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***Mouse primordial follicle culture as a  
model for fertility restoration***

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## Abstract

The aims of this study were to induce primordial follicle activation to generate a large amount of secondary follicles and to obtain mature oocytes from the activated follicles *in vitro*.

Ovaries of 3 days-old mice were cut into 18 equal pieces. Primordial follicles were cultured in 1) alginate matrix for 15 days in the presence or absence of Ascorbic Acid (AA) (2 concentrations: 100µg/ml and 200µg/ml). Tissue grown in presence of 100µg/ml of ascorbic acid showed conservation of basement membrane in the growing follicles. Primordial follicles cultured in ultra low attachment plates (ULA) were pretreated for 24 hours with 2) an enhancer of the activity of PI3K (activator 740Y-P) (50, 100 and 500µg/ml) or 3) PTEN inhibitor (bpV(pic)) (1, 5, 10, 25, 50 and 100µM) and cultured for 15 days. 4) The best concentrations were 500µg/ml Activator plus Inhibitor 1 or 10µM showing a synergistic effect. 5) The effect of Activin A (0, 50 or 100ng/ml) was evaluated on ovarian tissue pieces cultured for 15 days. 6) Finally, tissue pieces were pretreated for 24 hours with or without 500µg/ml Activator plus Inhibitor 1µM (Ac+I) and in order to help the activated follicles to reach the preantral stage, the pieces were cultured for 24 days with or without 100ng/ml Activin A. After 18, 21 and 24 days of repetitive secondary follicle harvesting from tissue culture, follicles were subsequently cultured in a standard mouse secondary follicle culture for 12 more days. The ability to resume meiosis, steroid production and the chromosomal abnormality rate of the grown oocytes were evaluated. Gene expression levels of *Gdf9*, *Mater*, *Amh* and *Cyp17* were studied to compare the quality of the *in vitro* grown secondary follicles to isolated secondary follicles from 13 day old ovaries.

The survival and growing rate of the ovarian tissue pieces were evaluated for all the tested conditions. Additionally, histological analysis of the cultured tissue pieces was performed for fresh tissue (3 day old ovary) and in cultured tissue pieces after 3, 15, 18 or 24 days of culture.

When ovarian tissue pieces were pretreated with Ac+I, follicle activation was induced and growing rate of the tissue pieces (60%) was higher than control (40%).

After the pretreatment for follicle activation, the tissue pieces were cultured in the presence of Activin A to induce follicle growth. The growing rate (80%) was higher than Control condition (40%) and Ac+I condition (50%). The histological analysis revealed a significantly higher ( $P < 0.05$ ) amount of secondary follicles in Ac+I and Ac+I+At conditions (around 15%) compared to Control (5%) and Activin-alone (10%) conditions. Secondary follicle quality was studied by using expression levels of key genes. The polar body (PB) rate was very variable (ranging from 0 to 100%) in all the *in vitro* treatments. However, on average PB rate of the *in vitro* treatments was lower than in the *in vivo* control. Moreover, Metaphase I (MI) and Metaphase II (MII) oocytes showed more Chromosomal abnormalities than in the *in vivo* condition.

In conclusion, Ascorbic acid keeps the basement membrane of *in vitro* grown follicles. Pretreating ovarian tissue briefly with activator and inhibitor induces follicular activation *in vitro*. The addition of Activin A during the culture promotes follicular growth, in consequence more secondary follicles are isolated. Although, *in vitro* grown secondary follicles look morphologically normal, the variability in gene expression levels of *Gdf9*, *Mater*, *Amh* and *Cyp17* and their low ability to resume meiosis indicates that the culture conditions needs to be further explored.

## i. INTRODUCTION

Due to the large amount of primordial follicles residing in the ovarian cortex and their ability to survive better the cryopreservation procedures, they are considered a large source of oocytes with potential oocytes use for fertility restoration (Hovatta 2004). Cryopreservation of Ovarian tissue as cortical strips has been performed for many cancer patients as an alternative to preserve their fertility (Donnez et al. 2004) which can be impaired as a consequence of the oncological treatments (chemotherapy and radiotherapy).

Since follicular viability after cryopreservation and thawing has been demonstrated in several studies (Newton et al. 1996, Sadeu et al. 2008, Sadeu et al. 2006) there are three strategies for the development of immature follicles from ovarian tissue: autotransplantation, xenotransplantation and *in vitro* culture (Kim et al. 2001). However, the transplanted strips of ovarian tissue might contain some cancer cells which can be reintroduced into the patient (i.e. in case of leukemia). Use of an *in vitro* culture would also eliminate the need to expose patients with hormone-sensitive cancers (i.e. breast cancer patients) to synthetic hormones as part of an IVF cycle and would give them the opportunity to restore their fertility avoiding the reintroduction of cancer cells. Therefore, an interesting strategy would be the development of a culture system that would be supports the growth of primordial follicles to obtain fertilizable oocytes (Smits et al. 2010).

### 1. Primordial follicle formation

The primordial germ cells (PGCs) originate during the very early embryonic stages. The PGCs migrate and proliferate in the genital ridges, where they differentiate into oogonia. In the female, the oogonia become the precursor for the follicle formation (Parker and Schimmer, 2006).

Primordial follicle formation (PFF) occurs during fetal life or few days after birth in most of mammalian species. For instance, in human, bovine and sheep the PFF occurs at day 140 (Rajkovic et al., 2006), at day 90 (Yang and Fortune, 2008) and at day 75 (McNatty et al. 1995) during fetal life, respectively. In contrast, in rat, mouse and hamster the PFF is only observed after birth a few hours later (Kezele and Skinner 2003), at day 3 (Rajkovic et al., 2006), and day 8 (Wang and Roy 2007), respectively.

### **1.1. Primordial follicle and its activating signaling**

Primordial follicles are the earliest stage of ovarian follicles and consist of a primordial oocyte surrounded by a single layer of flattened granulosa cells. The size of the primordial follicle pool is determined during the fetal life. During life, the majority of the primordial follicles are in a quiescent state with their oocyte arrested in prophase I of meiosis. These follicles progressively develop in a synchronized and coordinated fashion, until final ovulation.

One of the initial events in the primordial to primary follicle transition is the change in granulosa cells from a flattened to a cuboidal morphology; another event during this transition is the recruitment of theca cells from the stroma-interstitial cell population and subsequent proliferation of both granulosa and theca cells (Fortune 2003, Fortune et al. 2000).

It has been previously described that there are some factors which are involved in the primordial to primary follicle transition, such as: Kit Ligand (KL) (Parrott and Skinner 1999), basic fibroblast growth factor (bFGF) (Nilsson E. et al. 2001), leukemia inhibitor factor (LIF) (Nilsson E. E. et al. 2002), keratinocyte growth factor (KGF), bone morphogenetic protein 4 (BMP4) (Nilsson E. E. and Skinner 2003), platelet derived growth factor (PDGF) (Nilsson E. E. et al. 2006) and connective tissue growth factor (CTGF) (Schindler et al. 2010). Similarly, other studies have shown the role of selected pathways (like Tsc/mTORC1 signaling, PI3K signaling pathway) for the activation to occur (Adhikari et al. 2010, Li et al. 2010, Liu L. et al. 2007).

#### **1.1.1. Primordial follicle activation by the phosphatidylinositol 3 kinase (PI3K) signaling in oocytes**

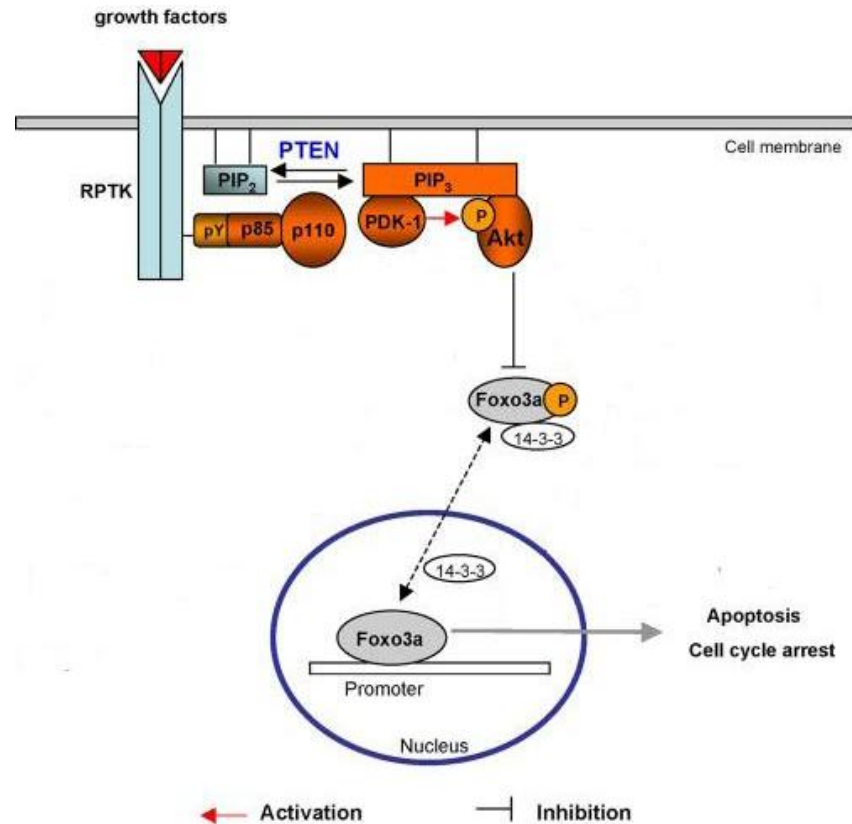
The PI3K signaling pathway consists of molecules (such as kinases, phosphatases, and transcription factors) that regulate cell proliferation, survival, migration, and metabolism (Cantley 2002). PI3Ks are lipid kinases that phosphorylate the 3'-OH group of the inositol ring of inositol phospholipids. The activation of the PI3K pathway results in the activation of Akt and the phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome ten) negatively regulates this process (Cantley 2002). The characterization of a functional PI3K pathway in growing oocytes was initiated

by *in vitro* studies where Akt and Foxo3a (FKHRL1), important components of the PI3K pathway, were found to be expressed in mouse and rat oocytes (Reddy et al. 2005).

The functional roles of the PI3K pathway in oocytes in controlling follicular activation were first revealed by deletion of *Pten* from oocytes of mice primordial follicles, leading to the overgrowth of immature oocytes, resulting in premature activation of the entire primordial follicle pool (Reddy et al. 2008). Thus, in mouse, ovaries lacking *Pten* in the oocytes developed premature ovarian failure (POF) (Reddy et al., 2008).

To evaluate if the PI3K pathway is needed in further developing follicles, transgenic mice in which *Pten* gene was conditionally deleted in the oocyte by the expression of the zona pellucida 3 (Zp3) promoter-mediated Cre recombinase were developed. It was observed that the resulting overactivation of PI3K signaling in oocytes did not affect the development of growing follicles suggesting that there is a stage-specific function of PTEN/PI3K signaling in mouse oocytes that controls follicular primordial activation (Jagarlamudi et al. 2009).

In mammals, Akt pathway phosphorylates three conserved serine or threonine residues of the forkhead transcription factors of the FOXO family; Foxo1, Foxo3a, and Foxo4, which leads to relocation of the Foxo peptides to the cytoplasm. Such relocalization suppresses their activities as transcription factors promoting apoptosis and cell cycle arrest (Figure 1) (Arden and Biggs 2002).



**Figure 1.** The PI3K signaling pathway. After ligand binding, the RPTK receptor is dimerized and autophosphorylated. The phosphorylated residues in the receptor now are capable to bind the SH2 domain of the regulatory subunit of PI3K (p85). The catalytic subunit of PI3K (p110) phosphorylates the 3-OH group of the inositol phospholipids. Due to the recruitment of PI3K to the inner membrane area of the cell, the production of PIP<sub>3</sub> from PIP<sub>2</sub> starts. As a natural negative regulator of this process, the phosphatase PTEN converts PIP<sub>3</sub> to PIP<sub>2</sub>. PDK1 and Akt (which are kinases containing PH domains) are recruited to PIP<sub>3</sub> and Akt is phosphorylated by PDK1. Akt can phosphorylates Foxo3a and induce a cytoplasmic relocalization of this transcription factor by binding to the 14-3-3 protein. Apoptosis and cell cycle arrest happen when unphosphorylated Foxo3a is located in the nucleus as a transcription factor.

Taken and modified from Adhikari *et al.* 2009

The role of Foxo3a in controlling the female fertility was discovered in the Foxo3a knockout (Foxo3a<sup>-/-</sup>) female mice. Those mice showed an age-dependent decline in reproductive function and became sterile by the week 15 of age due to the total depletion of follicles. Compared with wild-type controls, Foxo3a<sup>-/-</sup> ovaries were enlarged by postnatal day 14 with greater numbers of early-growing follicles while the primordial follicle reserve was totally depleted. Studies with wild-type mice have shown that Foxo3a is expressed mainly in the nuclei of oocytes of primordial



follicles and early primary follicles and its expression in oocytes of larger primary follicles and further developed follicles is dramatically down-regulated (Liu L. et al. 2007). A transgenic mouse model in which Foxo3a was expressed in oocytes of primary and large follicles was developed, where a retardation of oocyte growth and follicular development was observed, leading to infertility (Liu et al., 2007). This finding is in agreement with the role of Foxo3a in suppressing primordial follicle activation and suggests that down-regulation of Foxo3a expression in oocytes is a prerequisite for follicular activation (Castrillon et al. 2003, John et al. 2008).

For instance, Li et al. (2010) described that after a short-term incubation of mice and human ovaries containing primordial follicles with the PTEN inhibitor and the activator of the PI3K, a larger amount of developing follicles was observed in comparison with an untreated ovary.

### **1.1.2. Primordial follicle activation by Activins**

Activins, which are members of the TGF- $\beta$  superfamily are homodimers composed of two inhibin  $\beta$ -subunits named  $\beta$ A and  $\beta$ B. Three isoforms of Activin are known: Activin A ( $\beta$ A  $\beta$ A), Activin B ( $\beta$ B $\beta$ B) and Activin AB ( $\beta$ A $\beta$ B). Activin A is the isoform of activins most studied. The activin subunits are expressed in granulosa cells of growing follicles in rats, mice and humans (Drummond et al. 2002, Pangas et al. 2002).

Activins signal through a heteromeric assembly of membrane bound serine threonine kinase receptors that include a type II ligand binding receptor and a type I signaling receptor. Two type II receptors named ACVR2 and ACVR2B are the predominant activin binding receptors (Mathews 1994) (Mathews 1994). The type II receptors complex with one of three type I receptors, the activin receptor like kinases (ALKs) ALK2, ALK4, or ALK7 that results in the phosphorylation and activation of the transcription factors SMAD2 and SMAD3. SMAD2 and SMAD3 then mediate gene transcription via interactions with transcriptional co-activators and co-repressors (Moustakas et al. 2001).

It is known that Activin A plays a role in many cellular activities such as: cell proliferation, differentiation, and apoptosis through the transmembrane receptor mediated Smad pathways. *In vitro*, the activins can up-regulate FSH receptor expression, induce granulosa cell proliferation, and

enhance the responsiveness to FSH in preantral follicles coming from prepuberal mice ovaries (Yokota et al. 1997, Zhao et al. 2001). In contrast, Activin A does not stimulate granulosa cell proliferation and block the effect of FSH in preantral follicles coming from adult mice ovaries (Yokota et al., 1997).

Regarding to its effect in primordial follicle activation, contradictory data is available. For instance, during the time of follicle assembly and primordial to primary follicle transition, activin has been reported to inhibit the spontaneous activation of human primordial follicles *in vitro* (Ding et al. 2010) however it has also been shown that it regulates the initial follicle pool formation during the critical period of germline cyst breakdown in mice (Bristol et al., 2006).

Regarding follicle development, disruption of activin signaling, as in the mice with null mutations for the activin receptor ActRIIB (Matzuk et al. 1995) or in transgenic mice overexpressing follistatin (which binds and neutralizes Activin bioactivity) (Guo and Kyprianou 1998), follicle development is arrested, implying a role for activin in the cyclic recruitment of preantral follicles. Similarly, stimulation of follicular growth *in vitro* by activin has been demonstrated in pre-antral ovine (Thomas et al. 2003), caprine (Silva et al. 2006), human (Telfer et al. 2008) and rodent follicles (Smits et al. 1998, Zhao et al. 2001). However, there are also studies demonstrating its inhibitory role in follicular growth and development at this stage (Yokota et al., 1997). Additionally, the interaction between activin and the components of the extracellular matrix has been reported as a mechanism regulating follicular growth initiation and development (Oktay et al. 2000).

Systemically activin promotes the release of follicle stimulating hormone (FSH) from the anterior pituitary (Katayama et al. 1990), whereas in the ovary, activin promotes follicle growth by increasing granulosa cell proliferation and enhancing antral formation (Mizunuma et al. 1999, Zhao et al. 2001).

### **1.2. Primary and Secondary follicle development**

During transition from primordial to primary follicles, the granulosa cells (GCs) become cuboidal and start their proliferation as a monolayer surrounding the oocyte. The oocyte increases its diameter and the formation of the zona pellucida starts. The secondary follicles have more than 1

layer of granulosa cells and they additionally acquire another cell layer, the theca cells (which attach to the basement membrane of the follicle) (Rajkovic et al., 2006). The theca cells are important because they produce androgens, which are required for follicular growth (Wang et al., 1981; Roberts and Skinner, 1990) and are the substrate for later estradiol production during the antral phase.

## **2. Available models for primordial follicle culture**

### ***2.1. In vitro follicle culture***

Primordial follicles from different species have been cultured under different systems in order to obtain mature oocytes. Mouse primordial follicles were cultured within the whole neonatal ovary on a polycarbonate membrane (Eppig and O'Brien 1996, Jin et al. 2010, O'Brien et al. 2003) in a two-step system for 8 to 10 days followed by an enzymatic digestion to digest the ovary and isolate the preantral follicles which were cultured for 6 more days in culture treated plates.

