

Impact of various endocrine and paracrine factors on *in vitro* culture of preantral follicles in rodents

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Abstract

Folliculogenesis is a complex process regulated by various paracrine and autocrine factors. *In vitro* growth systems of primordial and preantral follicles have been developed for future use of immature oocytes, as sources of fertilizable oocytes and for studying follicular growth and oocyte maturation mechanisms. Rodents were often chosen for *in vitro* follicular culture research and a lot of factors implicated in folliculogenesis have been identified using this model. To date, the mouse is the only species in which the whole process of follicular growth, oocyte maturation, fertilization and embryo transfer into recipient females was successfully performed. However, the efficiency of *in vitro* culture systems must still be considerably improved. Within the follicle, numerous events affect cell proliferation and the acquisition of oocyte developmental competency *in vitro*, including interactions between the follicular cells and the oocyte, and the composition of the culture medium. Effects of the acting factors depend on the stage of follicle development, the culture system used and the species. This paper reviews the action of endocrine, paracrine factors and other components of culture medium on *in vitro* growth of preantral follicles in rodents.

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Introduction

Several endocrine and locally acting factors are involved during the complex process of ovarian follicular growth and oocyte maturation. *In vitro* follicular culture systems at various developmental stages allow the identification of these factors and the understanding of their mechanisms of action.

The development of *in vitro* follicular culture systems has not only had a major impact on ovarian physiological research but also on clinical practice. An efficient human ovarian tissue culture system could profoundly modify the treatment options for infertile couples by avoiding the need of intensive ovarian stimulation. Indeed, several hundred primordial and primary follicles can be obtained after a single ovarian biopsy in a young woman, which is enough to attempt several *in vitro* follicular cultures and oocyte maturation procedures. Optimal *in vitro* follicular culture models should result in competent mature oocytes that could be fertilized to obtain viable embryos. Another important outcome is to preserve the fertility of young woman at risk of premature ovarian failure by long-term storage of female germ-cells (Hardy *et al.* 2002, Demeestere *et al.* 2003): frozen-thawed ovarian tissue can later be

used to restore their fertility. In this case, follicular development can be supported *in vitro*, thanks to follicular culture techniques, or *in vivo*, thanks to autotransplantation techniques. However, both procedures require improvement before clinical application becomes routine.

Identification of factors promoting follicular growth and development or inducing atresia constitutes one of the main objectives of the research program on folliculogenesis. To date, the rodent model is the most advanced system for studying *in vitro* follicular development. At present, the mouse is the only species for which a complete *in vitro* culture process has been achieved from the primordial stage up to live offspring (Eppig & O'Brien 1996, O'Brien *et al.* 2003).

Rodent models present several advantages. As in humans, the follicular growth includes two different periods: a gonadotropin-independent and gonadotropin-dependent period. The development of the preantral follicles until the preovulatory stage spreads over a period of 85 days in humans (Gougeon 1996). The improvement of *in vitro* culture systems and the expansion of our knowledge of follicular biology are very difficult to achieve over such a long period of *in vitro* growth. In mice, this growth phase is completed within 10–12 days

in vitro. Furthermore, the isolation of the preantral follicles is easier in mice compared with humans due to the high density of the cortex in humans. The size and the total number of follicles are smaller in rodents (5000 vs 200 000 follicles in total in prepubertal ovaries from mice and humans respectively), but the fraction of follicles in growth phase is considerably higher, facilitating the isolation of a homogeneous population of growing follicles. The fraction of growing follicles is around 0.1 in adult mice, compared with 0.04 in humans (Gosden *et al.* 1993). Finally, much of our understanding of oocyte growth factors such as the growth differentiation factor-9 (GDF-9) or the bone morphogenic protein-15 (BMP-15) comes from experiments on rodents and has now been confirmed in humans (Erickson & Shimasaki 2001). The possibility to create mice deficient for specific genes represents considerably useful models for studying the impact of different factors on folliculogenesis. At last, the well-characterized technical aspects and the high reproducibility of results has led to the consideration of the preantral follicles culture system as a model for screening potentially ovotoxic substances (Cortvrindt & Smitz 2002).

Thus, despite some major differences in ovarian physiology between rodents and humans, mice constitute a relevant model for identifying the endocrine and local mechanisms controlling follicular development.

Follicular culture systems in mice

Several *in vitro* culture systems of preantral follicles from rodents have been developed leading to the production of fully competent oocytes and viable offspring (Roy & Greenwald 1985, Eppig & Schroeder 1989, Nayudu & Osborn 1992, Cortvrindt *et al.* 1996).

