COMPARISON OF MOUSE PREANTRAL FOLLICLE GROWTH AND SURVIVAL IN

THREE DIMENSIONAL VS. CONVENTIONAL CULTURE

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Comparison of Mouse Preantral Follicle Growth and Survival in Three Dimensional vs. Conventional Culture

by

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This is to certify that I have examined this copy of a master's thesis by

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To my family..

ABSTRACT

In vitro ovarian follicle culture is a new frontier in assisted reproductive technology with tremendous potential, especially for fertility preservation. Folliculogenesis within the ovary is a complex process requiring interaction between somatic cell components and the oocyte. Conventional two-dimensional culture of ovarian follicles disrupts spherical growth and spatial arrangements between oocyte and surrounding granulosa cells. Granulosa cell attachment and migration can leave the oocyte naked and unable to complete the maturation process. Recognition of the importance of these arrangements between cells has lead to research in to three-dimensional culture system. In the present work preantral follicles isolated from 14-day-old immature mouse ovaries were cultured either 3 dimensionally using matrigel or using 2D conventional culture. The result suggests that matrigel culture provides better milieu for growth and maturation of mouse preantral follicles.

ÖZET

In vitro over folikül kültürü, yardımcı üreme teknikleri teknolojisinde çok büyük potansiyel teşkil etmektedir. Yumurtalık içerisindeki foliküogenez, somatik hücre ve oosit arasında etkileşim gerektiren karmaşık bir süreçtir. Geleneksel 2 boyutlu kültür ortamı, oosit ve çevresinde yer alan granüloza hücreleri arasındaki küresel büyümeyi destekleyememektedir. Granüloza hücrelerinin bozulması ve göç etmesi oositi çıplak bırakmakta ve olgunlaşma sürecini bozmaktadır. Hücrelerin arasındaki bu düzenin öneminin tanınması üç boyutlu folikül kültürü ortamının araştırılmasına yol açmıştır. Bu çalışmada 14 günlük fare yumurtalıklarından izole edilen preantral foliküller kullanılmıştır. Bu foliküller iki boyutlu ve üç boyutlu kültür ortamında büyütülmeye çalışılmıştır. Bu çalışmanın sonucu üç boyutlu, matrigel, kültür ortamının preantral foliküllerin olgunlaşıp MII oosit olması için daha iyi bir ortam sağladığını göstermektedir.

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NOMENCLATURE

αMEM	Alpha-minimal essential medium
AMH	Anti-Müllerian hormone
ART	Assisted reproductive techniques
BMP	Bone morphogenetic protein
CL	Corpus Luteum
COC	Cumulus oocyte complex
ECM	Extracellular matrix
EGF	Epidermal growth factor
FSH	Follicle stimulating hormone
FSH-R	Follicle stimulating hormone receptor
GC	Granulosa Cell
GDF-9	Growth differentiation factor 9
GDF-9B	Growth differentiation factor -9B (also known as BMP-15)
GFR	Growth factor reduced
GH	Growth hormone
GH-R	Growth hormone receptor
GL	Granulosa luteal cells
GnRH	Gonadotropin releasing hormone
GV	Germinal vesicle
hCG	Human chorionic gonadotropin
HPO	Hypothalamus pituitary ovary Axis
HSA	Human serum albumin
IGF	Insulin like growth factor

IVF	In vitro fertilization
IVM	In vitro maturation
KL	Kit ligand (also known as stem cell factor, steel factor)
KO	Knock out
LH	Luteinizing hormone
LH-R	Luteinizing hormone receptor
MI	Metaphase I
MII	Metaphase II
OSE	Ovarian stromal epithelium
PCOS	Polycystic ovary syndrome
PGCs	Primordial germ cells
POF	Premature ovarian failure
SCF	Stem cell factor (also known as Kit Ligand and Steel Factor)
TGF-β	Transforming growth factor- beta
ZP	Zona Pellucida

Chapter 1

INTRODUCTION

Infertility is commonly defined as inability to conceive after one year of vaginal intercourse without use of protection. Infertility is a growing worldwide problem affecting 10% of all couples at fertile age. Over the last decades, patients resorting to in vitro fertilization clinics have been tremendously increased. One of the major sources of infertility is the side effects of radiotherapy and chemotherapy for cancer, which can cause the loss of gametes [1, 2, 3, 4]. As the survival rates of anti-cancer treatments increase, more and more patients depend on assisted fertilization techniques to get pregnant.