### ***2.2. Extracellular matrix (ECM) and alginate for in vitro follicle culture***

The ECM is composed of collagenases, laminin, fibronectin, proteoglycans and polysaccharides. The ECM provides a three-dimensional support for the follicular growth maintaining the connections between the oocyte and the somatic cells (Berkholtz et al. 2006). Alginate is an inert hydrogel (Augst et al. 2006) that has been used as a scaffold for primordial (Vanacker et al. 2011) and secondary follicle growth in rodents, primates and human (Jin et al. 2010, Kreeger et al. 2005, 2006, Shikanov et al. 2009, Xu et al. 2006a, 2006b, 2009)

In case of large mammalian species, the ovaries are too large to be cultured as a complete ovary; therefore, the cortex needs to be cut into small pieces. Moreover, the presence of the stromal cells around the primordial follicles improves the culture success Osborn et al. (1997). It has been also reported that individual primordial follicles can be isolated mechanically (Picton et al. 2003, Telfer et al. 2000) or by using enzymatic treatment (Abir et al. 1999, Oktay et al. 1997); some of those isolated primordial follicles are viable and can be grown *in vitro* for 24 hours within a

collagen gel (Abir et al, 1999) or 7 days in an alginate matrix (Vanacker et al., 2011) however they could not reach the late preantral stage. However, to obtain complete *in vitro* maturation of follicles and the release of a metaphase II oocyte, it is necessary to remove the follicle from the ovarian cortex (Abir et al., 2001; Telfer et al., 2008).

### **3. Strategies to activate primordial follicles *in vitro***

There are some factors and hormones which have been described to be important for follicle regulation and culture. Their effect has been evaluated in different animal models. Following there is a description of some of them.

#### **3.1. Effect of Follicle Stimulating Hormone (FSH)**

Contradictory effects have been reported regarding FSH. The addition of FSH has shown no effect on the development of primordial follicles in pieces of bovine cortex (Fortune et al. 1999) supported by the fact that FSH receptor is not expressed in primordial follicles (Oktay et al. 1997, Tisdall et al. 1995). By using knockout mice lacking the expression of FSH  $\beta$ -subunit or lacking the expression of FSH receptor (Abel et al. 2000, Dierich et al. 1998), it was demonstrated that the primordial follicles were able to develop up to the late preantral stage. In contrast other reports that FSH showed positive effects on 1) the primordial follicle formation by regulating the E-cadherin and N-cadherin expression in postnatal ovarian cells in hamster ovaries (Wang and Roy 2010), 2) the regulation of ovarian primordial follicle reserve (by enhancing the development and survival of these follicles in mice) (Allan et al. 2006); and 3) on somatic cell differentiation into early granulosa cells during the onset of primordial follicle development in hamsters (Roy and Albee 2000).

#### **3.2. Effect of Ascorbic acid**

The ascorbic acid (AA) was described as having beneficial effects on *in vitro* follicle development. For instance, by culturing isolated preantral follicles (mice, caprine, bovine and human) in the presence of AA, the collagen biosynthesis is promoted, the follicular basement membrane

protected and apoptosis reduced (Eppig et al. 2000, Murray et al. 2001, Rossetto et al. 2009, Telfer et al. 2008, Thomas et al. 2001). AA in combination with FSH promotes the early folliculogenesis in goats, supporting follicular survival and promoting the activation of primordial follicles and growth after long-term culture (Rossetto et al., 2009).

### **3.3. Effect of Insulin**

Insulin at 5µg/ml promotes the phosphorylation of Akt, the primordial follicle assembly and activation process via the InsR/PI3K/Akt signaling pathway in the 16.5 dpc fetal mouse ovaries cultured *in vitro*. In contrast, insulin has also been reported to repress the phosphorylation of Akt and the follicular assembly and activation process in ovaries of 3 days post partum (3dpp) mouse, cultured *in vitro*.

Although those results suggest that folliculogenesis is stage-specifically regulated by insulin via the Akt signaling pathway (Zhang et al. 2010), it has been reported that elevated unphysiological insulin levels leads to inappropriate differentiation of granulosa cells of secondary follicles grown *in vitro* (Eppig et al. 2000, Sanchez et al. 2011).

## **4. Cell proliferation and apoptosis**

Proliferating cell nuclear antigen (PCNA) has been studied in granulosa cells to provide evidences of the initiation of follicle growth while Caspase 3 is an important apoptotic marker to evaluate cell death.

PCNA is a protein (34KDa) which is synthesized in the early G1 phase and in the S phase from the cell cycle. PCNA serves as a cofactor for DNA polymerase delta and is involved in DNA damage repair during DNA synthesis. PCNA expression has been found in all follicular stages where granulosa cell proliferation and differentiation is seen (Tomanek and Chronowska 2006) and is a marker for healthy follicles (Kerr et al. 2006) .

In contrast to the proliferations of the follicular cells, caspases play a role in apoptosis in many cellular types. Most of them are proenzymes that need to be cleaved in order to be activated (Villa

et al. 1997). Among the caspases, caspase 3 is a key molecule in the ovarian apoptotic pathway, activating endonucleases for DNA fragmentation, which is mainly found in atretic follicles (Hartshorne et al. 2009). Recently reports indicate that the prepubertal primordial follicle loss in mice is not due to caspase 3, it might be related to another apoptotic pathways (Tingen et al. 2009).

## **5. Molecular characterization of developing follicles Oocyte, granulosa and theca cell gene expression**

There are specific proteins which play an important role in the regulation of folliculogenesis at all developmental stages. Disruptions in the function of those genes could lead to abnormal folliculogenesis, compromising oocyte/embryo quality, and consequently, fertility. Following is a list of genes selected for their importance in follicle development and oocyte/embryo quality.

### **5.1. *Gdf9* (Growth differentiation factor 9)**

GDF-9 is a family member of the transforming growth factor  $\beta$  superfamily and is expressed in growing and fully mature oocytes. *Gdf9* null female mice have been reported to be infertile and their folliculogenesis arrested at the primary stage (Dong et al. 1996, Vitt et al. 2000b). GDF-9 is considered as a factor involved in the primary to secondary follicle transition (McNatty et al. 1999). GDF-9 also contributes in the proliferation of granulosa cells, In the regulation of the expression of Prostaglandin synthase-2 and Hyaluronan synthase-2, enzymes which are involved in cumulus cell mucification and expansion (Gui and Joyce 2005, Wu and Matzuk 2002).

### **5.2. *Mater* (Maternal antigen that embryo requires)**

MATER, also known as NALP5, is a member of the NALP (NAVHT-, LRR-, and PYD-containing) family of proteins involved in both apoptotic and inflammatory signaling pathways (Dade et al. 2004,

Tschopp et al. 2003). Mater transcripts are first detected in primordial follicles but the protein is detected in oocyte from primary follicles and accumulates during oocyte growth being localized in the cytoplasm of the oocytes from the small primary follicles onwards (Tong et al. 2004). Mater-null females display a normal phenotype regarding folliculogenesis, ovulation and fertilization but their embryos do not develop beyond the 2-cell stage (maternal to embryo transition) (Tong et al., 1999).

### **5.3. *Amh* (Anti Mullerian hormone)**

The Anti Mullerian hormone (AMH) is a family member of the transforming growth factor  $\beta$  superfamily and is expressed in the granulosa cells in follicles at the primary stage to the early antral stage (Durlinger et al. 2002a, 2002b). The *Amh* null female mice are fertile but at the age of 13 months there are less primordial follicles compared with a normal ovary because the primordial follicles are recruited at a faster rate (Behringer et al. 1990, Durlinger et al. 1999)

Since AMH levels are related to estradiol levels in follicular fluid from human small antral follicles, it has been suggested that AMH is a co-regulator of steroidogenesis in granulosa cells (Andersen and Byskov 2006) by inhibiting aromatase activity on granulosa cells (Visser et al. 2006). Regarding to its relation with FSH, It have been suggested that AMH has an inhibitory effect on follicular sensitivity to FSH (Durlinger et al., 1999) playing a role in the process of follicular selection.

### **5.4. *Cyp17* (Cytochrome P450 17 $\alpha$ -hydroxylase-lyase)**

CYP17 is expressed in the theca cells in the ovary playing an important role in the control of the biosynthesis of steroid hormones (Roberts and Skinner 1990). CYP17 catalizes two important reactions in the ovary: 1) Hydroxylation of pregnenolone and progesterone at carbon 17 and 2) the conversion of pregnenolone and progesterone into C19 steroids (Patel et al. 2010).

## 6. Aims

1. The main aim was to design a culture system which supports the activation and growth of primordial follicles. Since follicle activation is an actively controlled process occurring in the ovary, it is hypothesized that *in vitro* activation could produce large numbers of mature follicles, even more than what are actually activated *in vitro*. To achieve this aim:

- a. Induction of primordial follicle activation was evaluated by manipulating the Akt/PI3K/Foxo pathway using the concept described by Li et al. (2010). Therefore, a PTEN inhibitor (bpV(pic)) and an enhancer of the activity of PI3K (activator 740Y-P).
- b. Promotion of small follicle growth was evaluated by the addition of Activin A to *in vitro* cultured tissue.

Evaluation parameters:

- i. Morphological analysis: histological sections of cultured tissue will be screened.
  - ii. Gene expression levels of *Gdf9*, *Mater*, *Amh* and *Cyp17* will be studied to compare the quality of the *in vitro* grown secondary follicles to isolated secondary follicles from 13 day old ovaries.
2. Because it has been shown that follicular growth further than preantral stages is difficult to achieve in cultured ovarian pieces, a second aim was to isolate secondary follicles from the cultured tissue and make them grow by culturing them in a standard mouse secondary follicle culture described by Cortvrindt and Smitz (2002). Survival, production of estradiol and progesterone before and after ovulation induction and their capacity of meiotic resumption will be evaluated.



## **ii. Materials and Methods**

### **1. Animals**

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

### **2. Primordial follicle culture**

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

Ovaries of 4 to 5 mice were used per repeat in the Experiments 1, 2, 3 and 4; and for the experiments 5 and 6, ovaries from 5 to 7 mice were used per repeat. Each ovary was cut into 18 pieces, the pieces were pooled and aleatorily distributed and cultured in 96-well plates (Costar) in 75µl of the different treatments under the following conditions: 37°C, 5% CO<sub>2</sub> and 100% humidity.

The basal culture medium was: α-MEM (Invitrogen) supplemented with 5µg/ml transferrine (Sigma), 5ng/ml selenium (Sigma), 10% FBS (Invitrogen) plus 10mIU/ml r-FSH (Ares-Serono). For all the experiments the media refreshment was done every 3 days by changing 30µl. The α-MEM medium contains 50µg/ml Ascorbic acid.

## **2.1. Assessment of cultured pieces**

For all experiments, the cultures were evaluated every 3 days. The evaluation was done under an inverted microscope. According to their morphological aspect and size, the ovarian tissue pieces were classified as follows:

1. "Growing" when at the end of the culture the tissue pieces were alive and larger than on the first day in culture.
2. "Non-growing" when at the end of the culture the tissue pieces were alive (still clear) but had not grown during the culture.
3. "Degenerated" when at the end of the culture the tissue pieces were dead (they became darker or had desintegrated).

The survival rate of the ovarian tissue pieces was calculated as the addition of the growing and non-growing tissue pieces over the initial amount of ovarian tissue pieces that were cultured. The growth rate of the ovarian tissue pieces was calculated as the number of ovarian tissue pieces that had increased in size out of the surviving fraction at the end of culture.

For the experiment 2 and 3, the amount of follicles within the ovarian tissue pieces were counted under an inverted microscope and the follicular diameter was estimated by using a micrometric ocular. According to their diameter the follicles were classified as follows: 40 – 89 $\mu$ m, primary follicles, 90 – 109 $\mu$ m, primary to secondary follicle transition and 110 – 130 $\mu$ m as secondary follicles. Then, the number of follicles per ovarian tissue piece was calculated as the total amount of counted follicles over the number of tissue pieces that survived by the end of the culture.

## **2.2. Culture in alginate matrix**

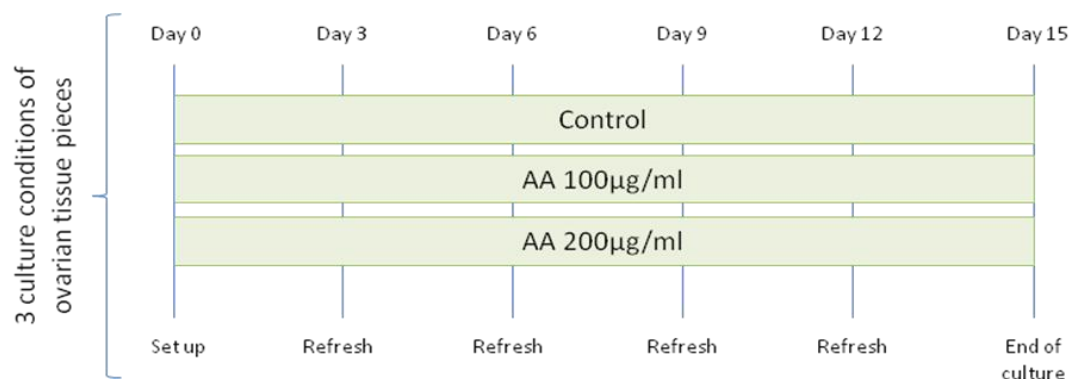
### ***Experiment 1: Effect of the Ascorbic acid (Figure 1)***

In order to keep the three-dimensional structure of the growing follicles alginate was used initially as a matrix to support growth of the ovarian tissue pieces.

The ovarian tissue pieces were embedded in small alginate beads (kindly donated by Prof. Lonnie Shea, Northwestern University Chicago, Illinois USA) 0.25% in PBS. To make the beads, 5 $\mu$ l droplets of alginate solution (containing one ovarian tissue piece) were released into a petri dish containing a 50mM CaCl<sub>2</sub> and 140mM NaCl solution for 2 minutes at 37°C. Then the beads were rinsed in Isolation medium for 30 minutes and cultured in 96-well plates (Costar) in 75 $\mu$ l of the basal culture medium supplemented with 5 $\mu$ g/ml insulin in the absence (Control) or presence of 100 or 200 $\mu$ g/ml ascorbic acid. The control medium was the basal medium supplemented with 5 $\mu$ g/ml insulin but without Ascorbic Acid (AA).

Ten beads were placed per plate. Four repeated experiments were done. Ovarian tissue pieces were fixed at day 0 and at day 15 for further histological analysis.

**Figure 1. Experimental set up to evaluate the effect of the Ascorbic Acid in primordial follicle culture.**



### 2.3. Culture in ultra low attachment plates

The use of alginate as a matrix to support the growing of ovarian follicles or tissue pieces has been described. The ultra low attachment (ULA) plates used under these experimental conditions are an alternative to preserve the three-dimensionality of the ovarian tissue pieces. The ULA surface is a unique covalently bonded hydrogel surface that is hydrophilic and neutrally charged. It minimizes cell attachment and spreading, protein absorption and enzyme activation. The surface is non-cytotoxic, biologically inert and non-degradable.

The ovarian tissue pieces were individually transferred to 96-well ULA plates (Costar) and cultured for 15 to 24 days under the further detailed conditions. The culture conditions (T°, CO<sub>2</sub>, humidity) were identical to those above. Basal medium supplemented with 5ng/ml.

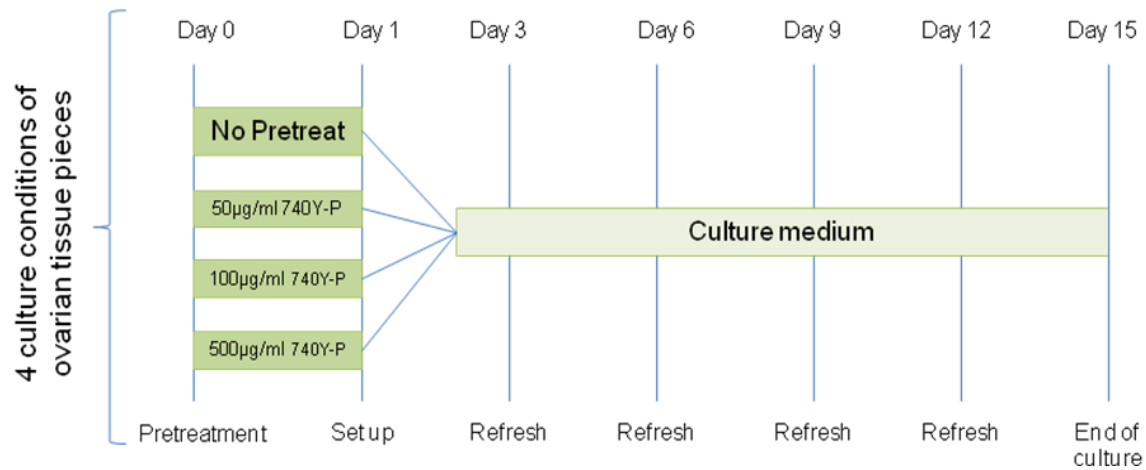
The idea of manipulating the PI3K pathway to mimic primordial follicle activation (The principle to use the 740Y-P activator and the bpV(pic), a PTEN inhibitor) was taken from Li et al. Jing Li, Kazuhiro Kawamura, Yuan Cheng, Shuang Liu, Cynthia Klein, Shu Liu, En-Kui Duan, and Aaron J. W. Hsueh. 2010. Activation of dormant ovarian follicles to generate mature eggs. Proc Natl Acad Sci U S A 107: 10280-10284

### ***Experiment 2: Effect of the 740Y-P activator (Figure 2)***

The activator 740Y-P is a cell-permeable phospho-peptide which enhances the enzymatic activity of PI3K, stimulating the Akt pathway known to be involved in the primordial follicle activation.

The ovarian tissue pieces were pre-pretreated either with 50, 100 or 500µg/ml of the 740Y-P activator for 24 hours in a hanging drop system. The culture medium used in this set-up was the basal culture medium supplemented with 5ng/ml insulin and 100µg/ml Ascorbic acid. Briefly, drops of 10 µL containing the activator in culture medium were distributed in the cover of a petri dish, quickly the ovarian tissue pieces were individually transferred to the drops in the petri dish. After 24 hours, the ovarian tissue pieces were washed 2 times in 75µl of culture medium without activator and individually transferred to each well in the ULA plates containing 75µl of culture medium. The No-pretreatment condition was our control, where the ovarian tissue pieces were cultured for 24 hours in a hanging drop system but in the absence of the Activator. Three repeats were done and 10 ovarian tissue pieces were cultured per plate.