These different culture systems have a common step – the isolation of preantral follicles from the ovary. Preantral follicles can be isolated mechanically or enzymatically (Eppig & Schroeder 1989, Demeestere *et al.* 2002). The isolation of preantral follicles using enzymes presents the advantage of collecting a large number of follicles per ovary. This technique also facilitates the procedure when ovarian cortex is dense as is the case in large mammals and humans (Roy & Treacy 1993). However, follicle integrity is not preserved and the structure is reduced to oocyte–granulosa-cell complexes (OGC) with an oocyte surrounded by one to three layers of granulosa cells (GCs) (Eppig & Schroeder 1989). In contrast, mechanical isolation of follicles allows intact preantral follicles to be obtained with a centrally located oocyte surrounded by two or three layers of membrane enclosed GCs and some theca cells attached to the basal membrane (Cortvrindt *et al.* 1996). The preantral follicles selected for *in vitro* culture generally measure 100–130 μm diameter in mice (Cortvrindt *et al.* 1996) and 130–160 μm in rats (McGee *et al.* 1999, Zhao *et al.* 2001a).

Isolated follicles have been cultured in systems that preserved the spherical follicular structure or in contrast, attached to a support. In spherical follicular culture systems, the follicles grow embedded within a collagen matrix (Torrance *et al.* 1989) or in non-adherent conditions (Nayudu & Osborn 1992). Both intact follicles and OGC can also be cultured on collagen-impregnated membranes (Eppig *et al.* 1996) or attached to the dishes (Cortvrindt *et al.* 1996).

Thus, different models of follicular culture have led to competent fertilizable oocytes after *in vitro* growth and maturation in mice. The choice of the culture system depends on the aim of the experiments and always involves some compromises (Smitz & Cortvrindt 2002). Furthermore, the effect of endocrine and locally acting factors described during *in vitro* follicular development varies regarding the culture system used and the species.

Based on the literature and on personal data, this manuscript focuses on the effect of some endocrine and paracrine factors on preantral follicular growth and differentiation during *in vitro* follicular culture using the rodent model.

Gonadotropins

The major endocrine factors that modulate follicular development are gonadotropins, namely follicle stimulating hormone (FSH) and luteinizing hormone (LH).

LH plays a key role in stimulating the enzymes responsible for androgen production in the theca cells and in initiating the final differentiation of the GCs. During ovulation, the LH surge induces a cascade of events leading to the resumption of meiotic maturation, the luteinization of the GCs, the expansion of the cumulus cells and finally the rupture of the follicle wall. Recently, it has been suggested that the action of LH during the ovulation process could be mediated by paracrine factors, members of the epidermal growth factor (EGF) family (Park *et al.* 2004, Ashkenazi *et al.* 2005). In LH receptor knockout mice (LuRKO) or LH β -subunit deficient mice, females are infertile. Follicles are able to reach the antral stage but they then degenerate (Zhang *et al.* 2001, Ma *et al.* 2004). This glycoprotein hormone is not essential to complete follicular development *in vitro*. However, the addition of low concentrations of LH during *in vitro* follicular culture enhances the antral cavity formation and improves the oocyte's meiotic maturation (Cortvrindt *et al.* 1998).

The essential role of FSH during antral follicular growth *in vivo* is well established. In FSH β deficient mice or FSH receptor knockout mice (FORKO), females are infertile (Kumar *et al.* 1997, Abel *et al.* 2000). All follicular stages, up to the preantral stage were observed but no further follicular development and ovulation have been described in FORKO mice. These experiments clearly indicate the essential role of FSH on early antral stage follicles for further growth and differentiation.

In vivo, FSH is essential for the steroidogenesis by stimulating aromatase enzyme activity (P450 aromatase), for the differentiation of the granulosa cells by inducing the expression of LH receptors and for the follicular antrum formation. FSH also regulates the transzonal connection between the oocytes and the surrounding GCs (Albertini *et al.* 2001). Furthermore, the presence of gonadotropins induces the expression of inhibitor of apoptosis proteins (IAP) by GCs *in vivo* and *in vitro* (Wang *et al.* 2003). Finally, FSH interacts with several growth factors to induce follicular growth such as kit ligand (KL), EGF, activin A, inhibin, BMP-15 or insulin-like growth factor (IGF-I). These intraovarian regulators mediate the effect of gonadotropins in regulating cellular interactions by autocrine and paracrine mechanisms (Erickson & Shimasaki 2001).

Assuming these major functions, FSH is usually added to the preantral follicular culture medium in mice and in large mammals (Nayadu & Osborn 1992, Cortvrindt *et al.* 1996, Mao *et al.* 2002). Both pituitary and recombinant FSH (rFSH) supported *in vitro* follicular growth. Liu *et al.* (2000) compared three different FSH preparations added during *in vitro* follicular growth: rFSH, urinary purified FSH (uFSH) and human menopausal gonadotropin (HMG). HMG induced a significant increase in follicular diameter and steroids secretion compared with rFSH or uFSH related to the presence of LH in the preparation.