Since late 1950's, man have the option of cryopreserving their sperm [5]. With the cryopreservation technique male patients had the opportunity to preserve their sperm prior to start of their cancer therapy. Today, cryopreservation is available for women as well. Current tools to conserve a women's fertility include cryopreservation of embryos, mature and immature oocytes and ovarian tissues. The last approach is the only procedure that can be applied to prepubertal girls.

Since pre-mature follicles are the ones that constitute big part of the ovarian reserve it is important to find a technique to in vitro grow these follicles in to antral stage.

In this study a in vitro growth milieu has been developed in which a preantral follicle can be grown to a mature oocyte with a polar body.

Chapter 2 provides necessary background and literature review on female reproductive biology. The female ovarian structure, hormones and growth factors required for healthy oocyte growth are reviewed.

Chapter 3 describes the study conducted to find a better milieu for the growth of preantral follicles. Research conducted to find this milieu, methodology that is used and results together with discussion is given at this chapter.

The thesis is concluded with a short discussion on the possible future research work.

Chapter 2

LITERATURE REVIEW

2.1 Human Ovary and Its Structure

Ovaries are part of the mammalian female reproductive system. They are a pair of oocyte-producing glands that are homologous to the testes in the male reproductive system. There are two almond shaped ovaries located in the pelvis of every women; one on the right and the other on the left side. They are approximately 2cm wide and 4cm long with weight of 2 to 4 grams. They sit just above the fallopian tubes on the area called ovarian fossa, lateral wall of the pelvis. Each ovary is attached to a fallopian tube through fimbria. Right under the outermost layer of ovarian stromal epithelium is a dense connective tissue called tunica albuginea. The follicles, surrounding fibers of collagen, elastin and fibroblasts form the ovarian cortex which is situated just under the tunica albugenia. Under the ovarian cortex there is ovarian medulla, which contains lymphatic and blood vessels.

The ovarian cortex of young girls is consisted of many ovarian follicles whereas in the cortex of older women these follicles are replaced by fibers as a result of ovulation and atresia. The number of follicles is highest prior to birth and declines gradually until menopause.

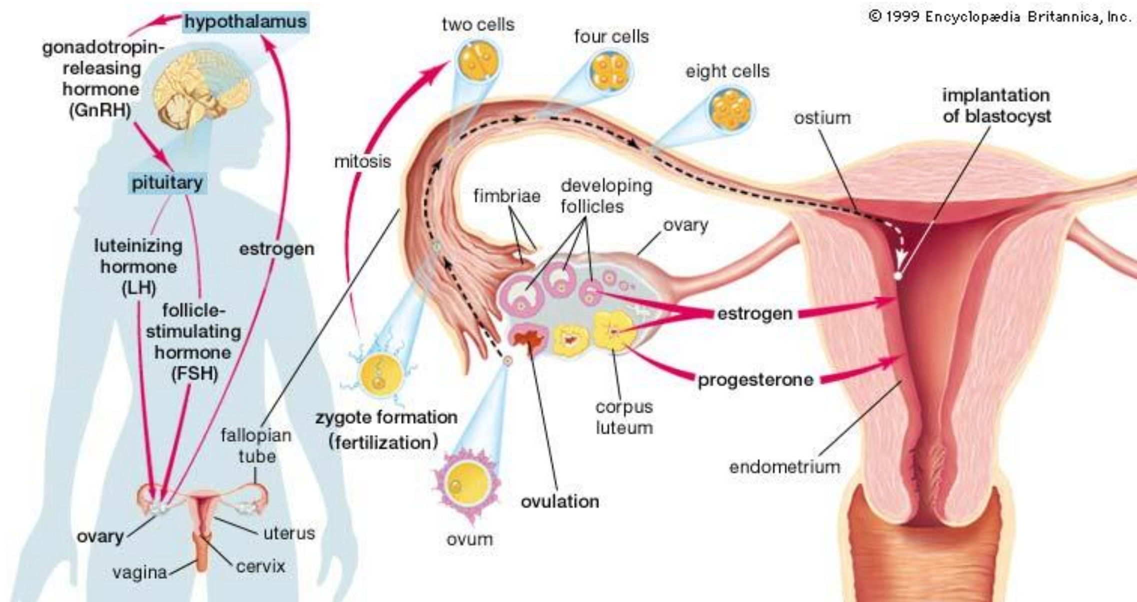


Figure 1: Human ovary structure and hormone cycle during ovulation. *Credit Encyclopedia Britannica.*

Ovaries have two different functions; producing ova and producing female hormones. Every month either left or right ovary will produce a single mature oocyte for fertilization. Although only one oocyte will be fully mature, during the ovulation period, every month approximately ten to twenty ovarian follicles will begin the maturation process.