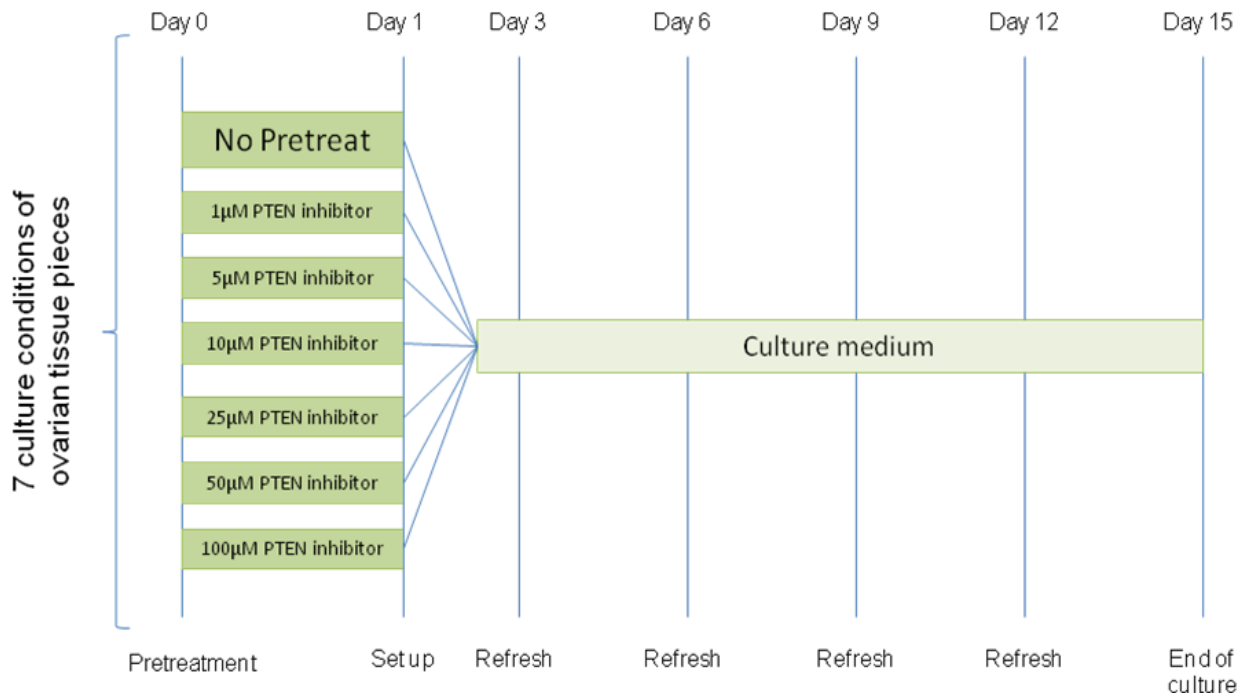
**Figure 2. Experimental set up to evaluate the effect of the Activator in primordial follicle culture.**



**Experiment 3: Effect of the bpV(pic), a PTEN inhibitor (Figure 3)**

The bpV(pic), a PTEN inhibitor, prevents the conversion of PIP3 back to PIP2. The accumulation of PIP3 in the cell will stimulate the Akt pathway. The ovarian tissue pieces were pre-pretreated either with 1, 5, 10, 25, 50 or 100 µM of the PTEN inhibitor for 24 hours in a hanging drop system. Then, the pieces were treated and cultured as described above. The No-pretreatment condition was our control, where the ovarian tissue pieces were cultured for 24 hours in a hanging drop system but in the absence of the PTEN inhibitor. Four repeats were done and 20 ovarian tissue pieces were cultured per plate.

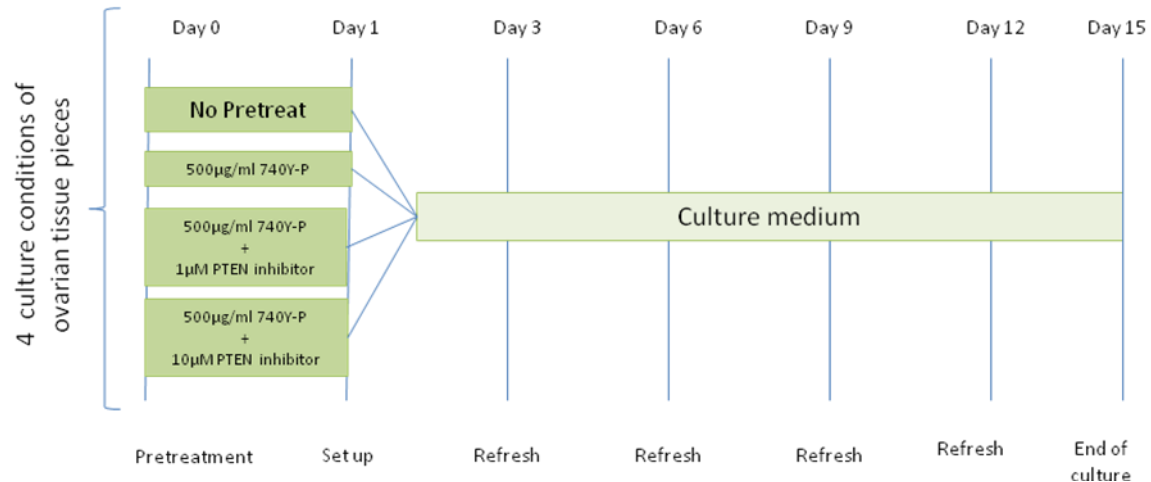
**Figure 3. Experimental set up to evaluate the effect of the PTEN Inhibitor in primordial follicle culture**



***Experiment 4: Effect of the PTEN inhibitor plus the 740Y-P activator (Figure 4)***

The ovarian tissue pieces were pre-pretreated either with 500µg/ml of the 740Y-P activator, or with the combination of both PTEN inhibitor (1 or 10µM) plus 500µg/ml of the 740Y-P activator for 24 hours in a hanging drop system. The ovarian tissue pieces were treated and cultured as describe above. The No-pretreatment condition was our control, where the ovarian tissue pieces were cultured for 24 hours in a hanging drop system but in the absence of the Activator (500µg/ml) and the PTEN inhibitor. Three repeats were done and 20 ovarian tissue pieces were cultured per plate.

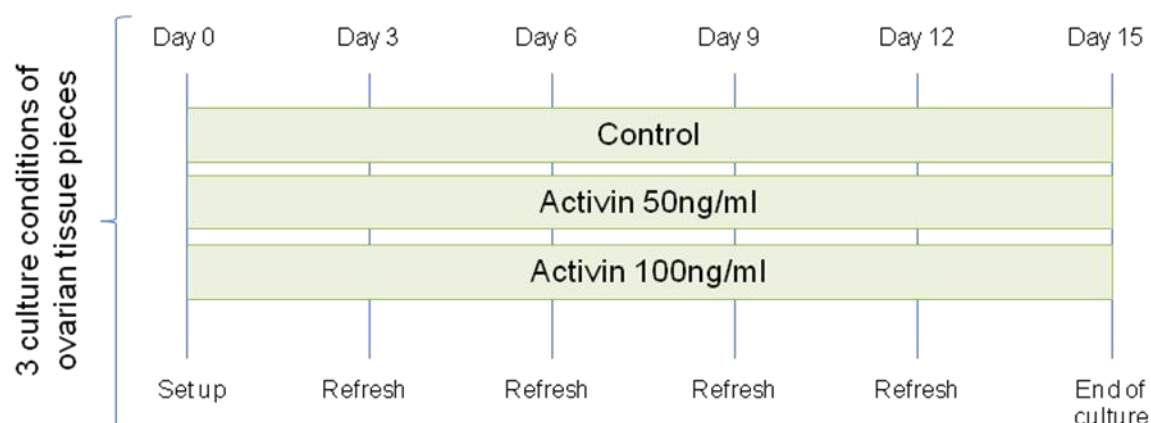
**Figure 4. Experimental set up to test the effect of the PTEN inhibitor and the 740Y-P activator in primordial follicle culture.**



**Experiment 5: Effect of Activin A (Figure 5)**

The ovarian tissue pieces were cultured in 75 µl of the basal culture medium supplemented with 5ng/ml insulin and 100µg/ml Ascorbic acid in the presence or absence of 50ng/ml or 100ng/ml rh-Activin A (kindly donated by Prof. Teresa Woodruff, Northwestern University Chicago, Illinois USA). As a control the ovarian tissue pieces were cultured in 75µl of the culture medium in the absence of Activin A. Three repeats were done and 20 ovarian tissue pieces were cultured per plate.

Figure 5. The figure is showing the experimental set up used to evaluate the effect of Activin A on primordial follicle development.



**Experiment 6: Effect of Activin A supplementation on a background of PTEN inhibitor plus the 740Y-P activator treatments (Figure 6 and 7)**

The ovarian tissue pieces were first pre-pretreated either with the combination of both PTEN inhibitor (either at 1 or 10 $\mu$ M) and 500 $\mu$ g/ml of the 740Y-P activator for 24 hours in a hanging drop system. Briefly, drops of 10  $\mu$ L containing the inhibitor plus the activator in culture medium were distributed in the cover of a petri dish, quickly the ovarian tissue pieces were individually transferred to the drops in the petri dish. After 24 hours, the ovarian tissue pieces were washed 2 times in 75 $\mu$ l of culture medium without inhibitor and activator and individually transferred to each well in the ULA plates containing 75 $\mu$ l of the culture medium. The ovarian tissue pieces were cultured in the basal culture medium supplemented with 5ng/ml insulin and 100 $\mu$ g/ml Ascorbic acid in the presence or absence of 100ng/ml Activin A.

The No-pretreatment condition was our control, where cultured for 24 hours in a hanging drop system and the ovarian tissue pieces were incubated in the basal control medium supplemented with 5ng/ml insulin and 100 $\mu$ g/ml AA (in the absence of the 740Y-P activator and PTEN inhibitor).



Three repeats were done and 20 ovarian tissue pieces were cultured per plate.

For this experiment, the treatments are described as:

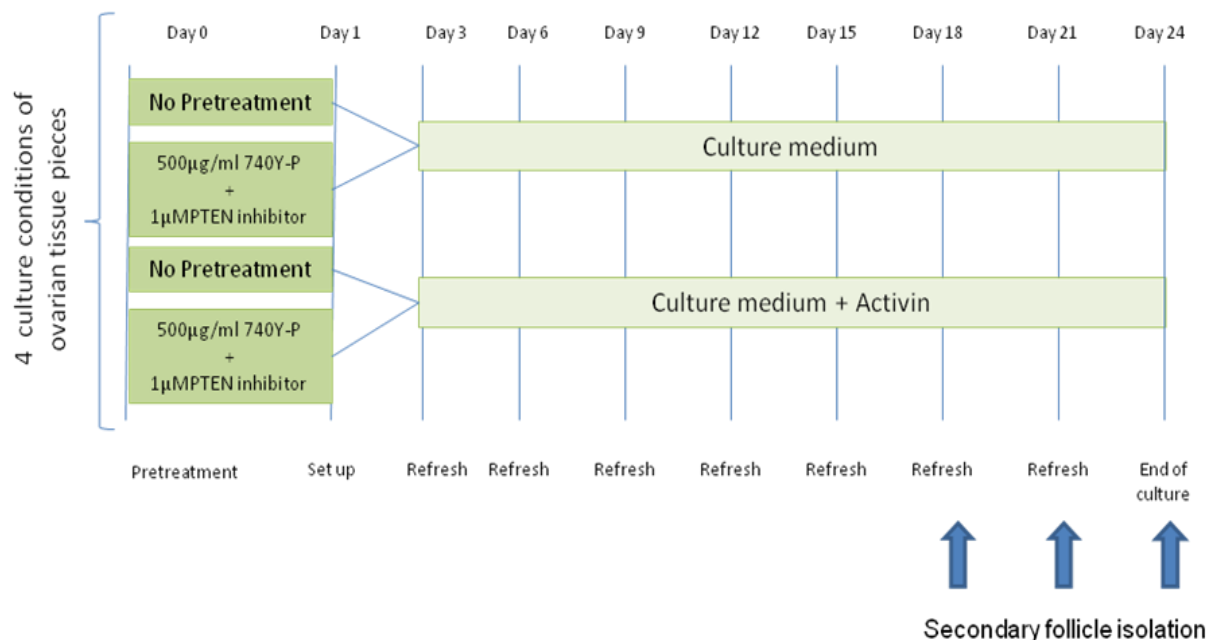
Control: Ovarian tissue pieces were pretreated with control medium (not pretreated with PTEN inhibitor plus the Activator)

Activin: Ovarian tissue pieces were not pretreated with PTEN inhibitor plus the Activator but were cultured in the presence of Activin A.

Ac+I: Ovarian tissue pieces were pretreated with PTEN inhibitor plus the Activator for 24 hours.

Ac+I+At: Ovarian tissue pieces were pretreated with PTEN inhibitor plus the Activator for 24 hours but were cultured in the presence of Activin A.

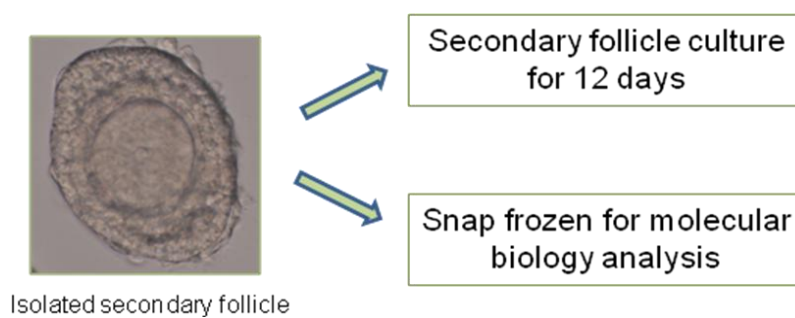
**Figure 6. Experimental set up to test the effect of the PTEN inhibitor plus the 740Y-P activator in combination with Activin A in primordial follicle tissue culture.**



After 18, 21 and 24 days in tissue culture, the ovarian tissue pieces containing secondary follicles were taken out of the plates and placed in a one-well petri dish containing isolation medium. By using 30G x ½" needles, the secondary follicles were carefully isolated. After the isolation procedure the remaining ovarian tissue was put back in its original well to allow the further growth of the activated follicles (transitional and primary stages) contained in that piece.

The secondary follicles isolated were further allocated to two procedures: 1) half of them were cultured in a secondary follicle culture system for more 12 days, 2) the other half was snap frozen for further molecular biology analysis (study the gene expression for important markers of early follicle development) (Figure 7).

**Figure 7. Handling of the isolated secondary follicles at day 18, 21 and 24 *in vitro*.**



### 3. Secondary follicle culture

Secondary follicles were isolated from the cultured ovarian tissue pieces in experiment 6 (Figure 8) and cultured individually as described previously (Cortvrindt and Smitz 2002). Only follicles with diameters between 110 and 130  $\mu\text{m}$  were individually transferred to 96-well plates which allow attachment to flat plastic bottom (Costar, Escolab) and cultured for 12 days under the following conditions: 37°C, 5% CO<sub>2</sub>, 100% humidity. Culture medium consisted of  $\alpha$ -MEM (Invitrogen), 5% FBS (Invitrogen), 5 ng/ml insulin (Sigma), 5  $\mu\text{g}/\text{ml}$  transferrin (Sigma) and 5 ng/ml sodium selenite (Sigma), 10 mIU/ml r-FSH and 10 mIU/ml r-LH (added once at the beginning of the culture) (both from Ares-Serono). Follicles were cultured in 75  $\mu\text{l}$  of culture medium refreshed every 3 days by replacing 30  $\mu\text{l}$  of spent by fresh medium.

As *in vivo* controls, secondary follicles with diameters between 110 $\mu\text{m}$  and 130 $\mu\text{m}$  were mechanically isolated out from ovaries of 13-day old mice (F1 hybrids: C57Bl/6j x CBA/ca).

### **3.1 Criteria of follicle selection**

On day 1 of culture the secondary follicles were selected based on their oocyte-granulosa cell connections and the presence or absence of theca cells surrounding the follicle. Only those follicles which had 75 to 100% of oocyte-granulosa cell connection and 3 or more theca cells surrounding the follicle were chosen for intact follicle culture as single units (one follicle per well).

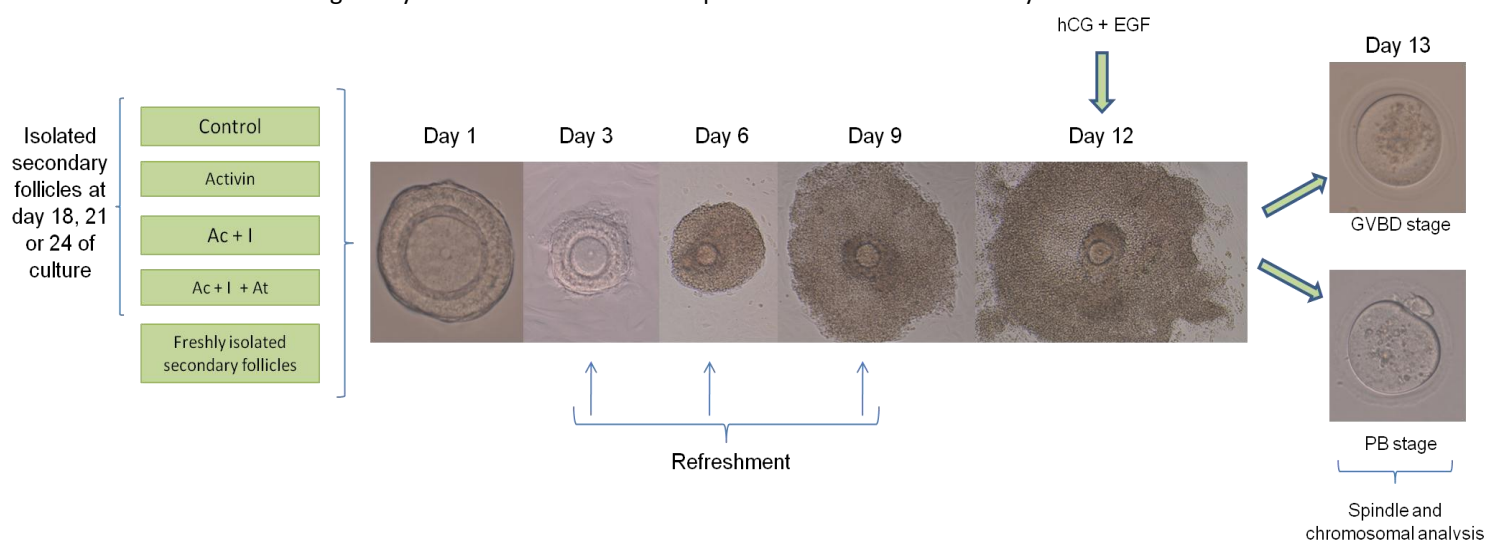
#### **a. Assessment of meiotic resumption**

On Day 12 of culture, ovulation was induced by the addition of 1.2 IU/ml human chorionic gonadotrophin (hCG) (Ares-Serono) plus 4ng/ml recombinant human epidermal growth factor (EGF; Roche Diagnostics, Mannheim, Germany). After 16 hours, the mucification was scored and the cumulus-oocyte-complex (COCs) were taken out of the follicle to start the denudation. The denudation procedures was done by using a glass Pasteur pipette, the COC was taken in and out of the pipette until the oocyte was freed of all cumulus cells. After denudation, the oocytes were evaluated under an inverted microscope and classified as: Germinal vesicle (GV), where a round spot of nuclear material was seen; Germinal vesicle break down (GVBD), where the spot of nuclear material cannot be longer seen and polar body (PB), where a small cell was seen in the perivitelline space. After denudation, all the oocytes were fixed for spindle and chromosomal analysis as described below.