Cortvrindt *et al.* (1997) showed that a minimal concentration of 10 mUI/ml of FSH is essential during *in vitro* culture of intact preantral follicles. In the absence of FSH, only 17% of the follicles survived. In this culture system, full differentiation of the preantral follicles *in vitro* can be achieved only in the presence of FSH at least from the late preantral stage (Adriaens *et al.* 2004). Using the same culture model, Mitchell *et al.* (2002) also reported a follicular survival rate of only 10% in the absence of FSH during *in vitro* growth. A dose–response curve for the effect of FSH during intact preantral follicular culture showed an increase in the follicular mean growth rate with increasing concentrations of FSH to a maximum of 100 mUI/ml (Nayadu & Osborn 1992). When the follicles were cultured in the presence of 1000 mUI/ml FSH, the proportion of follicles ovulating was significantly lower than in the presence of 100 mUI/ml FSH (Mitchell *et al.* 2002). Excessive exposure to FSH could result in FSH receptor down-regulation, leading to a suboptimal follicular response (LaPolt *et al.* 1992).

On the other hand, mice OGC have been cultured in the absence of FSH and serum up to the final oocyte maturation process (Eppig & Shroeder 1989). The same authors even described a negative effect of FSH on the growth of OGC in the presence of insulin (Eppig *et al.* 1998a). However, in the same culture system but in the presence of serum, the oocyte growth was increased after culture in medium supplemented with FSH compared with culture without FSH (Eppig & O'Brien 1998b). In accordance with these observations, studies in the rat did not show any FSH effect on the follicular development

when the culture medium was not supplemented with serum. However, follicular culture with both FSH and cyclic guanosine monophosphate (cGMP), as antiapoptotic factor, resulted in an increase of the follicle diameter (McGee *et al.* 1997). Administered with other growth factors such as IGF-I or activin A, FSH also had a significant stimulatory effect on follicular development and steroidogenesis in serum free conditions (Liu *et al.* 1998).

The actions of FSH during *in vitro* preantral follicular growth thus appear to be dependent on the culture conditions and particularly on the presence of serum in the culture medium: in the absence of serum, FSH can achieve its mitogenic and steroidogenic effects in the presence of other growth or survival factors.

Variations in the effects of FSH *in vitro* were also observed regarding the species. The addition of FSH to the culture media of porcine and bovine GCs decreased apoptosis (Guthrie *et al.* 1998, Yang & Rajamahendran 2000), while FSH as well as LH or IGF-I were ineffective in the prevention of spontaneous apoptosis when added to the culture medium of isolated rat GCs (Hsueh *et al.* 1994). However, FSH attenuated apoptosis during *in vitro* culture of intact rat preovulatory follicles (Chun *et al.* 1994). Recently, Yacobi *et al.* (2004) described opposite effects of gonadotropins on apoptosis depending on the cell types: in culture of rat preovulatory follicles, the addition of FSH or LH to the serum free medium resulted in a decrease of DNA fragmentation in GCs but in an increase of DNA fragmentation in theca cells following the activation of caspase-3 and -7, involved in the apoptosis process.

Although gonadotropins are essential factors regulating follicular development, several growth and endocrine factors locally produced by the follicles are able to amplify or attenuate FSH action (Fig. 1). One of those endocrine factors are androgens, that are produced by the theca interna and diffuse to the mural GCs where they are converted to estrogens by cytochrome P450 aromatase through FSH stimulation (Tetsuka *et al.* 1995). Inhibition of aromatase P450 activity during mice preantral follicular culture enhanced follicular differentiation and oocyte maturation rates. In these conditions, estradiol secretion was suppressed and androgens accumulated in the medium (Hu *et al.* 2002). Other authors confirmed that androgens have a direct stimulatory effect on *in vitro* follicular development in rodents, suggested by the absence of effect of neither the anti-estrogen nor the anti-progesterone antiserum (Murray *et al.* 1998).

Serum and ITS (insulin, transferrin, selenium)

Most culture systems use fetal calf serum (FCS) or hypogonadal (hpg) mouse serum (containing extremely low levels of FSH and LH) as the protein source to support follicular growth *in vitro*. The identification of the different growth factors and carrier proteins, present in the serum and essential for follicular development, is still a challenge in

