturation. Actually, this report questions a lot, and particularly concerning the difference found between fresh and frozen prepubertal and adult follicles at the same stage in term of *in vitro* secretion.

To conclude, folliculogenesis is still poorly understood. The culture of individual ovarian follicles is one approach to the problem that makes it possible to determine the secretion profile of maturing follicles independent of the ovarian environment. Our study demonstrated that, in culture, follicles with similar morphological aspects are quite heterogeneous in their ability to grow and to produce hormones. The reason why such differences exist is still not clear and requires further investigations. As long as such differences cannot be explained, it does not appear justified to pool samples prior to hormone determination. AMH production during *in vitro* folliculogenesis was measured for the first time. Follicular growth, hormone secretion profile and oocyte maturation appeared not to be closely correlated. However, we observed that inhibin B and estradiol secretions became effective only after follicles reached 250 μm in size and that no AMH was produced in follicles larger than 600 μm in diameter. No estradiol secretion was detected in follicles isolated from frozen–thawed ovarian tissue and from adult ovaries. In adult follicle AMH levels were three times higher than in prepubertal follicles. Follicular hormone production seems to be highly specific to each follicle. Mice constitute a relevant model to improve our understanding of *in vitro* follicle culture. The present study suggests that it could be useful to investigate hormonal levels *in vitro* in culture medium during human follicle culture.

Disclosure statement

The authors have nothing to disclose.

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