#### **b. Steroid measurement**

At day 12 and day 13, before and after the hCG+EGF stimulus respectively, 30 $\mu$ l of culture media was collected and frozen for Estradiol and Progesterone measurement. Kits for Estradiol and Progesteron were used for steroid measurement in a COBAS automated analyzer (Roche Diagnostic GmbH, Mannheim, Germany). Steroids concentration were measured in the Hormonology Laboratory at UZ Bruzzel by Johan Schiettecatte.

**Figure 8. Secondary follicles isolated from cultured ovarian tissue pieces after 18, 21 and 24 days in culture and freshly isolated secondary follicles were cultured for 12 days.** The media refreshment was done every 3 days (at day 3, 6 and 9) and on day 12 they received the hCG+EGF stimulus. 16 hours after stimulation, GV, GVBD and PB stage oocytes were fixed for further spindle and chromosomal analysis.



#### 4. Spindle and chromosome analysis

This method was originally published by Mattson and Albertini (1990). GVBD and PB stage oocytes were fixed in a microtubule-stabilizing buffer (0.1M PIPES, 5mM  $MgCl_2 \cdot 6H_2O$ , 2.5mM EGTA, 1mM Dithiothreitol, 50% deuterium oxide, 0.1% Triton X-100, 0.01% PMSF, 1 $\mu$ M Taxol and 2% formaldehyde) for 45 minutes at 37°C and processed for staining of the meiotic spindle and chromosomes. After fixation, the oocytes were sequentially incubated in mouse monoclonal anti-alpha tubulin antibody (1:100, Sigma-Aldrich) for 45 minutes, with Alexa Fluor 488-labelled goat anti-mouse antibody (1:200, Molecular Probes) for 45 minutes at 37°C and with ethidium homodimer-2 to counter stain the chromosomes (1:2000, Molecular Probes) for 15 minutes. In between incubations, the oocytes were washed 3 times for 15 minutes in Washing-blocking solution (PBS containing 0.02% sodium azide, 0.2% powdered milk, 2% normal goat serum, 1% BSA, 0.1M glycine and 0.01% Triton X-100). Stained oocytes were mounted in 90% glycerol-PBS solution and analyzed under an Olympus IX70 Fluoview confocal microscope system equipped with an Argon-Krypton laser (488-568nm) and band filter 510-540 and long pass filter 610 to visualize Alexa Fluor 448 and Ethidium homodimer-2, respectively.

Secondary follicles isolated from 13 day old mouse ovary were culture in a regular secondary follicle culture for 12 days. The ovulated oocytes were used as a in vivo controls.

## **5. Histological analysis**

### ***Selection of pieces for histological analysis***

For histological analysis at day 15, 18 or 24 of culture, ovarian tissue pieces out of the surviving fraction, were taken aleatorily to be fixed.

Ovarian tissue pieces from culture days 0 and 15 were fixed from the experiment 1; from day 0, 3 and 18 for the experiment 4; from day 0 and day 15 for the experiment 5 and from day 0, 3 and 24 for experiment 6. The ovarian tissue pieces were fixed in formalin 4% overnight at 4°C. Subsequently, they were transferred into 70% ethanol at 4°C until they were processed and embedded into paraffin blocks. The ovarian tissue pieces were sectioned at 5µm thickness on a sliding microtome (This work was performed by Katty Billooye).

For hematoxylin-eosine-saffran staining (HES), the sections were deparaffinized, hydrated and stained. Primordial, primary and secondary follicles were counted. To avoid counting the same follicle more than once, only follicles with visible oocyte nucleolus were counted.

### ***Follicle classification***

The early stage follicles were classified as: 1) Primordial follicle, oocyte surrounded by flattened granulosa cells (GC), 2) Primordial to Primary follicle transition, oocyte surrounded by a mixture of flattened and cuboidal GC, 3) Primary follicle, oocyte surrounded by one layer of cuboidal GC, 4) primary to secondary follicle transition, oocyte surrounded by one and a half layer of GC, 5) secondary follicle, oocyte surrounded by 2 layers of GC, 6) medium secondary follicle, oocyte surrounded by two and a half to 3 layers of GC.

## 6. Immunohistochemistry

Ovarian tissue pieces from day 0 and day 15 were fixed for the experiment 1; from day 0, 3 and 18 for the experiment 4; from day 0 and day 15 for the experiment 5 and from day 0, 3 and 24 for experiment 6. 5µm sections were cut for immunolocalization of proliferation cell nuclear antigen (PCNA) and caspase 3. The sections were incubated overnight at 37°C and then for 2 hours at 60°C, at which point they were deparaffinized, hydrated and rinsed in running water.

### ***For PCNA***

Endogenous peroxidase activity was blocked by 3% H<sub>2</sub>O<sub>2</sub> in methanol for 30 minutes at room temperature. Sections were washed in PBS plus 1% Triton X-100 for 5 minutes and preincubated with 10% normal goat serum plus 0.8% BSA for 30 minutes at room temperature and then incubated with the first antibody, monoclonal mouse anti rabbit PCNA (Novocastra, Prosan) overnight at 4°C. The second antibody goat anti-mouse peroxidase (Amersham Pharmacia Biotech) was applied for 30 minutes at room temperature. Binding of the primary antibody was detected with diaminobenzidine (DAB; Vector Laboratories, Burlingame) according to the manufacturer's instructions. Sections were counterstained with hematoxylin. Negative control sections were stained without the first antibody.

Primordial follicles were considered PCNA positive if at least one pregranulosa cell was positive. Primary, primary to secondary transition and secondary follicles were considered positive if 50% or more of their granulosa cells were PCNA positive.

### ***For caspase 3***

This work was done at the lab of Prof. Wim Declercq (VIB Laboratory in Zwijnaarde - Gent). The sections were treated in citrated buffer for 10 minutes at 95°C. After cooling the sections to room temperature, they were washed with distilled water and PBS for 5 minutes. Endogenous peroxidase activity was blocked by 0.1% H<sub>2</sub>O<sub>2</sub> in PBS for 20 minutes at room temperature. Sections were preincubated with normal goat serum for 20 minutes at room temperature and then

incubated with the first antibody, monoclonal anti-active caspase3 (Bio-Connect B.V. – Cell Technology Inc.) overnight at 4°C. The second antibody goat anti-rabbit peroxidase (DAKO) was applied for 1 hour at room temperature. Binding of the primary antibody was detected with DAB (DAKO) according to the manufacturer's instructions. Sections were counterstained with hematoxylin. Negative control sections were stained without the first antibody.

## **7. Gene expression analysis of isolated follicles**

### **7.1. *In vitro* collections**

In the experiment 6, at day 18, 21 and 24 days after culture ovarian tissue pieces containing secondary follicles from all the treatments were placed in Isolation medium. *In vitro* grown secondary follicles were mechanically isolated, classified according to their diameter (between 110 and 130µm), pooled per 5 follicles and snap frozen in liquid nitrogen (LN<sub>2</sub>) for further molecular biology analysis.

### **7.2. *In vivo* collections**

As *in vivo* controls, secondary follicles from 13 day-old mice (F1 hybrids: C57BL/6j x CBA/Ca) were mechanically isolated in Isolation medium. Follicles between 110 and 130µm were isolated and pooled per 5 follicles and snap frozen in LN for further molecular biology analysis.

### **7.3. *RNA isolation and cDNA synthesis***

This protocol was originally designed by Adriaenssens et al. (2009). Total RNA was extracted from pools of five follicles (of *in vivo* and *in vitro* grown follicles) using the total RNA extraction kit (RNeasy; Qiagen, Antwerp, Belgium) on the QiaCube robot (Qiagen) according to the manufacturer's instructions with minor modifications. During the lysis step, Polyd A was added to

improve the efficiency of the extraction and luciferase mRNA was added as exogenous control. A DNase step was used to remove residual genomic DNA and then, extracted mRNA was eluted in 14µL of RNase free water.

Total mRNA was reverse transcribed into cDNA with the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) containing a blend of oligo (dT) and random primers, according to manufacturer's instructions. 10µl of extracted RNA from each pool of 5 follicles was supplemented with reverse transcription (RT) mix containing: 5µl of water, 4µl of the 5X iScript reaction mix and 1µl of the iScript Reverse transcriptase per sample. The cycling conditions were as follows: 5 minutes at 25°C, 30 minutes at 42°C and 5 minutes at 85°C. Reaction was carried out in 20µl and the samples were at the end diluted to a final volume of 60µl.

#### **7.4. Real time PCR**

cDNA quantification by Real-time PCR was performed on a LightCycler 480- apparatus (Roche Diagnostics). Primers sequences for mouse *Gdf9*, *Amh*, *Mater*, *Cyp17*, *18S* and *Luciferase* are detailed in Table 1. The methods used to measure current genes were originally designed by Sanchez et al. (2009)

For the genes *Gdf9*, *Amh*, *Mater*, *18S* and *Luciferase*, the total volume for the amplification reaction was 10µl, containing 2µl cDNA, and 8µl of PCR mix containing 5µl SYBR Green PCR Master Mix 2x (Roche Diagnostic), 1µl of 0.6µM primer mix and 2µl of nuclease free water per sample. The amplification conditions were as follows: 10minutes at 95°C, followed by 50 cycles at 95°C for 10 seconds and 60°C for 30 seconds, followed by the acquisition of the melting curve (95°C per 5s and 60°C for 1 min by a continuous fluorescence measurement).

For *Cyp17*, the total volume for the amplification reaction was 10µl, containing 2µl cDNA, and 8µl of PCR mix containing 5µl Probe PCR Master Mix 2x (Roche Diagnostic), 1µl of 0.6µM primer mix, 0.1µl of Probe and 1.9µl of nuclease free water per sample. The cycling conditions were as follows: 10 minutes at 95°C, followed by 50 cycles at 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 1 second.



Reactions were performed in triplicate for each sample. Standard curves for *Gdf9*, *Amh*, *Mater*, *18S* were constructed using 10-fold serial dilutions of a corresponding synthetic oligo (ranging from 1 to  $10^6$  copies) while for *Luciferase* the serial dilutions were from 100 to  $10^{-3}$  pg and were run in each reaction plate.

The number of copies for each gene within each sample was normalized in relation to copies of exogenous or endogenous control measured in the same sample. *Gdf9* and *Mater* were normalized in relation to copies of exogenous control luciferase and calibrated for the number of oocytes per sample. *Amh* was normalized in relation to copies of endogenous control *18S*. All the graphs will represent the normalized amount of copies per sample.

**Table 1. Primer sequences used for PCR analysis**

Gene	Sequence (5'-3')	Fragment size (bp)	Probe	Location	Accession No.
<b><i>Gdf9</i></b>	F: taccgtccggctcttcagt	93	-	364-382	NM_008110.1
	R: ttaaacagcagggtccaccatc			436-456	
<b><i>Mater</i></b>	F: caatgccctgtcttaacctg	71	-	3239-3259	NM_011860.2
	R: tgttcttctactcgggcata			3290-3309	
<b><i>Amh</i></b>	F: tgctagtctacatctggctga	120	-	380-401	NM_007445.2
	R: gtccagggtatagcactaacagg			477-499	
<b><i>Cyp17</i></b>	F: ggactgcatcacgataaaa	70	20	1345-1364	NM_007809.3
	R: tggatctaagaagcgctcag			1395-1414	
<b><i>18S</i></b>	F: tcaagaacgaaagtccgagg	488	-	1026-1045	NR_003278.1
	R: ggacatctaaggcatcaca			1494-1513	

## 8. Statistical Analysis

A comparison of percentages of survival rate, growing rate, follicle classification in the cultured ovarian tissue pieces and Polar body rate was done by arcsine transformation and analyzed by one-way ANOVA. Group comparison was performed with Tukey Post test.

Differences in gene expression levels for *Gdf9*, *Mater* and *Amh* between the treatments and the *in vivo* condition were compared using one-way ANOVA. Group comparison was performed with Tukey Post test.

To analyze the number of follicles per cultured ovarian tissue pieces, the normal distribution was assessed by the Kolmogorov-Smirnov test. Since our data showed a normal distribution, we decided to analyze it by one-way ANOVA. Group comparison was performed with Tukey Post test.

Differences were considered statistically different when  $P < 0.05$ . All the statistical analysis was done using the GraphPad Prism program, version 4 for Windows (GraphPad software, San Diego California - USA).

## ii. Results

It has been reported that individual primordial follicles can be isolated mechanically (Telfer et al., 2000; Picton et al., 2003) or by using enzymatic treatment (Oktay et al., 1997; Abir et al., 1999). Some of those isolated primordial follicles are viable after isolation and it has been reported in human that they can be grown *in vitro* for 24 hours within a collagen gel or for 7 days in an alginate matrix (Abir et al., 1999; Vanacker et al., 2011) but they could not reach large preantral stages. An alternative approach for primordial follicle culture is to grow them within fragments of the ovarian cortex, Osborn et al. (1997) had shown that the presence of the stromal cells around the primordial follicles improves the culture success.

In the beginning of our experiments, some ovarian tissue pieces were able to grow, however after 15 days of culture histological evaluation showed that no follicular basement membrane was observed. In an attempt to maintain the follicular basement membrane, Ascorbic Acid (AA) concentration in medium was increased from 50µg/ml to 100µg/ml. It has been described that AA can promote collagen biosynthesis, give protection to the basement membrane and reduce apoptosis (Eppig et al., 2000; Thomas et al., 2001; Telfer et al., 2008; Murray et al., 2001; Rossetto et al., 2009) having beneficial effects on *in vitro* follicle development.

### **1. Effect of the ascorbic acid on primordial follicle culture (Figure 9, 10)**

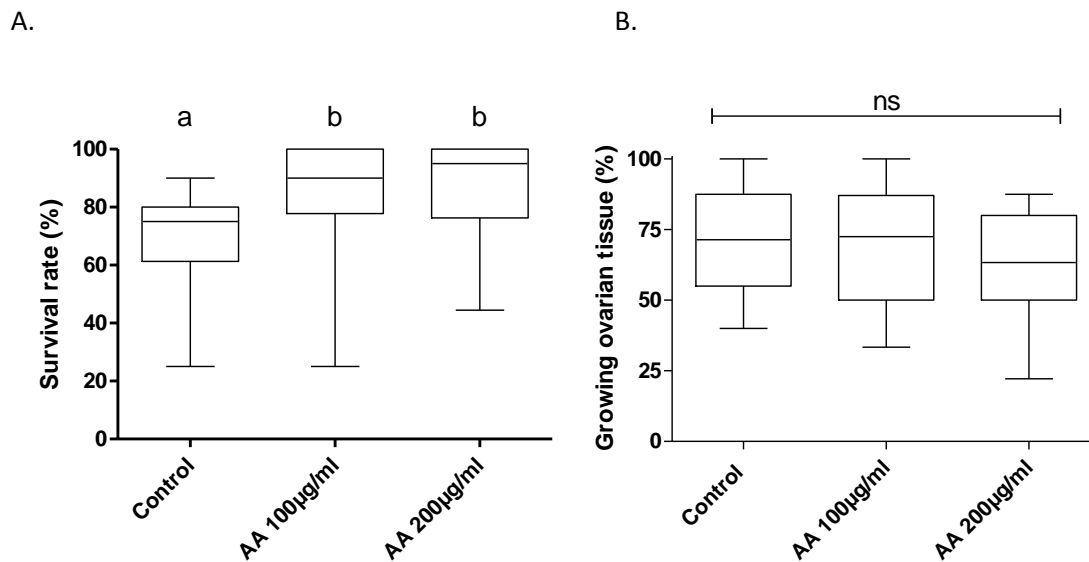
Tissue pieces cultured in presence of AA 100µg/ml or 200µg/ml demonstrated a significantly higher survival rate (growing +non growing tissues) than in controls ( $P<0.05$ ), 84%, 86% and 69% respectively (Figure 9A). Out of the surviving fraction, no differences were observed between the tissue pieces cultured in the presence of Ascorbic acid and the Control condition (Figure 9B). The mean growing rate was 66%. Some crystallization was observed within the beads cultured in AA 200µg/ml.