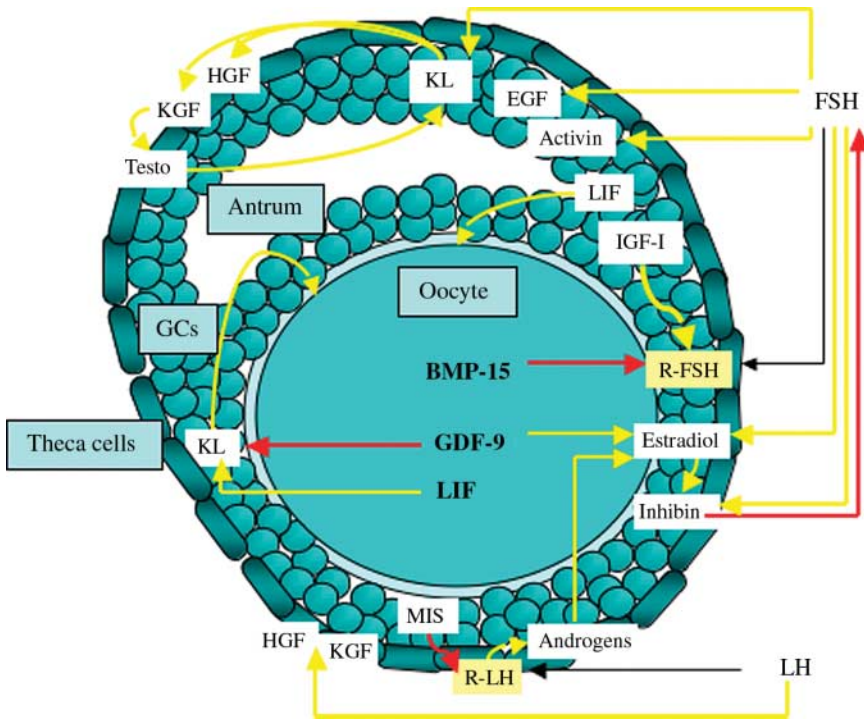


Figure 1 Factors involved in the control of follicular development: negative (red arrow) and positive (yellow arrow) effect. GDF, growth differentiation factor-9; KGF, keratinocyte growth factor; GH, growth hormone; IGF-I, insulin growth factor-I; KL, kit ligand; LIF, leukemia inhibitory factor; EGF, epidermal growth factor; BMP-15, bone morphogenetic protein-15; HGF, hepatocyte growth factor; MIS, mullerian-inhibiting substance; FSH, follicle-stimulating hormone; LH, luteinizing hormone; R-LH and R-FSH, receptors for LH and FSH; Testo, testosterone; GCs, granulosa cells.

cell culture. The presence of high concentrations of serum during preantral follicular culture constitutes inadequate conditions for *in vitro* physiological studies. Indeed, serum provides numerous known and unknown proteins that could interact with the other components added in the medium. Furthermore, serum should be avoided from *in vitro* follicular culture medium regarding further clinical application of the procedure.

Serum elimination from follicular culture medium induced apoptosis compromising the subsequent follicular development. Using an intact preantral follicle culture model, Mitchell *et al.* (2002) showed that all follicles cultured in human serum albumin (HSA) or normal mouse serum, degenerated after 9 days of *in vitro* culture. In contrast, hpg mouse serum and FCS both supported follicular growth in the presence of FSH. The follicular survival rate was not significantly different when FCS or hpg mouse serum was added to the culture medium (follicular survival rate was of 93 and 87% respectively). However, a lower proportion of oocytes were able to mature in response to human chorionic gonadotropin (hCG) when the follicles were cultured with hpg mouse serum than when cultured with FCS, suggesting on the other hand that factors lacking in the hpg mouse serum may also influence further oocyte competence.

Extrinsic and intrinsic pathways of apoptosis induction in GCs are not yet clearly defined but they include lack of growth factors, or presence of cytotoxic stimuli such as fas ligand (FasL). Previous studies showed that the presence of $\geq 1.25\%$ of serum is enough to completely block the action of FasL (Quirk *et al.* 2000). Below this concentration, other factors should be added to the culture

medium to reduce the apoptotic process. Different factors have been used to reduce apoptosis during follicular *in vitro* culture in serum free conditions (McGee *et al.* 1997, Eppig *et al.* 2000, Murray *et al.* 2001). Treatment of cultured rat preantral follicles with 8-bromo-cGMP did suppress apoptosis (McGee *et al.* 1997). However, the antiapoptotic effect of 8-bromo-cGMP was not observed in cultured mice OGC (Eppig *et al.* 2000).

Ascorbic acid (vitamin C) reduced apoptosis during *in vitro* growth of mice OGC and intact preantral follicles in serum free conditions (Eppig *et al.* 2000, Murray *et al.* 2001). Added to serum containing medium during intact bovine preantral follicular culture in a three-dimensional system, ascorbic acid also increased the percentage of follicles that maintained basement membrane integrity, demonstrating the multiple roles of this vitamin during folliculogenesis in rodents but also in domestic species (Murray *et al.* 2001, Thomas *et al.* 2001).