After 15 days in culture, histological analysis revealed that the mean number of primordial follicles in the fresh tissue (89%) (Ovarian tissue from 3 day old ovaries) was significantly higher compared to the 2 conditions of AA (60% as a mean). However, the number of primary follicles in the fresh tissue pieces (4%) was significantly lower compared to the 2 conditions of AA (20% as a mean). The

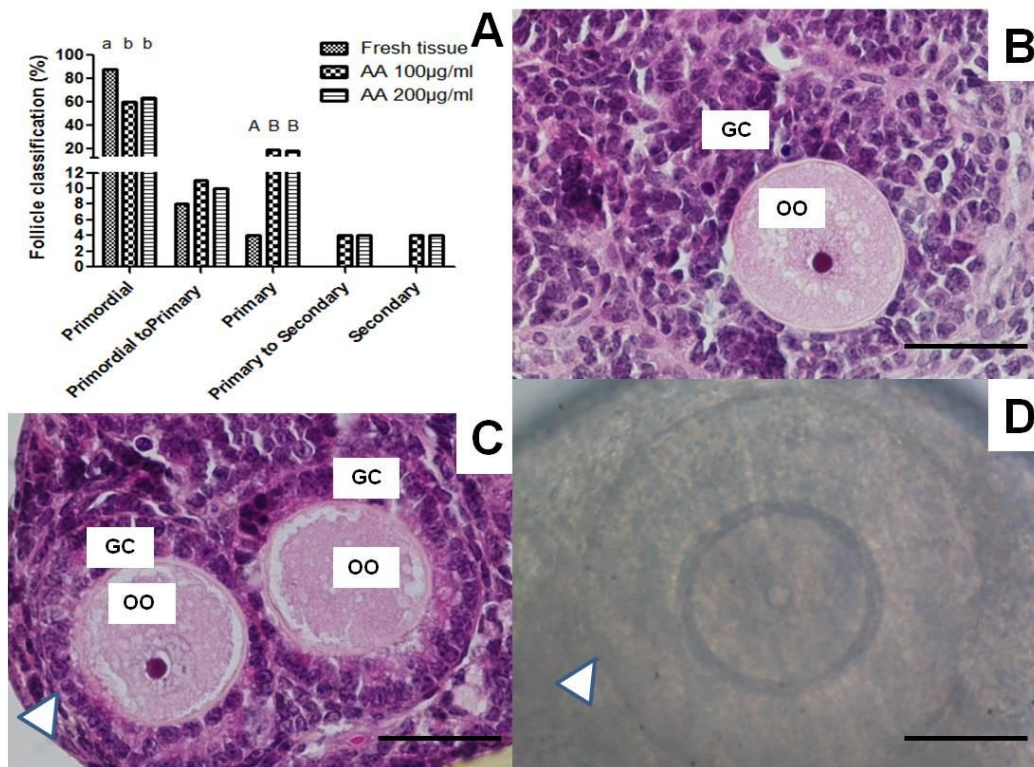
mean number of healthy primordial, primordial to primary transition, primary, primary to secondary transition and secondary follicles was not statistically different between the 2 conditions of AA (Figure 10A) ( $P < 0.05$ ). In the control condition (ovarian tissue pieces cultured in the absence of AA) (Figure 10B), oocytes surrounded by granulosa and stromal cells were observed, however no follicular structures (follicles enclosed by a basal membrane and theca cells attached) were visible. In contrast, in the ovarian tissue pieces treated with ascorbic acid (Figure 10 C and D), the basement membrane was observed in all growing follicles.

Since both AA concentrations preserved well the basal membrane and crystals started to form at  $200\mu\text{g/ml}$  AA, it was decided to include AA  $100\mu\text{g/ml}$  in the basal culture medium for all the subsequent experiments.

**Figure 9. Effect of the Ascorbic acid on the primordial follicle culture.** Survival rate (A) and growing rate (B) of the ovarian tissue pieces cultured in control media or in media supplemented with either  $100\mu\text{g/ml}$  or  $200\mu\text{g/ml}$  AA. Four repeats were done and at least 157 ovarian tissue pieces were evaluated per treatment. Different letters represent significant differences ( $P < 0.05$ ).



**Figure 10. Effect of Ascorbic acid on follicle structures.** Classification of the follicles counted in fresh tissue (Ovarian tissue from 3 day old ovary) and in cultured ovarian tissue pieces after 15 days in culture (n=3000) (A). Bars represent the percentage of follicles calculated per treatment and classified based on their morphology. Sections from fixed embedded and hematoxylin-eosin-saffrane (HES) stained ovarian tissue pieces cultured for 15 days. In the absence of AA (B) only oocytes surrounded by granulosa cells in a disorganized way, but no follicular structures were observed. In the presence of AA (C) follicular structures were observed, two primary to secondary follicle transition with a clear basement membrane are shown. Follicular morphology was observed in a cultured ovarian tissue piece under an inverted microscope. White head arrows indicate the basement membrane; OO: oocyte, GC: granulosa cells. Scale bar 50 $\mu$ m. Different letters represent significant differences (P<0.05).



## 2. Effect of the activator 740Y-P on primordial follicle activation

The cell permeable phospho-peptide 740Y-P was tested. This compound is known to enhance the enzymatic activity of the PI3K enzyme to convert PIP2 into PIP3 promoting the primordial follicle activation (Li et al., 2010). On average, the survival rate was more than 82% and equally good in the different concentrations tested (50, 100 and 500 $\mu$ g/ml) and they were not significantly different (P>0.05). Out of the surviving fraction, the growing rate was around 40% for all tested conditions, being no significantly different (P>0.05). Therefore results are not shown.

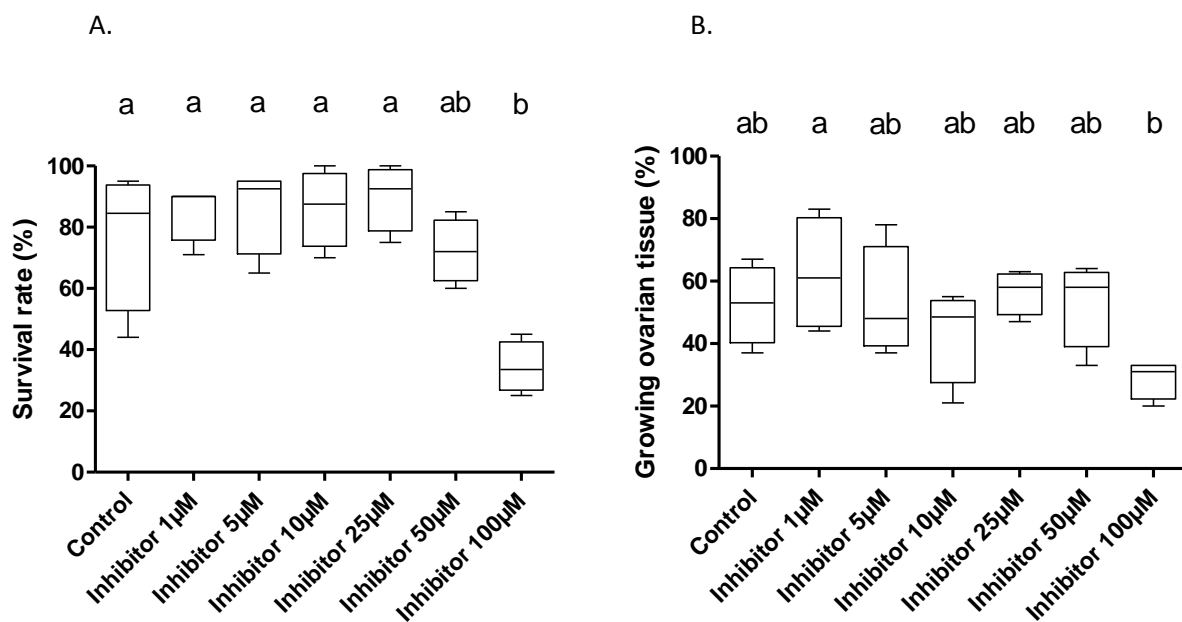
### 3. Effect of bpV(pic), a PTEN inhibitor, on primordial follicle activation (Figure 11)

When mouse ovaries are incubated with bpV(pic), a PTEN inhibitor, the primordial follicles become activated (*Li et al.*, 2010). In this experiment, different concentrations of this compound were evaluated in order to find an adequate the concentration which keeps the tissue survival rate high (Figure 11A) and allows activation of primordial follicles (Figure 11B) within cultured ovarian tissue pieces.

After 15 days in culture a drop in the survival rate (Figure 11A) was observed at 50 $\mu$ M (70%) and 100 $\mu$ M PTEN inhibitor (30%), being only significant ( $P < 0.05$ ) for pieces treated with 100 $\mu$ M PTEN inhibitor. No differences between 50 $\mu$ M and 100 $\mu$ M were observed. Out of the surviving fraction, some tissue pieces grew (Figure 11B) in all the treatments; there were no differences between inhibitor concentrations except for 1 $\mu$ M PTEN inhibitor compared with 100 $\mu$ M PTEN ( $P < 0.05$ ), which had the lowest growing rate (30%).

Regarding the proportion of follicles per cultured tissue piece, the highest proportion of follicles was observed under 1 $\mu$ M and 10 $\mu$ M (ns) (Table 2).

**Figure 11. Effect of the bpV(pic) inhibitor on the cultured ovarian tissue pieces.** Survival rate (A) and growing rate (B) of the cultured ovarian tissue pieces pretreated for 24 hours with control media or different concentrations of the bpV(pic) Inhibitor. Four repeats were done and at least 40 ovarian tissue pieces were evaluated per treatment. Different letters represent significant differences ( $P < 0.05$ ).



**Table 2. Number of follicles per cultured ovarian tissue piece.** Numbers in the table represent the number of follicles (addition of primary, primary to secondary transition and secondary follicles) per tissue piece calculated after 15 days in culture. There were no significant differences between the treatments however the highest values were found between the treatment Inhibitor 1 $\mu$ M and Inhibitor 10 $\mu$ M. One way ANOVA ( $P>0.05$ ).

Treatments	Number of follicles per cultured tissue piece (Mean $\pm$ SD)
Control	2.42 $\pm$ 0.4
Inhibitor 1 $\mu$ M	3.18 $\pm$ 0.55
Inhibitor 5 $\mu$ M	2.84 $\pm$ 0.74
Inhibitor 10 $\mu$ M	2.98 $\pm$ 0.6
Inhibitor 25 $\mu$ M	2.74 $\pm$ 0.45
Inhibitor 50 $\mu$ M	2.23 $\pm$ 0.91
Inhibitor 100 $\mu$ M	2.54 $\pm$ 0.41

#### ***4. Effect of combined PTEN inhibitor and 740Y-P activator on the activation of primordial follicle in culture (Figure 12, 13)***

Based on our previous experiments, two concentrations of the bpV(pic) inhibitor (1 and 10 $\mu$ M) and one concentration of the activator 740Y-P (500 $\mu$ g/ml) were selected to test their synergistic effect. The survival rate was over 90% and not significantly different between the treatments (Figure 12A) ( $P>0.05$ ). Out of the surviving fraction (Figure 12B), the pieces treated with the combination of Activator 500 $\mu$ g/ml plus inhibitor 1 $\mu$ M showed a higher growing rate compared to control but no statistically different to the Activator condition and Activator 500 $\mu$ g/ml plus inhibitor 10 $\mu$ M condition.

At day 3 and 18 of culture, tissue pieces were taken aleatorily from the surviving fraction and fixed for further histological analysis.

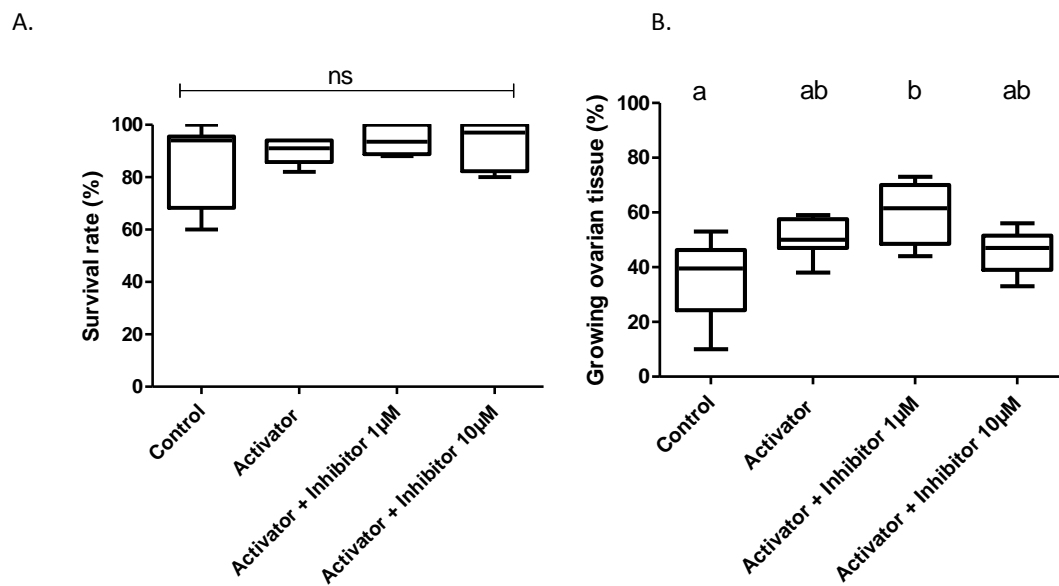
At day 3, the amount of primordial and primordial to primary transition was recorded. In the fresh tissue (ovarian tissue from 3 day old ovary) showed 90% of primordial follicles, while 100%, 91%, 81% and 78% of primordial follicles were recorded for the Control, Activator alone, Activator + Inhibitor 1 $\mu$ M and Activator + Inhibitor 10 $\mu$ M, respectively (No significant differences). Fresh tissue showed 8.8% of primordial to primary follicle transition, the Activator condition showed

10% of transitional follicles and the control condition were not observed any. The amount of primordial to primary follicle transition was significantly higher in the conditions combining activator and inhibitor (19% and 21%, 1 or 10  $\mu$ M Inhibitor, respectively) compared to Fresh tissue (10%), Control (0%) and Activator (9%) conditions.

The number of follicles per tissue piece after 18 days in culture was significantly lower ( $P < 0.05$ ) in control than in the treatments (Table 3).

At day 18 of culture, the amount of primordial follicles in the *in vitro* conditions was significantly lower ( $P < 0.05$ ) than in fresh tissue (ovarian tissue from 3 day old ovary) than, while the amount of primordial to primary follicle transition, primary and primary to secondary transition follicles was significantly higher ( $P < 0.05$ ) in the *in vitro* conditions than in the fresh tissue. There were no differences in the amount of primordial, primordial to primary transition, primary and primary to secondary transition follicles (Figure 13) between all the *in vitro* conditions but the amount of secondary follicles was significantly higher ( $P < 0.05$ ) in the treated pieces (7%) compared to control condition (1%).

**Figure 12. Effect of the activator 740Y-P in combination with the bpV(pic) Inhibitor on the cultured ovarian tissue pieces.** Survival rate (A) and growing rate (B) of the cultured ovarian tissue pieces pretreated for 24 hours with control media, 500 $\mu$ g/ml Activator or the combination of the 500 $\mu$ g/ml Activator plus 1 $\mu$ M or 10 $\mu$ M of the Inhibitor. Three repeats were done and at least 96 ovarian tissue pieces were analyzed per treatment. Different letters represent significant differences ( $P < 0.05$ ).

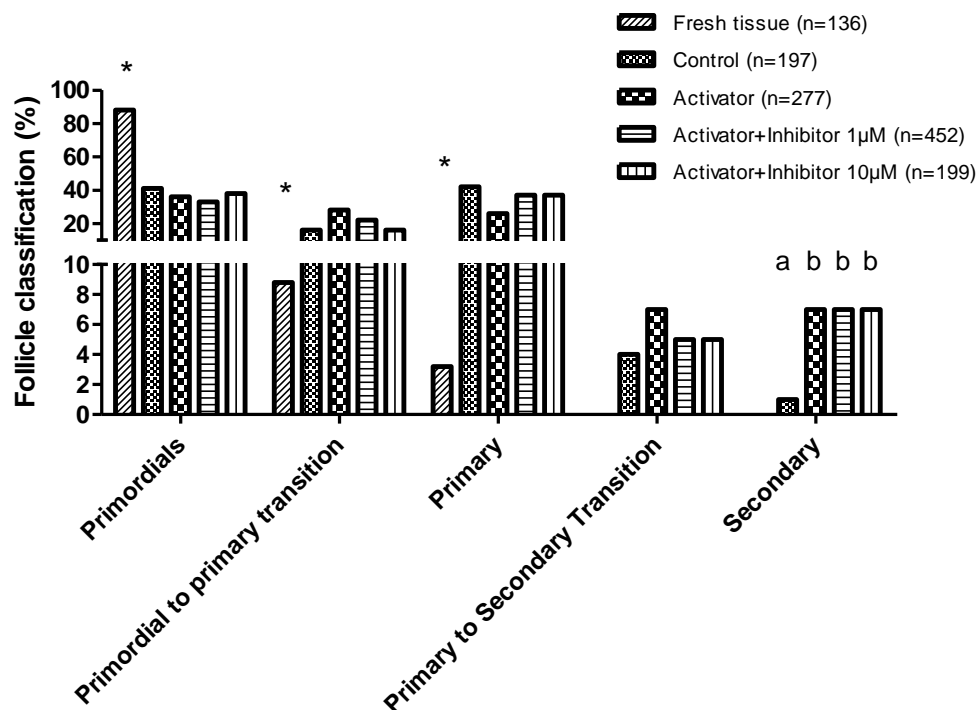




**Table 3. Number of follicles calculated per cultured ovarian tissue piece after 18 days of culture.** Numbers in the table represent the number of follicles (addition of primary, primary to secondary transition and secondary follicles) per tissue piece calculated after 18 days in culture. Different letters state significant differences. One way ANOVA ( $P < 0.05$ ).

Treatments	Number of follicles per cultured tissue piece (Mean $\pm$ SD)
Control	1.84 <sup>a</sup> $\pm$ 0.45
Activator 500 $\mu$ g/ml	3.02 <sup>b</sup> $\pm$ 0.27
Activator+ Inhibitor 1 $\mu$ M	3.28 <sup>b</sup> $\pm$ 0.73
Activator + Inhibitor 10 $\mu$ M	2.95 <sup>b</sup> $\pm$ 0.98

**Figure 13. Follicular classification by histology found at day 18 of tissue culture under the different tested conditions.** Bars represent the percentage of follicles calculated per treatment and classified based on their morphology. One way ANOVA was done independently per follicle classification including the fresh tissue in every analysis. Different letters represent significant differences ( $P < 0.05$ ). “n” represents the number of follicles analyzed per treatment.



After 18 days in culture, one plate per condition and per experiment was kept and cultured for 3, 6 or 9 more days in order to obtain more secondary follicles. At day 18, 21 and 24 secondary

follicles were isolated and when at the end of culture a total sum was made, the mean number of secondary follicles per cultured tissue piece was significantly higher in the treatment Activator + Inhibitor 1 $\mu$ M (2.03 follicles per cultured tissue piece) than in the Control (0.66 follicles per cultured tissue piece) and Activator + Inhibitor 10 $\mu$ M (1.15 follicles per cultured tissue piece); however, it was not significantly different from the Activator condition (1.39 follicles per cultured tissue piece) (Table 4).