The standard concentration of serum added to the follicular culture medium is 5% of FCS or hpg mouse serum. Using intact preantral follicular culture model in mice, we investigated the effect of reducing the FCS concentration in the culture medium. The culture medium used for these experiments was supplemented with rFSH, rLH and insulin, transferrin, selenium (ITS) as previously described (Demeestere *et al.* 2002). Reducing the concentration of serum from 5% to 1% in the culture medium did not significantly affect the follicular survival rate after 12 days of *in vitro* culture but decreased the oocyte maturation rate after *in vitro* maturation induced by hCG and EGF (Table 1). However, estradiol levels measured in the culture medium collected every 3 days were higher when

Table 1 Effect of different media during *in vitro* culture of mouse preantral follicles on subsequent oocyte *in vitro* maturation. Intact preantral follicles were isolated from ovaries of 12–14 day-old mice and cultured in MEM supplemented with 100 mIU/ml r-FSH, 10 mIU/ml r-LH, 5 µg/ml transferrin, 5 ng/ml selenium, 0 or 5 µg/ml of insulin (ITS) and 1 or 5% fetal calf serum (FCS). Half of the medium was replaced every 3 days. After 12 days, culture medium was replaced by maturation medium (MEM supplemented with r-FSH, r-LH, ITS, 1.5 IU/ml r-hCG, 5 ng/ml EGF and 5% FCS). Oocyte-cumulus complexes (OCC) were collected and denuded to observe nuclear maturation stage: metaphase II (MII), germinal vesicle (GV), germinal vesicle breakdown (GVBD).

	With insulin		Without insulin
	5% FCS	1% FCS	1% FCS
<i>n</i> (follicles)	99	100	99
OCC (%)	90 (97.7)	80 (93)	86 (96.6)
MI I (%)	75 (83.3) ^a	45 (56.2) ^b	52 (60.4) ^b
GV (%)	2 (2.2)	13 (16.1)	15 (17.4)
GVBD (%)	12 (13.3)	20 (25)	20 (23.2)

a vs b: $P < 0.05$.

serum concentration was reduced during *in vitro* follicular growth. The values reached significance after 6 and 9 days of *in vitro* culture (data not shown). These results confirm previous studies in bovine reporting a decrease of estradiol secretion by GCs cultured into serum-coated wells compared with those cultured in serum free conditions (Gutierrez *et al.* 1997). The reason for the decrease of estradiol levels in the presence of serum is still unclear. However, the decrease of estradiol production in the presence of serum is not related to premature luteinization as suggested by the absence of serum effect on progesterone secretion by the GCs (Gutierrez *et al.* 1997).

Insulin is usually added to the culture medium at supra-physiological concentration (5 µg/ml) as survival factor, together with transferrin and selenium (ITS). Added to cell cultures, insulin increases the uptake of metabolic precursors as amino acids and glucose. However, Eppig *et al.* (1998a) demonstrated that in the absence of serum, insulin alone had no effect on the development of OGC and subsequent oocyte competence. The author even observed deleterious effects of the combination of insulin and FSH in the culture medium. Other studies using culture medium supplemented with 5% FCS showed that the presence of insulin had no beneficial effect (Mitchell *et al.* 2002). In contrast, other authors showed that the association of gonadotropins and insulin was considered optimal during intact preantral follicular culture for follicular proliferation, differentiation and to generate mature oocytes (Liu *et al.* 2002).

During intact follicular culture in mice using a low serum concentration medium (1%), we did not observe any difference in the follicular survival rate or the subsequent oocyte maturation rate in the presence or in the absence of insulin (Table 1).

We further evaluated the embryo development using a simplified culture medium (with 1% FCS without insulin)

compared with a control medium (with 5% FCS and insulin). The embryo cleavage rate, obtained after *in vitro* growth, maturation and fertilization of oocytes from intact follicles cultured in the simplified medium was significantly reduced compared with those cultured in the control medium, without affecting the blastocyst development rate (Table 2). The total cell number per blastocyst, assessed using a double labelling staining technique, decreased when the follicles were cultured in simplified medium compared with those cultured in control medium, to the detriment of the trophectoderm cells (Table 2). The controversial results concerning the role of insulin during follicular culture could be related to the presence of other survival factors such as selenium or transferrin in the medium or to species differences.

Proliferating cells require iron, which is carried and delivered by a metal-binding glycoprotein, transferrin, with subsequent intracellular transport via the transferrin receptor. Transferrin receptors were previously described in GCs of rats and humans, with an increasing expression parallel to follicular maturation (Aleshire *et al.* 1989). The authors even suggested that the follicular maturation process was closely related to the high levels of cellular transferrin and of its receptor.

Sodium selenite can be added to the culture medium to activate enzymes involved in metabolic detoxification and free radical scavenging. However, the absence of sodium selenite in culture medium in mice did not significantly decrease the viability of intact preantral follicles (personal data not shown). It is important to emphasize that the action of various factors on the preantral follicular development and steroid secretion *in vitro* does not necessarily reflect the subsequent embryo potential.