**Table 4. Number of isolated secondary follicles per tissue piece.** Numbers in the table represent the sum of secondary follicles isolated at day 18, 21 and 24 of culture per tissue piece. Different letters represent significant differences ( $P < 0.05$ ).

Treatments	Number of total isolated secondary follicles per cultured tissue piece (Mean $\pm$ SD)
Control	0.66 <sup>a</sup> $\pm$ 0.4
Activator alone	1.39 <sup>ab</sup> $\pm$ 0.65
Activator + Inhibitor 1 $\mu$ M	2.03 <sup>b</sup> $\pm$ 0.22
Activator + Inhibitor 10 $\mu$ M	1.15 <sup>a</sup> $\pm$ 0.02

### ***5. Effect of Activin A in the primordial follicle culture (Figure 14)***

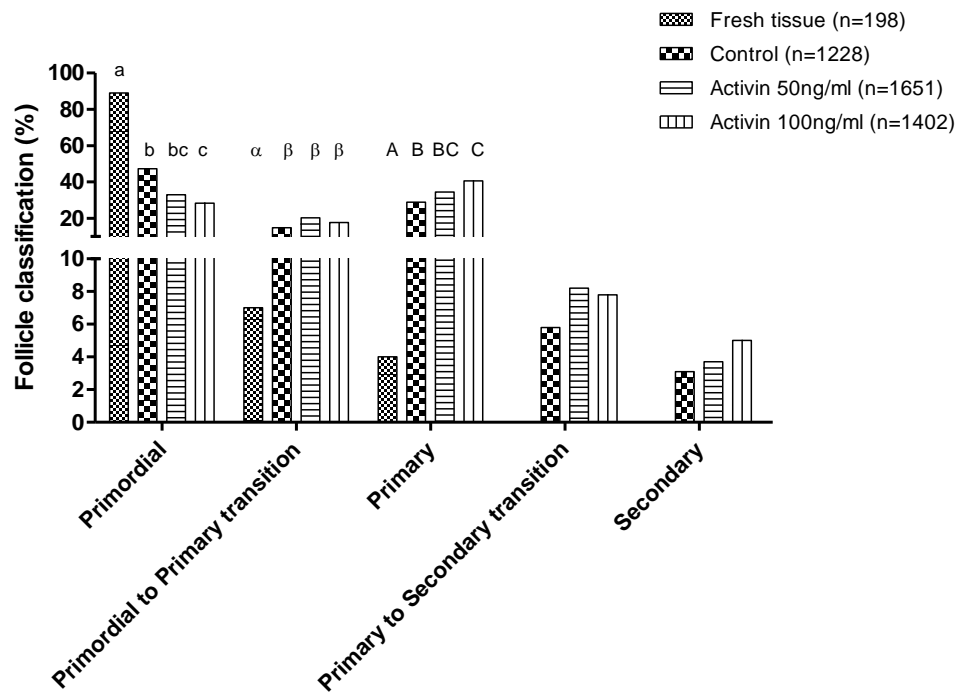
The regulation of the initial follicle pool formation in mice by Activin A has been described (Bristol et al., 2005). In this experiment, 2 concentrations of Activin A were tested on follicular development. The survival rate of tissue pieces was more than 83% in all the conditions, no significant differences between conditions were observed ( $P > 0.05$ ). From surviving ovarian tissue pieces, 52% grew during 15 days in culture while from the pieces treated with Activin (50 or 100ng/ml) the growing rate was 78% on average. However, there were no significant differences between the treatments ( $P > 0.05$ ).

At day 15 of culture, ovarian tissue pieces were fixed for histological analysis. The amount of primordial follicles in fresh tissue (85%) (Ovarian tissue from 3 day old ovary) was significantly higher ( $P<0.05$ ) than in the *in vitro* conditions. Moreover, it was significantly lower ( $P<0.05$ ) in the ovarian tissue pieces treated with 100ng/ml Activin A (30%) compared to the control (47%). No differences were observed between the two Activin concentrations.

In contrast, the amount of primordial to primary transition and primary follicles in fresh tissue was significantly lower ( $P<0.05$ ) than in the *in vitro* conditions. Furthermore, it was significantly higher ( $P<0.05$ ) in the ovarian tissue pieces treated with 100ng/ml Activin (42%) versus control (29%). No differences were observed between the two Activin concentrations.

Regarding the amount of secondary follicles, no differences were observed between the treatments.

**Figure 14. Follicular classification by histology found in fresh tissue and at day 15 of culture.** Bars represent the percentage of follicles calculated per treatment and classified based on their morphology. One way ANOVA was done independently per follicle classification including the fresh tissue in every analysis. Different letters represent significant differences ( $P<0.05$ ). "n" represents the number of follicles were analyzed per treatment.



## **6. Effect of the PTEN inhibitor and the 740Y-P activator alone or in combination with Activin A on outcome parameters (Figure 15, 16 and 17)**

### **6.1. In primordial follicle culture**

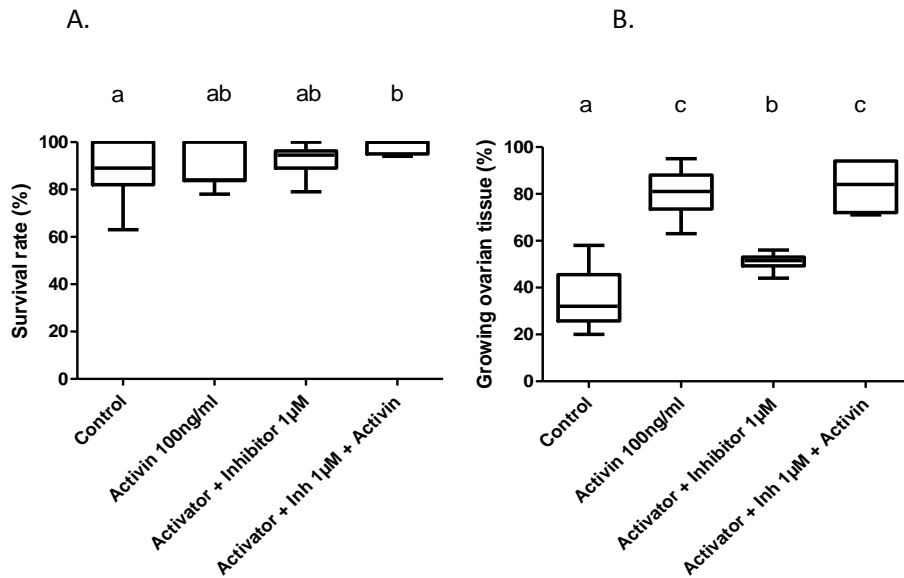
#### **6.1.1. Observations on pieces in culture**

The survival rate (Figure 15A) of the tissue pieces was over 85% for all the conditions, however, the treatment Activator plus Inhibitor 1 $\mu$ M plus Activin (Ac+I+At) was significantly higher ( $P<0.05$ ) than the control but not different from the Activin and Activator plus Inhibitor 1 $\mu$ M (Ac+I). From the surviving fraction (Figure 15B), pieces pretreated with or without Ac+I but cultured in the presence of Activin A 100ng/ml had a growing rate (80%) significantly higher ( $P<0.05$ ) than in the Control and Ac+I (where the growing rate was around 50%).

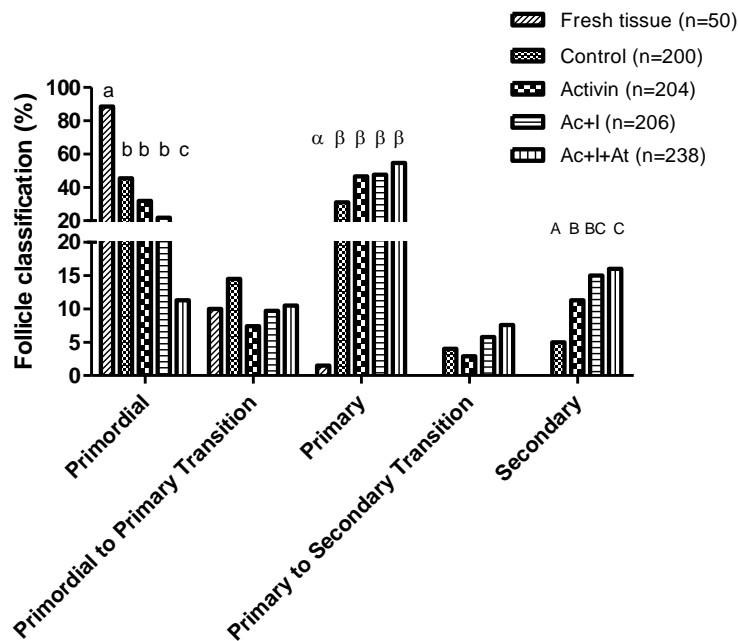
#### **6.1.2. Histology of cultured pieces**

On day 3, the amount of primordial, primordial to primary transition and primary follicles were not different among treatments and compared to the fresh tissue (ovarian tissue from 3 day old animal). After 24 days in culture (Figure 16), there were still morphologically normal primordial follicles remaining in the tissue in all the treatments. The amount of primordial follicles was significantly higher ( $P<0.05$ ) in the fresh tissue (88%) compared to *in vitro* treatments. Nevertheless, the pieces treated with Ac+I+At (11%) showed a significantly lower ( $P<0.05$ ) amount of primordial follicles compared to Control (45%), Activin (30%) and Ac+I (20%) treatment. There were no differences between the amount of primordial to primary follicle transition and primary to secondary follicle transition between the *in vitro* treatments compared to the fresh tissue. However, a significantly higher ( $P<0.05$ ) amount of primary follicles was observed in the *in vitro* treatments than in the fresh tissue (where no secondary follicles were observed); however no significant differences were observed among the *in vitro* treatments. A significantly higher ( $P<0.05$ ) amount of secondary follicles was observed in Ac+I and Ac+I+At conditions compared to Control and Activin conditions.

**Figure 15. Effect of Activin A in combination with the activator and the inhibitor on the cultured ovarian tissue pieces.** Survival rate (A) and growing rate (B) of the cultured ovarian tissue pieces were pretreated for 24 hours with control media or with the combination of 500µg/ml Activator plus 1µM of the Inhibitor and were further cultured in the presence or absence of Activin A. Four repeats were done and at least 100 tissue pieces were analyzed per treatment. Different letters represent significant differences, one way ANOVA (P<0.05).



**Figure 16. Classification of the follicles by histology 24 days after culture under different treatments.** Bars represent the percentage of follicle calculated per treatment and classified based on their morphology. Different letters represent significant differences, X<sup>2</sup> square (P<0.05).



## 6.2 Gene expression of *Gdf9*, *Mater*, *Amh* and *Cyp17* (Figure 18)

Secondary follicles were isolated from fresh and cultured ovarian tissue and the expression pattern of selected genes was analyzed by quantitative RT-PCR.

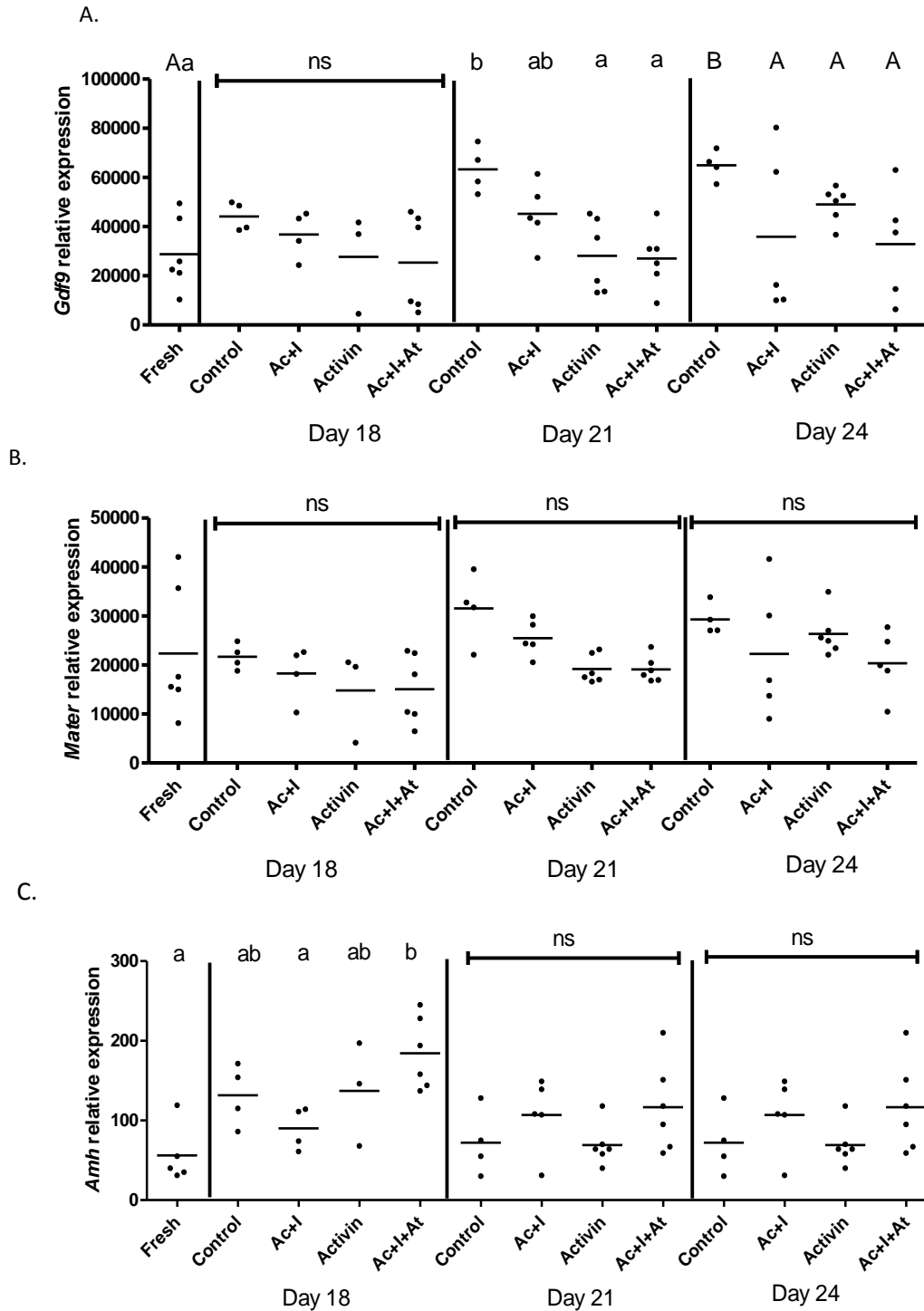
It was observed that *Gdf9* transcript levels in secondary follicles isolated after 18 days of culture were not different between the *in vitro* conditions and compared to the freshly isolated secondary follicles (fresh condition) (Figure 17A). However, samples at day 18 of isolation showed a large variability between the samples in the treatments where Activin was included (Activin and Ac+I+At). For *Gdf9* Transcript levels in isolated follicles from the control group at day 21 and day 24 of culture were significantly higher ( $P < 0.05$ ) compared to *in vivo* condition. The gene expression values were 3 fold higher in the *in vitro* control groups than in the fresh condition. In the secondary follicles isolated at day 24, a large variability was observed in samples pretreated with activator and inhibitor.

For *Mater* transcripts levels, no differences ( $P > 0.05$ ) were observed among the *in vitro* conditions compared to fresh condition (Figure 17B). However, the isolated secondary follicles at day 24, showed a big variability between the samples pretreated with activator and inhibitor. Interestingly, transcript levels in the control condition tend to be higher in follicles isolated at day 21 and 24.

For *Amh* transcript levels, the secondary follicles isolated at day 18 after culture, significant differences ( $P < 0.05$ ) were observed between Ac+I+At compared to Ac+I and fresh condition (Figure 17C). It was not different from control and activin condition. *Amh* transcripts from secondary follicles isolated at day 21 and 24 after culture did not show significant differences between the *in vitro* conditions compared to fresh condition.

*Cyp17* transcripts were present in 100% of the samples in the fresh, control and Activin conditions, and in 93% and 95% in Ac+I and Ac+I+At, respectively.

**Figure 17. Relative quantification of total mRNA for *Gdf9* (A), *Mater* (B) and *Amh* (C) isolated from *in vitro* grown secondary follicles and freshly isolated secondary follicles. Gene expression between the *in vitro* treatments and the fresh condition were compared. Each dot represents 4 to 5 follicles. One way ANOVA was done independently per day of isolation including the fresh control in every analysis. Different letters mean significant differences ( $P < 0.05$ ). ns: No significant differences between the treatments per day of isolation.**



### 6.3 Meiotic competence of isolated secondary follicles (Figure 19)

Secondary follicles were isolated from ovarian tissue pieces cultured for 18, 21 and 24 days and cultured in a standard culture system for mouse secondary follicles. As in vivo control, freshly isolated secondary follicles from 13 day old ovaries were cultured simultaneously.

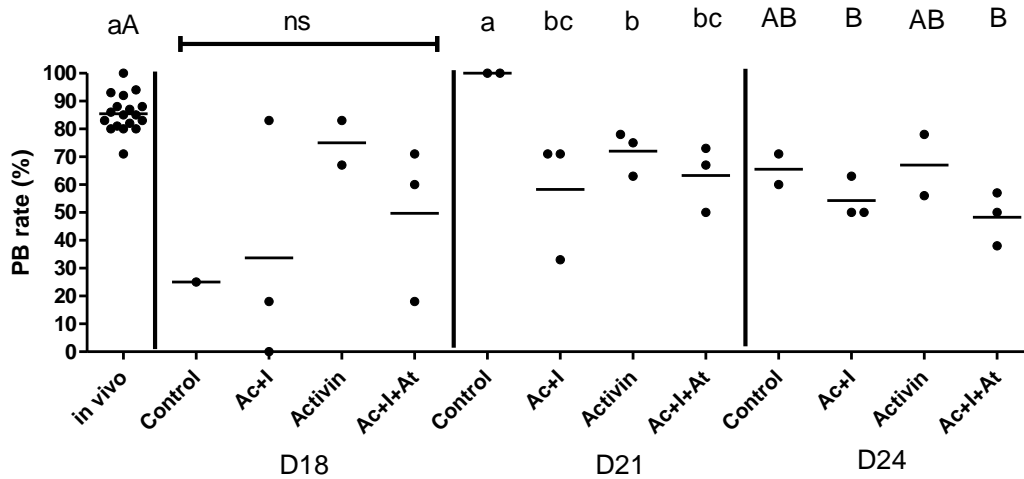
The initial follicular diameter did not differ between treatments and the days of isolation. The PB rate after 12 days in secondary follicle culture was very variable among the days of isolation and among the treatments, going from 0 to 100%. The PB rate of the oocytes from the follicles that were isolated at day 18 was significantly lower than the in vivo control ( $P < 0.05$ ) but no differences were observed among the *in vitro* treatments (Figure 18). In general, the PB rate of the oocytes from *in vitro* grown secondary follicles isolated at day 21 and 24 after culture was significantly lower than in the in vivo control ( $P < 0.05$ ). Oocyte diameters in isolated secondary follicles from all *in vitro* treatments were significantly ( $P < 0.05$ ) larger than from in vivo condition (Figure 19). Nevertheless, the initial oocyte diameter was not significantly different between the *in vitro* conditions compared to in vivo control.