Table 2 Cleavage and blastocyst (BI) development rates after *in vitro* fertilization of oocyte-cumulus complexes (OCC) obtained after *in vitro* preantral follicular growth using two different culture media. Intact preantral follicles were isolated from ovaries of 12–14 day-old mice and cultured in MEM supplemented with 100 mIU/ml r-FSH, 10 mIU/ml r-LH, 5 µg/ml transferrin, 5 ng/ml selenium, and 5 µg/ml of Insulin (ITS) and 5% fetal calf serum (FCS) (basal medium) or without insulin and 1% FCS (simplified medium). After 12 days, culture medium was replaced by maturation medium (MEM supplemented with r-FSH, r-LH, ITS, 1.5 IU/ml r-hCG, 5 ng/ml EGF and 5% FCS). OCC were collected and fertilized with capacitated sperm. Embryo were cultured in simplex optimised medium with potassium (KSOM) supplemented with 1 mg/ml of bovine serum albumin. Blastocyst cell number was evaluated using double labelling technique.

	Basal medium with 5% FCS	Simplified medium with 1% FCS and no insulin
<i>n</i> (OCC)	242	208
2-cells/OCC (%)	113 (46.7) ^a	77 (37) ^b
B1/2-cells 120 h (%)	54 (47.8)	39 (50.6)
<i>n</i> (blastocyst)	44	32
TE (range)	27.9 ± 1.08 ^a (13–42)	22.9 ± 1.4 ^b (7–41)
ICM (range)	17 ± 0.7 (8–27)	17.6 ± 1.06 (8–31)
Total (range)	45.1 ± 1.7 ^c (24–68)	39.9 ± 2.49 ^d (21–65)

a vs b, c vs d: $P < 0.05$.

TE, trophectoderm; ICM, inner cell mass.

Paracrine factors

Since the last decade, many peptides interacting with the follicular growth and cell differentiation during *in vitro* culture of preantral follicles have been identified (Table 3).

Some factors such as tumor necrosis factor- α (TNF- α), or interleukin-6 (IL-6) have been shown to promote apoptosis and therefore have negative effects on *in vitro* follicular development (Chun & Hsueh 1998). During *in vitro* culture of early antral rat follicles, the addition of TNF- α suppressed the antiapoptotic effect of FSH in a dose dependent manner (Kaipia *et al.* 1996).

Other factors have also been shown to induce a negative effect on *in vitro* follicular development. GCs, theca cells and interstitial cells have specific receptors for leptin, the hormone of the obese gene, inducing an inhibition of steroid production and cellular proliferation during FSH-dependant follicular growth (Kikuchi *et al.* 2001). Administration of leptin during preantral follicular culture blocked the stimulatory effect of the growth hormone (GH) and the IGF-I on GC proliferation. The leptin inhibitory effect on steroid production has also been observed using rat and human GCs culture in the presence of FSH and IGF-I (Zachow & Magoffin 1997, Agarwal *et al.* 1999). Other extraovarian factors have receptors in somatic and germ cells and can directly influence the reproductive function. Thyroid dysfunction is frequently involved in infertility. The mouse preantral follicle culture system was used to assess the influence of 3,3',5-triiodothyronine (T₃) during early folliculogenesis. In the presence of FSH, T₃ inhibited the follicle's ability to form an antral cavity, reduced the estradiol release and affected the oocyte meiotic competence (Cecconi *et al.* 2004).

The transforming growth factor- β (TGF- β) superfamily includes different factors such as activin, inhibin, growth differentiation factor-9 (GDF-9), BMPs or the anti-müllerian hormone (AMH) that influence follicular development (Lin *et al.* 2003). The pattern of AMH expression in the ovary is comparable in humans and mice, and was observed in the GCs from the primary to the early antral follicular stage (Weenen *et al.* 2004). This hormone regulates the cyclic recruitment of preantral follicles. AMH deficient female mice have an increased number of growing follicles in spite of a lower serum FSH level (Durlinger *et al.* 1999). During *in vitro* mice preantral follicular culture, the presence of AMH suppressed the stimulatory effect of FSH (Durlinger *et al.* 2001). In contrast, AMH was also reported to enhance FSH stimulatory effect of rat preantral follicles (McGee *et al.* 2001). These contradictory results were not clearly explained and could be due to serum factors influencing the AMH effect.

GDF-9 and BMP-15 are expressed by oocytes from primary to antral follicles and play an essential role in the initial follicular recruitment. Both GDF-9 and BMP-15 are able to stimulate GCs proliferation in preantral follicles, through an FSH dependent mechanism (Hayashi *et al.* 1999, Shimasaki *et al.* 2004). Added during rat GCs

culture in the presence of FSH, BMP-4 and BMP-7, they induced an increase in estradiol production and a decrease in progesterone production. BMPs seem to play an important role in modulating the FSH action and in avoiding premature luteinization (review by Shimasaki *et al.* 2004). Paracrine factors secreted by the GCs also regulate many aspects of early folliculogenesis. Among them, the proto-oncogene c-kit, encoded at the white spotting (W) locus in the oocyte and its product KL, encoded at the steel locus (Sl) in the GCs appeared to be one of the key factors of the follicular growth initiation. However, Kit-KL interaction also operates throughout preantral and antral follicular growth and differentiation. Both KL and leukemia inhibitory factor (LIF) act on the oocyte to modulate its survival and maturation at the meiotic stage. Added during preantral follicle culture, KL stimulated the production of testosterone as well as GCs proliferation and oocyte maturation (Reynaud *et al.* 2000).

Activin A has been shown to enhance GCs proliferation during *in vitro* rat OGC culture (Li *et al.* 1995). During antral follicular development, activin A induces a dose dependent suppression of apoptosis DNA fragmentation (Chun & Hsueh 1998). Activin A also acts as a regulator in the progression of the cohort of small follicles through the later stage. The presence of activin A inhibits the stimulatory effect of FSH and GH in a reversible way during culture of small preantral follicles isolated from adult mice ovaries (Mizunuma *et al.* 1999). Moreover, the authors demonstrated that activin A is the factor secreted by secondary follicles responsible for the maintenance of small preantral follicles at a dormant stage. In contrast, using coculture across polycarbonate membrane, Spears *et al.* (2002) did not identify activin A as an inhibitory factor secreted by dominant follicles. Those contradictory results may be due to the difference of age of the mice and of the developmental stage of the follicles selected for culture.

Other factors identified in the ovary were shown to promote preantral follicular development *in vitro*. Functional receptors for EGF and TGF- α , an EGF analogue, are expressed in the ovary and have been shown to be up-regulated by gonadotropins (Fujinaga *et al.* 1994). However, both EGF and TGF α have been described as potent inhibitors of aromatization in cultured GCs in humans and during *in vitro* growth of mouse follicles (Steinkampf *et al.* 1988, Boland & Gosden 1994). The physiological concentration of EGF to which mouse follicles are exposed is around 1 ng/ml. The presence of EGF ranging from 1 to 20 ng/ml in the preantral follicular culture medium induced a dose-dependent decline in estradiol production (Boland & Gosden 1994). Above a concentration of 5 ng/ml EGF, the number of follicles reaching the antral stage decreased. However, at physiological concentrations, EGF increased the number of follicles reaching the antral stage. Treatment of GCs and preovulatory follicles with EGF inhibited the spontaneous onset of apoptotic DNA cleavage found during culture by 40–60%

Table 3 Effect of different growth/paracrine factors added to the culture medium during *in vitro* preantral follicular growth.

	Species	Culture medium supplements				Effect of the growth/paracrine factors	References
		Protein	Survival factors	Hormone	Growth/paracrine factors		
Intact follicles	Mouse	5% MS	ITS/IGF-I	(FSH)	Relaxin hypoxantine dcAMP	No effect on follicular growth and antrum No effect on follicular growth	Hartshorne <i>et al.</i> (1994)
	Mouse	5% hpgMS	T	FSH	EGF	↑ follicular growth ↓ E2 production	Boland & Gosden (1994)
	Mouse	5% MS	T	FSH/hCG	EGF-Androst. TGF-α	No effect on follicles diameter and DNA content ↓ follicular growth ↓ E2 secretion	Almabobi <i>et al.</i> (1995)
	Mouse	BSA	ITS	(FSH)	Activin A	↑ follicles diameter and E2 secretion ↑ effect of FSH and GH but not of IGF-I ↑ follicles diameter and E2 secretion	Liu <i>et al.</i> (1998)
	Mouse	5% FCS	ITS	FSH/LH	GH IGF-I Activin A	↑ follicles diameter when associate with FSH ↑ inhibin secretion ↓ survival rate ↓ oocyte maturation rate (FSH 100 mUI/ml) ↓ antiapoptotic effect of FSH	Smitz <i>et al.</i> (1998)
	Rat	None	ITS/cGMP	FSH	TNF-α Il-6		Chun & Hsueh (1998)
	Rat	None	ITS/cGMP	(FSH)	GDF-9	↑ follicles diameter ↑ protein content	Hayashi <i>et al.</i> (1999)
	Rat	None	ITS+/cGMP	(FSH)	KGF	↓ apoptosis ↑ follicles diameter and inhibin secretion	McGee <i>et al.</i> (1999)
	Mouse	5% FCS	ITS	FSH	Kit-ligand	↑ cytoplasmic maturation ↑ GCs proliferation (if no serum)	Reynaud <i>et al.</i> (2000)
	Mouse	BSA	ITS	–	Activin A/GH	↑ GCs and TC proliferation	Kobayashi <i>et al.</i> (2000)
	Rat	None	ITS+	FSH	AMH/activin A TGF-β	↑ follicles diameter but no effect on apoptosis	McGee <i>et al.</i> (2001)
	Mouse	5% IMS	–	FSH	AMH	No effect on follicular diameter but ↑ apoptosis ↓ stimulatory effect of FSH	Durlinger <i>et al.</i> (2001)
	Mouse	BSA	ITS+	(FSH)	Leptin	↓ stimulatory effect of GH and IGF-I with FSH	Kikuchi <i>et al.</i> (2001)
	Rat	BSA	ITS	FSH	IGF-I	↑ follicular diameter at a concentration up to 10 ng/ml	Zhao <i>et al.</i> (2001b)
Mouse	1% FCS	TS	FSH	IGF-I	↑ E2 secretion ↑ subsequent embryo development	Demeestere <i>et al.</i> (2004)	
Mouse	5% FCS	ITS	FSH	T ₃	↓ follicles diameter and ↓ estradiol secretion ↓ PB1 formation during oocyte maturation	Cecconi <i>et al.</i> (2004)	
OGC	Rat	None	IT	FSH	Activin A	↑ follicles diameter (↑ GCs proliferation)	Li <i>et al.</i> (1995)