Regarding the steroid production at day 12 in culture, it was observed, the Estradiol production in all the *in vitro* control was similar to the *in vivo* condition. The switch to progesterone output after hCG+EGF stimulus (day 13) was also observed in all the *in vitro* conditions and it was similar to *in vivo* control.

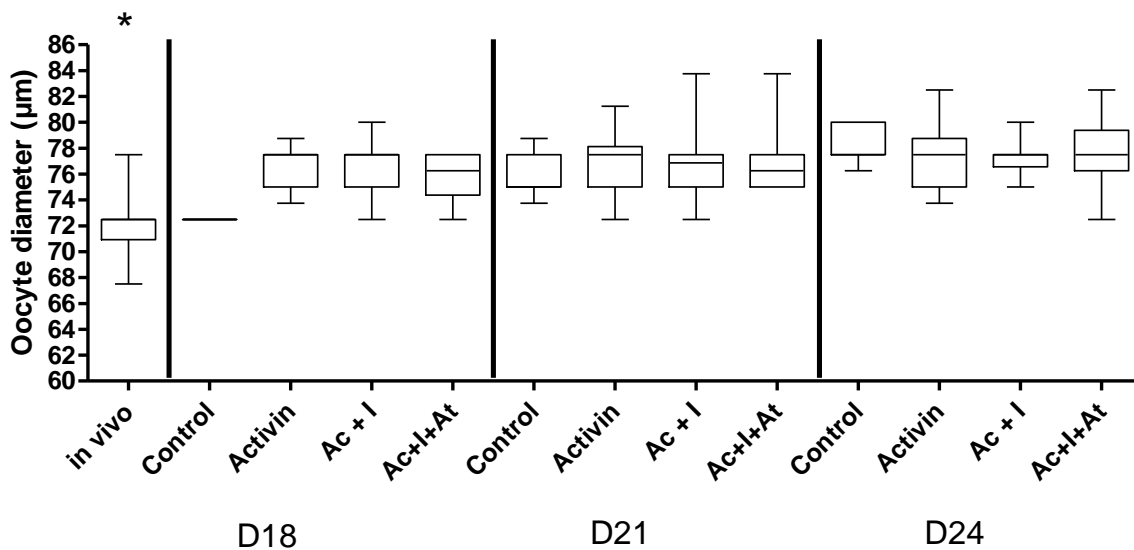


**Figure 18. Oocyte maturation rate after 12 days in a standard culture system for mouse secondary follicle.**

Oocytes from *in vitro* grown secondary follicles isolated after 18, 21 and 24 days of culture and oocytes from *in vivo* controls were analyzed. PB rates between the treatments and the *in vivo* control was compared. One way ANOVA was done independently per day of isolation including the *in vivo* condition in each analysis. Different letters mean significant differences ( $P < 0.05$ ). ns: no significant. Each dot represents at least 4 oocytes, except for the Control on Day 18 of isolation where the dot represents only a single oocyte. Lines represent mean PB rate.



**Figure 19. Diameters of *in vitro* ovulated oocytes after the secondary follicle culture in relation to treatment and isolation day.** Diameter of PB oocytes between the treatments and the *in vivo* condition was compared. One way ANOVA was done independently per day of isolation including the *in vivo* condition in each analysis. (\*): The *in vivo* control was statistically different from all the *in vitro* treatments ( $P < 0.05$ ).



#### 6.4 Spindle morphology and chromosome metaphase plate

Chromosomal abnormalities in GVBD stage oocyte were analyzed only in metaphase I (MI) and PB stage oocytes were analyzed in metaphase II (MII) oocytes (Figure 20). Description of the abnormalities is detailed in table 5 and 6, respectively. The chromosomal abnormalities in MI were not significantly different in any of the *in vitro* treatments when compared to the *in vivo* condition. However, all *in vitro* treatments had more chromosomal abnormalities than *in vivo* condition. Notably, the untreated (control) condition had the highest abnormality rate.

The chromosomal abnormalities in MII oocytes were significantly higher ( $P < 0.05$ ) in all *in vitro* treatments (except for Control condition) compared to *in vivo* condition. Oocytes cultured in Ac+I treatment had the highest abnormality rate. Interestingly, the oocytes from *in vitro* grown secondary follicles from Activin and Ac+I+At conditions showed a lower rate of chromosomal abnormalities compared to Ac+I condition.

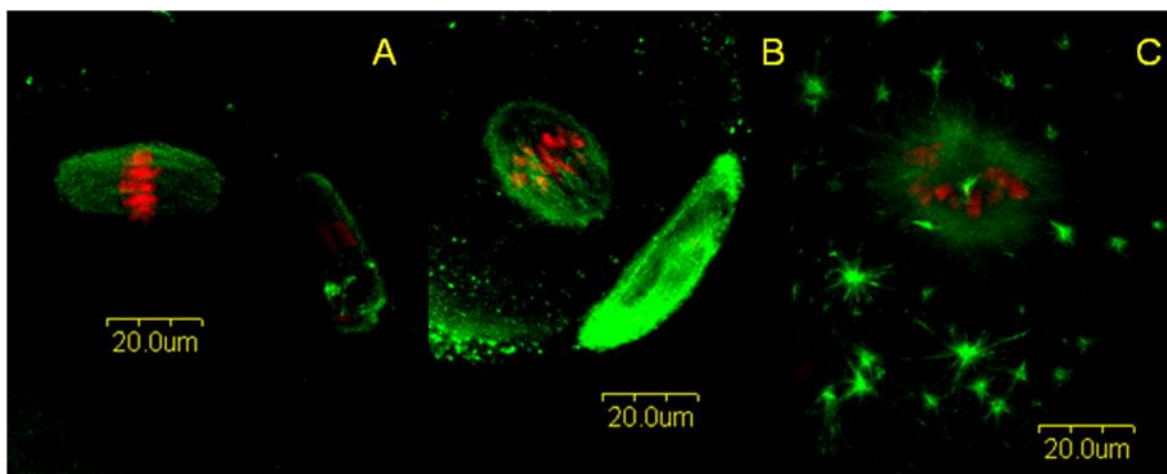
**Table 5. Chromosomal abnormalities observed in Metaphase I (MI) oocytes.** The total amount of MI oocytes from secondary follicles isolated at day 18, 21 or 24 of culture were analyzed per condition. The percentage of abnormalities was calculated as the amount of abnormal MI oocytes (with one or two abnormalities) over the total amount of analyzed MI oocytes per condition.

	Secondary follicles isolated from cultured ovarian tissue pieces				In vivo condition: Freshly isolated secondary follicles from 13 day old mouse ovary
	<i>In vitro</i> conditions				
<b>Abnormalities:</b>	Control	Activin	Ac+I	Ac+I+At	in vivo
Unalignment of chromosomes	3	4	5	10	4
Spindle disorganization	0	3	2	2	0
Round spindle shape		2	1	0	1
PB retention	1	0	0	2	0
One abnormality	4	6	4	12	3
Two abnormalities	0	2	2	1	1
<b>Total MI analyzed</b>	5	20	13	23	13
Percentage of abnormalities in MI	80%	40%	46%	57%	31%

**Table 6. Chromosomal abnormalities observed in Metaphase II (MII) oocytes.** The total amount of MII oocytes from secondary follicles isolated at day 18, 21 or 24 of culture were analyzed per condition. The percentage of abnormalities was calculated as the amount of abnormal MII oocytes (with one or two abnormalities) over the total amount of analyzed MII oocytes per condition. Different letters represent significant differences ( $P < 0.05$ ).

	Secondary follicles isolated from cultured ovarian tissue pieces				In vivo condition: Freshly isolated secondary follicles from 13 day old mouse ovary
	Treatments				
	Control	Activin	Ac+I	Ac+I+At	in vivo
Unalignment of chromosomes	2	6	15	7	7
Spindle disorganization	1	1	4	1	1
Round spindle shape	1	3	2	0	0
One abnormality	2	6	11	8	8
Two abnormalities	1	2	5	0	0
Total MII analyzed	14	22	28	25	61
Percentage of abnormalities in MII	21% <sup>a</sup>	36% <sup>b</sup>	57% <sup>b</sup>	32% <sup>b</sup>	13% <sup>a</sup>

**Figure 20. Representative images of a normal metaphase II oocyte (A), unaligned metaphase II oocyte (B) and the presence of microtubule asters in the cytoplasm of metaphase I oocytes.** Spindle alpha-tubulin is stained in green and chromosomes are stained red. Oocytes were obtained 16 hours after hCG+EGF stimulus. Scale bar 20 $\mu$ m.



## 6.5. Cell proliferation and apoptosis in cultured tissue pieces

In fresh ovarian tissue from 3 day old mouse, PCNA staining was observed in 30% of the primordial follicles. In cultured follicles, PCNA staining was observed in the granulosa cells at all the follicular stages, from activated primordial follicles to secondary follicles. In ovarian tissue pieces at day 3 of culture (Figure 21A), more than 89% of the primordial follicles were PCNA positive, while it was detected in less than 50% of the granulosa cells from primary follicles in all the evaluated treatments in the experiment 4 and 6.

In ovarian tissue pieces cultured for 18 (Experiment 4) and 24 days (Experiment 6) (Figure 21 B and C) primordial, primary and secondary follicles were PCNA positive.

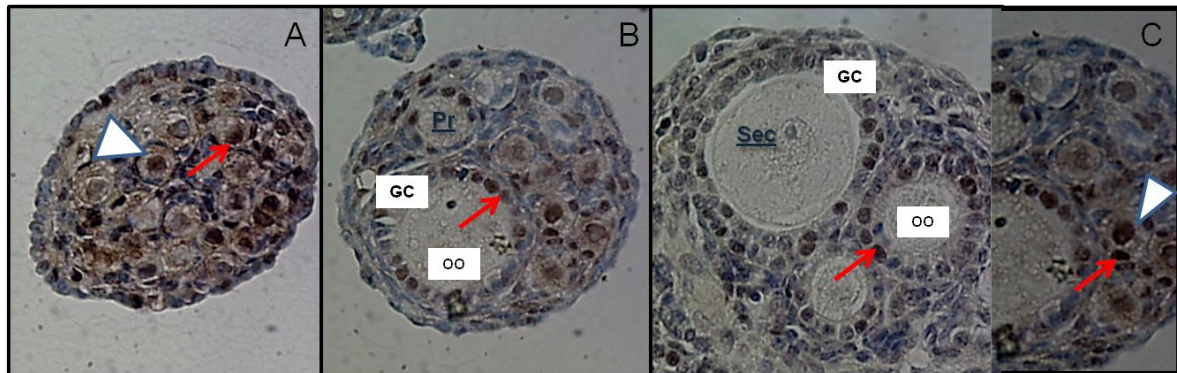
In the experiment 4, after 18 days of culture (Figure 21B), 100% of the observed primordial follicles (n=44) in all the treatments (Control, Activator, Activator + Inhibitor 1 $\mu$ M and Activator + Inhibitor 10 $\mu$ M) were PCNA positive. However, primary follicles (n=38) in all the treatments showed 50% to 60% of the granulosa cells PCNA positive.

In the experiment 6, after 24 days of culture (Figure 21C), 100% of observed primordial follicles (n=8) in all the treatments (Control, Activin, Ac+I and Ac+I+At) were PCNA positive. However, primary follicles (n=24) in all the treatments showed 51% to 60% of the granulosa cells PCNA positive. Additionally, secondary follicles (n=6) showed 71% to 81% granulosa cells PCNA positive in Activin, Ac+I and Ac+I+At condition while Control condition showed 45% of PCNA positive cells.

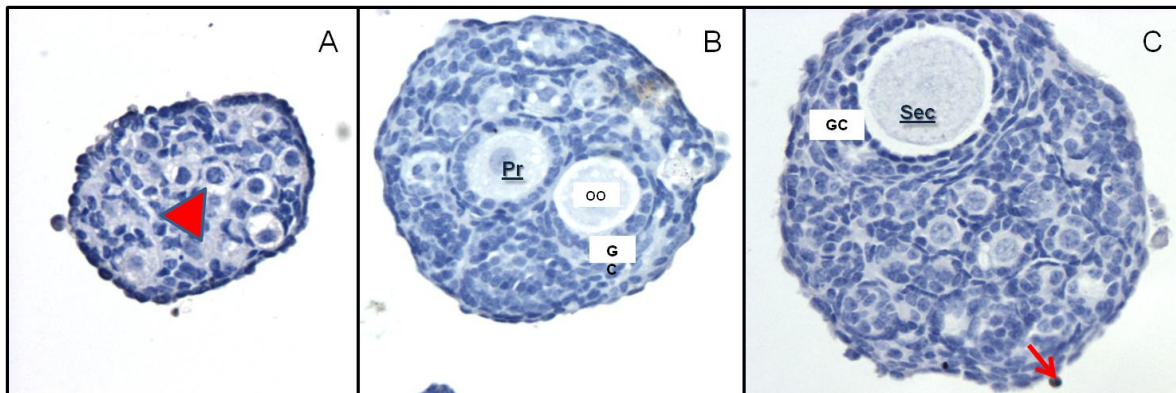
Interestingly, PCNA expression was detected in most of the oocytes of primordial follicles and in some oocytes from primary and secondary follicles.

Positivity for caspase 3 was not observed within the ovarian tissue pieces after 3, 18 or 24 days in culture (figure 22 A, B and C). However, some cells located in the outer part of the cultured tissue pieces showed piknotic nuclei.

**Figure 21. PCNA staining in ovarian tissue pieces cultured for 3, 18 and 24 days.** Ovarian tissue pieces after 3 days in culture showed primordial follicles PCNA positive (A). After 18 days in culture primary and primordial follicles showed PCNA positivity (B). After 24 days in culture primordial, primary and secondary follicles showed PCNA positivity. White head arrow is indicating a primordial follicle, Pr: Primary follicles, Sec: Secondary follicles. Red arrows are indicating examples for PCNA cell positivity. OO: oocyte, GC: granulosa cell.



**Figure 22. Caspase 3 staining in ovarian tissue pieces cultured for 3, 18 and 24 days.** Ovarian tissue pieces after 3 days in culture showed healthy primordial follicles (A). After 18 (B) and 24 (C) days in culture primordial, primary follicles and secondary follicles showed normal morphology. Red arrow head is indicating a primordial follicle, Pr: Primary follicles, Sec: Secondary follicles. Red arrow is indicating an example for caspase 3 cell positivity. OO: oocyte, GC: granulosa cell. Red arrow is a caspase 3 positive cell.



## iv. Discussion

Folliculogenesis is a process where primordial follicles grow till they reach the preovulatory stages, the maintenance of the basement membrane during the early stages and the communication between the oocyte and the granulosa cells are play an important role in this process.

### **Effect of Ascorbic Acid (AA) on ovarian tissue culture**

In our previous experiments, using a medium low concentration of AA it was impossible to maintain the basement membrane. Previous research by others had reported that to preserve the integrity of the basement membrane and improve the survival of the ovarian tissue pieces during culture, a higher concentration of AA had to be added to the culture medium. AA plays a role in the regulation of extracellular matrix and the maintenance of follicle health during long-term culture (Murray et al. 2001) and reduced theca cell death *in vitro* (Thomas et al. 2001). Additionally, FSH in combination with AA maintained follicular survival and promoted the primordial follicle transition (Rossetto et al. 2009). When AA was increased from 50 to 100µg/ml we observed an improvement in the follicular integrity and survival in the cultured ovarian tissue pieces. The proportion of growing follicles increased in pieces treated with AA compared to a 3 day old ovary (mostly primordial follicles) however it was not reflected in the growing of the tissue pieces in culture. Therefore, AA maintains the basement membrane after 3 weeks in culture but does not improve the growing of the tissue pieces in culture.

The maintenance of the three-dimensionality of primordial and primary follicles in culture is critical for follicle *in vitro* growth. In order to keep the three-dimensionality of early preantral and preantral follicles (cultured individually or within the ovarian tissue pieces *in vitro*), strategies to prevent tissue adhesion, such as: the use of extracellular matrix (ECM) (Berkholtz et al. 2006, West et al. 2007a, West et al. 2007b), membrane inserts (Scott et al. 2004), and gel encapsulation (Jin et al. 2010, Kreeger et al. 2005, Kreeger et al. 2006, Shikanov et al. 2009, Vanacker et al. 2011, Xu et al. 2006a, Xu et al. 2006b, Xu et al. 2009) have been described.

In an attempt to preserve the three-dimensional structure, very small pieces of neonatal ovaries with only primordial follicles were cultured in an alginate matrix as scaffold. This promoted the growth and survival of primordial follicles after an *in vitro* culture period of 15 days. Additionally,

ultra low attachment (ULA) plates were used for the same purpose. With both systems, follicles were kept assembled and some primordial follicles developed and reached the preantral stage. However, there are some advantages for one system over the other. For instance, if the alginate matrix is too rigid, it might influence ovarian tissue expansion and in the transport of nutrients and hormones between the ovarian tissue piece and the surrounding culture medium. Furthermore, besides the fact that alginate encapsulation is a time-consuming technique, secondary follicle isolation requires an extra enzymatic step to release the tissue piece from the matrix, which might induce stress on the preantral follicles and ovarian tissue pieces. In contrast, ovarian tissue pieces cultured in ULA plates are always in direct contact with the culture medium, there are no problems of limitations in expansion, the ovarian tissue pieces are easily handled for secondary follicle isolation. Therefore, ULA plates offered a more attractive technique for ovarian tissue culture.

#### **Activation of primordial follicles**

The aim of our experiments with the primordial follicles is to obtain a larger number of secondary follicles to transfer into a routine standard culture system for secondary follicle. In order to achieve this aim, we tried to activate a larger number of primordial follicles by using two compounds: bpV(pic) inhibitor (a PTEN inhibitor) and 740Y-P activator (a PI3K activator). The 740Y-P activator enhances the enzymatic activity of PI3K increasing the intracellular PIP3 levels; together with the prevention of PIP3 conversion to PIP2 by the PTEN inhibitor, the increased PIP3 has been shown to induce primordial follicle activation in mouse and human (Li et al. 2010). The same doses used by Li et al. (2010), 500µg/ml for Activator and 100µM for Inhibitor were tested in a preliminary study with our tissue culture system, however after 3 days in culture it was observed that a large amount of tissue became degenerated. Therefore it was decided to perform a dose-finding study and select the best combination for our later studies.

Ovarian tissue pieces pretreated with 500µg/ml activator did not improve the survival and growing rate of the pre-treated tissue pieces. However, in a further experiment when the number of follicles per cultured tissue piece was evaluated, it was observed a significantly higher number of follicles compared to the control condition. This suggests that the activator had an effect in follicle activation.

When human tissue pieces were exposed for a short period of time (24 hours) to PTEN inhibitor at a dose of 100 $\mu$ M, minimal adverse effects were observed after xeno-transplantation (Li et al., 2010). However, in our culture system, when mouse ovarian tissue pieces were incubated with the same inhibitor concentration for 24 hours, the number of ovarian tissue pieces surviving were significantly 3 fold lower than in the control condition (without PTEN inhibitor). PTEN is known to be a tumor suppressor gene inducing apoptosis in tumor cells (Vásquez et al, 2000). If cells are exposed to PTEN inhibitor, cell apoptosis response is inhibited.

It was subsequently determined that under *in vitro* culture conditions the ovarian tissue pieces pretreated for 24 hours with substantially less PTEN inhibitor (1-10 $\mu$ M) had a good survival rate (over 80%). Furthermore, more follicles per cultured tissue piece were observed than in the control condition after 15 days of culture. It suggests that the 1-10 $\mu$ M inhibitor might be inducing follicle activation.

After the selection of the best concentration for the inhibitor and the activator, their synergistic effect was tested. For this experiment, the ovarian tissue pieces were culture for 18 days (3 more days than in the previous experiments) to allow that growing follicles can reach the secondary follicle stage. Results on *in vitro* cultured tissue (in contrast to transplantation by Li et al., 2010) revealed that mouse ovarian tissue pieces pre-treated with 1 $\mu$ M PTEN inhibitor plus 500 $\mu$ g/ml 740Y-P activator had the highest growing rate. Furthermore, in this treatment, more follicles in transition from primordial to primary stage were counted in the ovarian pieces fixed after 3 days in culture, indicating that there was more primordial follicle activation in the pretreated ovarian tissue pieces. Moreover, more secondary follicles per cultured ovarian tissue unit were also obtained in Activator + Inhibitor 1 $\mu$ M condition compared to Control, Activator and Activator + Inhibitor 10 $\mu$ M conditions after 18 days of culture.

#### **Effect of Activin A in activated primordial follicles**

Activin A, being a member of the TGF beta superfamily of growth factors, with multiple potential beneficial effects on the growth of small follicles, was chosen to be tested in the primordial follicle tissue culture system. The addition of Activin A in culture medium induces inhibition of the activation of human primordial follicles *in vitro* (Ding et al. 2010). However, activin has also been shown to regulate the initial follicle pool formation during the critical period of germline cyst



breakdown in mice (Bristol-Gould et al. 2006). As we are taking ovaries from mice 3 days after birth, there could still be follicle formation happening, however we did not attempt to study this nevertheless its very interesting aspect.

Activin A stimulated the growth of the mouse ovarian tissue pieces; the growing rate was higher (80%) compared to the control condition (around 50%) as observed at 15 days of culture. Moreover, significantly higher amounts of primary follicles were found after treatment with Activin A in comparison to control condition. Comparing the effects of Activin (100ng/ml) on follicle distribution at day 15 with day 24 of culture on the histological sections, more secondary follicles were observed after 24 days of culture, lower amounts of primordial to primary transition and primary to secondary follicle transition. Therefore, this data indicates that Activin A supports follicular growth. The promotion of follicular growth induced by Activin A has been previously reported in pre-antral ovine (Thomas et al., 2003), caprine (Silva et al., 2006), human (Telfer et al., 2008) and rodent follicle culture (Smits et al., 1998; McGee et al., 2001, Zhao et al., 2001).

The study hypothesis (based on Li et al., 2010) was that the Activator + Inhibitor would activate more follicles than control (where 20% of the primordial follicles are activated). However, more activated follicles can go two ways: either they grow further into larger stages, or they die. Therefore as a help to get the activated follicles surviving it was decided to add Activin A to the culture medium to sustain the surplus of activated follicles throughout the first stages of folliculogenesis. The histological analysis of the tissue pieces on day 3 of culture did not demonstrate significant differences in the amount of primordial, primordial to primary transition and primary follicles between the treatment conditions (Activin, Ac+I, Ac+I+At) compared to the control condition. This suggests that Activin A had not effect on primordial follicle activation, but also than the Activator and inhibitor conditions did not have an effect on follicle activation.

However, after 24 days in culture, Activin induced follicular growth in the ovarian tissue pieces pretreated with 1 $\mu$ M PTEN inhibitor plus 500 $\mu$ g/ml 740Y-P Activator. The amount of primordial follicles was significantly lower in the treatment Ac+I+At compared to the Control, Activin and Ac+I treatments.

As it was observed in the ovarian tissue pieces cultured for 18 days that there were approximately 40% of primary follicles present in the tissue pieces it was decided to prolong their growth period

in the hope to harvest them as secondary follicles at a later stage. Secondary follicles were thus isolated after 18, 21 and 24 days of culture. The total amount of isolated secondary follicles (i.e. after, 18, 21 or 24 days of culture) was significantly higher in Ac+I+At compared to control and Activin alone treatment, but was not significantly different to the Ac+I treatment. Therefore, Ac+I+At treatment induced the production of more secondary follicles than in the other *in vitro* conditions.

### Gene expression

The expression pattern of isolated secondary follicles cultured from primordial follicles (from 3 day old mice ovaries) for 18, 21 or 24 days was analyzed. Genes known to be important in the regulation of folliculogenesis such as: *Gdf9*, *Mater* and *Amh* (Dong et al., 1996; Vitt et al., 2000; Durlinger et al., 2002 and Tschopp et al., 2003) and a gene known to be an important theca cell marker, *Cyp17* (Robert and Skinner, 1990; Patel et al., 2010) were studied.

Secondary follicles isolated after 18, 21 and 24 days from tissue cultured showed expression levels of *Gdf9* and *Mater* transcripts, similar to isolated secondary follicles from a day 13 ovary (stage at which secondary follicles are normally isolated for individual follicle culture). However, expression levels of *Gdf9* were significantly higher in the isolated secondary follicles from the *in vitro* control condition (no activating treatment) at day 21 and 24 compared to the secondary follicles from 13 days-old mouse control. The *Mater* transcripts levels had a similar expression profile for the same condition, which suggested a correlation between both, however no correlation was observed between expression levels of the two genes. Although, there is no literature about the effects of the overexpression of *Gdf9* transcripts in secondary follicles, it has been reported that overexpression of *Gdf9* in mature oocytes correlates with the reduction of steroid hormone levels and a disruption in the expression of *Star*,  $3\beta$ HSD and  $17\alpha$ OH (Lan et al. 2003).

*Amh* transcripts from isolated secondary follicles after 18, 21 and 24 days in culture from all the *in vitro* conditions (except for Ac+I+At after 18 days in culture) showed a similar expression level to the freshly isolated secondary follicles from day 13. The elevated expression of *Amh* transcripts in Ac+I+At condition, might be due to the presence of larger amounts of small growing follicle, then, the secondary follicles present in the tissue piece will express more *Amh* transcripts to inhibit the growth of small follicles. The same explanation on the expression levels of *Amh* in Ac+I+At can be

applied to all the days where there is a *Amh* expression tends to decrease over time for all the treatments.

The theca cells marker, *Cyp17*, was present in 93% of the *in vitro* grown secondary follicles isolated from cultured tissue after 18, 21 and 24 days and in 100% of the freshly secondary follicles of day 13 mice. CYP17 is present in steroidogenic theca interna cells (Vitt et al. 2000a) which suggests the ability of the growing *in vitro* follicles to start steroid biosynthesis. A relation has been shown for a long time in GDF-9 knock out animals between a deficient GDF-9 and abnormal recruitment of theca cells (producers of *Cyp17* marker. As GDF-9 expression is within normal ranges, although with a huge variability between individuals, it suggests that the GDF-9 produced *in vitro* may have a normal paracrine effect upon the theca acquisition.

To our knowledge, the only report on gene expression of *Gdf9* and *Amh* was done on primary follicles obtained from tissue culture (10 days in culture) where the transcripts values turned also out to be like freshly isolated primary follicles (Sadeu et al. 2008). Therefore, this is the first time that key transcripts such as *Gdf9*, *Mater*, *Amh* and *Cyp17* are measured in completely *in vitro* cultured follicles grown from primordial stages.

### **Meiotic resumption, chromosome and spindle analysis and steroid production**

The *in vitro* grown secondary follicles were isolated and cultured in a routine secondary follicle culture to assess their ability to resume meiosis and to evaluate the steroid production before and after hCG+EGF stimulus.

Some oocytes from all the *in vitro* treatments resumed meiosis. Oocytes from *in vitro* grown secondary follicles cultured for 18 days showed a maturation rate similar to *in vivo* condition; however, there was a large variation in the maturation rate. In contrast, oocytes from *in vitro* grown secondary follicles cultured for 21 or 24 days the maturation rate was significantly lower in all the *in vitro* treatments compared to *in vivo* control. Interestingly, oocytes from *in vitro* grown follicles isolated at day 21 showed 100% of maturation rate. Long term cultures had been described to be a possible reason for the inability of the oocyte to resume meiosis (Obata et al. 2002) or to obtain a lower maturation rate after 21 days in culture compared to the maturation rate of oocytes cultured for 11 days (Obata et al. 2007).

The oocyte diameter (from GVBD and PB oocytes) from the *in vitro* grown secondary follicles was larger than the *in vivo* control oocytes. It is known that Kit ligand-C Kit signaling pathway is involved in oocyte growth (Packer et al. 1994) and its signaling cascade is mediated through the PI3K pathway (Liu K. et al. 2006). This suggests that manipulation of the PI3K pathway in primordial follicles might in some way induce oocyte growth via Kit ligand. This partially explains why larger oocytes were observed in the treatments where primordial follicles were activated; however, this does not explain why oocytes in the other treatments were also larger than in the *in vivo* control. The latter implies that, most likely, there is an *in vitro* effect of the long term culture which makes the oocytes to overgrow.

The chromosomal abnormalities found in MI were not statistically different compared to *in vivo* control; however, the *in vitro* control condition showed the highest abnormality rate. Due to the low number of evaluated oocytes (n=5) for this condition it is difficult to make any conclusion for this experiment. Chromosomal abnormalities in MII treated oocytes were higher than in the *in vivo* control, which may indicate that pretreatment with the activator and the inhibitor is inducing abnormalities in MI and MII oocytes. There is no literature about the effect of this compound inducing chromosomal abnormalities; however, Li et al. (2010) reported a lower fertilization rate of the oocytes retrieved from ovaries pretreated with the activator and the inhibitor (50%) compared to oocytes retrieved from superovulated females (80%) which might be related to some abnormalities in the oocyte.

It is known that for the maintenance of a stable meiotic spindle, a high MPF activity, high MAPK activity, the presence of the cytostatic factor and MAPK-associated proteins are required (Kubiak et al. 1993, Ma et al. 2005). Moreover, the absence of CDK2AP2 or MAPK1IP1 in the oocytes induces spindle disorganization and cytoplasmic aster formation (Lefebvre et al. 2002) while the absence of MAD2 and BCL2 reduce the accuracy of chromosome segregation in meiosis and increase the apoptosis in oocytes, respectively (Ma et al. 2005). However, aged oocytes gradually decrease the expression of spindle stabilizing proteins (Ma et al. 2005, Segers et al. 2008). Therefore, the abnormality rate observed in MI and MII oocytes might indicate that some of these spindle stabilizing proteins and meiotic checkpoints might be affected by the prolonged *in vitro* culture.

Regarding the steroid production at day 12 in culture some observations were made on a few cultures. The Estradiol production in all the *in vitro* conditions was similar to the *in vivo* condition.

The switch to progesterone output after hCG+EGF stimulus (day 13) was also observed in all the *in vitro* conditions and was similar to *in vivo* condition. At this point a preliminary conclusion is that entirely *in vitro* grown follicles seem not to be affected in their capacity to produce steroids, preserving the physiological function of granulosa and theca cells throughout folliculogenesis.

### **Cell proliferation and apoptosis**

The expression of PCNA protein in granulosa cells was used to provide evidence of granulosa cell proliferation, which would mean follicular growth. In the present study, PCNA staining was observed in the granulosa cells at all the follicular stages observed in tissue culture. This suggests that under current *in vitro* conditions the follicles grow and remain proliferative active after a relative long period of time in culture.

PCNA stained most of the oocytes of primordial follicles and in some oocytes from primary and secondary follicles. This finding is not related to cell proliferation because oocytes in meiotic arrest do not replicate but PCNA is an auxiliary protein of DNA polymerase delta (involved in DNA repair) (Downey et al. 1990).

Regarding caspase 3 activity in the ovarian tissue pieces, caspase 3 positivity was not found in the cultured ovarian tissue pieces 3 days after culture however nuclear piknosis was observed in some granulosa and stromal cells from ovarian tissue pieces after 18 or 24 days in culture. There is a possibility that a nonclassical apoptosis pathway is involved in this process, i.e. caspase 7 activity instead of caspase 3 (Tingen et al. 2009). Another possible explanation for this failure in caspase 3 detection is the technical limitations in the timing between the release of caspase 3 (after the apoptosis has started) and the fixation of our tissue pieces.

In conclusion, increase the concentration in ascorbic acid into tissue culture medium allows to preserve the basement membrane of *in vitro* grown follicles. The synergistic effect of the activator and the inhibitor induces follicle activation *in vitro*. Nevertheless addition of Activin A during the culture promotes follicular growth, in consequence more secondary follicles can be isolated. Although, *in vitro* grown secondary follicles are morphologically normal and the gene expression pattern is similar compared to fresh condition, their ability to resume meiosis is affected leading in chromosomal abnormalities in MI and MII oocytes.

One of our aims was to activate primordial follicles and obtain more secondary follicles than those found in a 13 day old ovary (in vivo control). In our best condition, Ac+I+At, an average of 26 secondary follicles per ovary were obtained, which compared to the in vivo control (30 to 40 secondary follicles per ovary) is still a relatively low number. Furthermore, the quality of those follicles needs still to be improved, as meiosis resumption is low and chromosomal abnormalities are highly present.

### **Future perspectives**

The tissue culture system presented in this study needs to be improved to obtain more growing follicles in less time. The addition of some other factors (Kit ligand, GDF-9) known to be involved in follicular growth might improve the culture conditions, however this requires further evaluation. Once the maturation rate is improved, the developmental capacity of the oocytes remains to be tested by *in vitro* fertilization. A more detail study to evaluate the chromosomal abnormalities by evaluating the expression of spindle stabilizing proteins and meiotic checkpoints in MI and MII oocytes needs to be performed.

In this study, we analyzed the total RNA amount of our genes of interest; however, the study of the polyadenylated transcripts could provide us a better view of what is occurring in the *in vitro* grown follicles compared to freshly isolated follicles. The polyadenylated transcripts will give us relevant information of the mRNAs that would translated into proteins.

## v. Abbreviations

740Y-P	cell-permeable phospho-peptide PDGFR740Y-P
AA	ascorbic acid
Ac	Activator 740Y-P
Ac+I	Activin + Insulin
Ac+I+At	Activin + Insulin + Activator
ActRIIB	Activin Receptor Type IIB
ACVR	activin receptor
ALKs	activin receptor like kinases
AMH	Anti Mullerian hormone
At	activin
bFGF	basic fibroblast growth factor
BMP4	bone morphogenetic protein 4
bpV(pic)	Dipotassium bisperoxo (picolinate) oxovanadate (V)
cDNA	complementary DNA
COCs	complex-oocyte-cumulus
CTGF	connective tissue growth factor
CYP17	Cytochrome P450 17 $\alpha$ -hydroxylase-lyase
DAB	<i>diamino</i> benzidine cromogen
ECM	extracellular matrix
EGF	epidermal growth factor
EGTA	ethylene-bis(oxyethylenitrilo)tetraacetic acid
FBS	fetal bovine serum
FKHRL1	forkhead transcription factor L1

FOXO	forkhead box protein O
FSH	follicle stimulating hormone
GCs	granulosa cells
Gdf9	Growth differentiation factor 9
GV	germinal vesicle
GVBD	germinal vesicle break down
hCG	human chorionic gonadotrophin
HES	hematoxylin-eosine-saffran staining
I	Inhibitor
IVF	<i>In vitro</i> fertilisation
KGF	keratinocyte growth factor
KL	kit ligand
LN <sub>2</sub>	liquid nitrogen
MAPK	Mitogen-activated protein kinases
MATER	Maternal antigen that embryo requires
MI	metaphase I
MII	metaphase II
MPF	Mitogen-promoting factor
mRNA	Messenger RNA
NALP	NAVHT-, LRR-, and PYD-containing
PB	polar body
PBS	Phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDGF	platelet derived growth factor
PFF	primordial follicle formation



PGCs	primordial germ cells
PI3K	phosphatidylinositol 3 kinase
PIPES	Piperazine-1,4-bis(2-ethanesulfonic acid)
PMSF	Phenylmethylsulfonyl fluoride
PTEN	phosphatase and tensin homolog deleted on chromosome ten
TGF- $\beta$	transforming growth factor $\beta$
Tsc/mTORC1	Tuberous sclerosis protein/ mammalian target of rapamycin 1
ULA	ultra low attachment
Zp3	zona pellucida 3
$\alpha$ -MEM	Minimum Essential Medium Alpha Medium

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