AMH, anti-mullerian hormone; MS, mouse serum; hpgMS, hypogonadal mouse serum; IMS, immature mouse serum; BSA, bovine serum albumin; ITS + , insulin, transferrin, selenium, linoleic acid; EGF, epidermal growth factor; Androst., Androstenedione; TGF-α, transforming growth factorα; TNF-α, tumor necrosis factor-α; Il-6: interleukin-6; GDF-9: growth differentiation factor-9; KGF: Keratinocyte growth factor; GH: growth hormone; IGH-I: insulin growth factor-I; T₃: thyroid hormone; GCs: granulosa cells; TE: theca cells; OGC: oocyte–granulosa-cell complex; PB1: first polar body. Under ‘Hormone’ column, ‘FSH’ indicates experiment conducted in the presence of FSH, ‘(FSH)’ indicates experiment conducted in absence of FSH.

through tyrosine kinase pathways. In contrast, EGF had no effect on the suppression of apoptosis on preantral follicles (McGee *et al.* 1997).

Keratinocyte growth factor (KGF), also known as fibroblast growth factor-7, is a single polypeptide produced by the theca cells in rodents and bovine. The KGF receptors are expressed in GCs of growing follicles (Parrott & Skinner 1998). Added during rat preantral follicular culture, KGF suppressed apoptosis and enhanced the GCs differentiation (McGee *et al.* 1999).

The intraovarian IGF system is also largely involved in the ovarian physiology. When added during *in vitro* culture of preantral follicles, IGF-I has been shown to stimulate follicular growth in synergy with FSH in various species (Zhou *et al.* 1991, Gutierrez *et al.* 2000, Louhio *et al.* 2000, Zhao *et al.* 2001b). In the rat, IGF-I added during *in vitro* preantral follicular culture increased significantly the follicular diameter, the DNA content and promoted the functional integrity of the follicles. IGF-I also stimulated steroidogenesis during mice preantral follicular culture, without any advantage on the subsequent oocyte maturation rate. However, embryo development and blastocyst cell numbers were enhanced when follicles were cultured in the presence of IGF-I and FSH (Demeestere *et al.* 2004). IGF binding proteins (IGFBPs) have been involved in the physiological mechanism of follicular dominance and atresia (Fortune *et al.* 2004). However, the expression of IGFBPs strongly varies between species. In the rat, IGFBP-4 mRNA increases during the follicular atretic process and its expression decreases in the presence of FSH during *in vitro* follicular culture. Furthermore, IGFBPs affinity can be modulated by phosphorylation and proteolysis. Recently, interest was focused on IGFBP-4 protease called pregnancy-associated protein A (PAPP-A). The expression of PAPP-A by the GCs of healthy follicles is stimulated by the gonadotropins (Hourvitz *et al.* 2002). Recently, Matsui *et al.* (2004) identified the oocyte derived factor BMP-15 as an inhibitor of FSH-induced PAPP-A production *in vitro*. Those experiments using the rat model confirmed other results obtained in domestic species suggesting that PAPP-A is essential for the selection of dominant follicles (Fortune *et al.* 2004).

Conclusion

Follicular growth and maturation are complex processes controlled by endocrine factors such as gonadotropins and locally produced factors. The reproduction of the whole follicular development *in vitro* constitutes a real challenge with major potential applications in clinical practice. Progress has been made in the adaptation of the follicular culture medium since the last decade in rodents, mammals and humans. However, the mouse is the only species where offspring were obtained after *in vitro* growth, maturation and fertilization of oocytes from the

primordial stage. This species constitutes an efficient model to assess the effects of the different *in vitro* components on the follicular development and to improve our knowledge on folliculogenesis.

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