After the mature oocyte is released, it begins its journey in the oviducts where it travels into the uterus. The mature oocyte moves in fallopian tube with the help of wave-like contractions. Inside of the fallopian tube is covered with cilia which helps the sperm to move towards the egg. The fertilization occurs inside the fallopian tubes close to the ovary. It takes five to six days for the fertilized egg to reach the uterus where it is implanted.

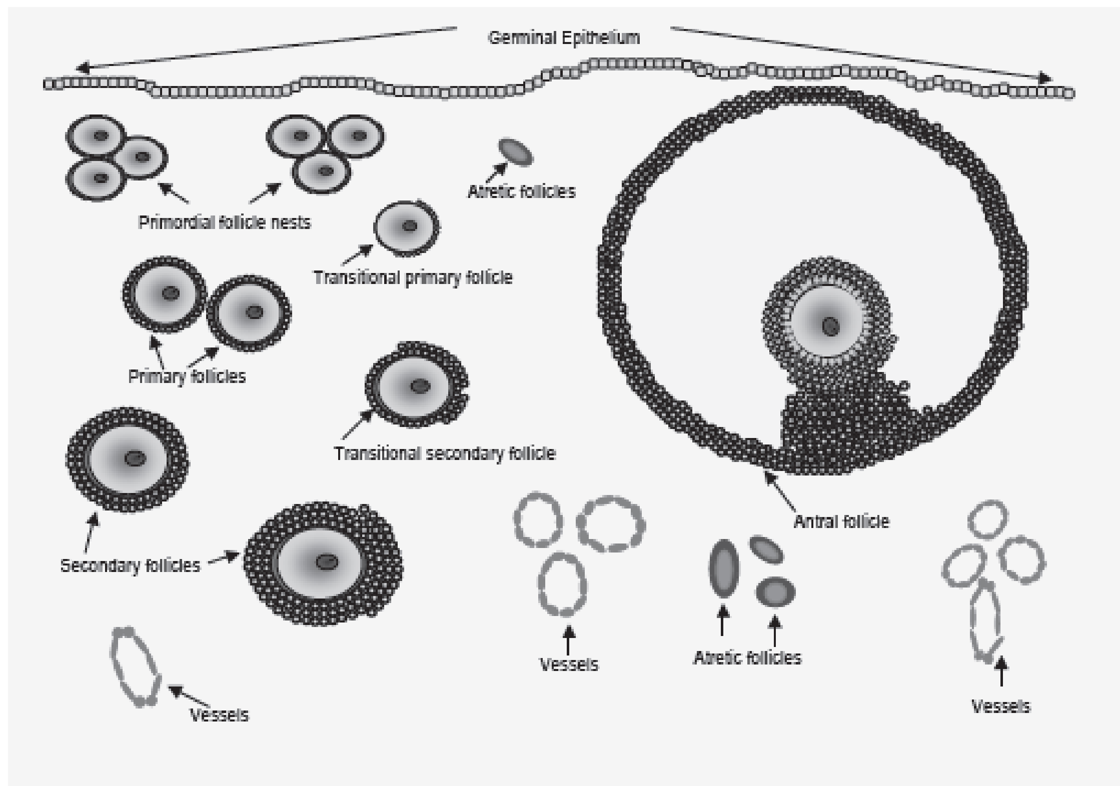


Figure 2: Basic morphological components of ovary.

2.2 Oogenesis

The creation of an ovum (egg cell) is called oogenesis. The process involves several procedures including oocytogenesis and ootidogenesis. Oogenesis starts in embryonic life as primordial germ cells are starting to form in embryonic epiblast. At around 5 to 6 weeks of gestation, under the influence of cytokines such as transforming growth factor β (TGFβ) and kit-ligand (KL), germ cells migrate to the genital ridge where they develop into oogonia. Here, primordial oocytes continue to expand through mitotic cell division before they enter meiosis and become oocytes. At this point, the oocyte is surrounded in one layer of pre-granulosa cells enclosed by a basal membrane and referred to as a primordial follicle [7]. As the oocyte enters meiosis it becomes

arrested in the prophase stage of the first mitotic division and, as a result, transformed to a primary oocyte. Here, the oocyte is surrounded by a single layer of squamous granulosa cell. Following puberty, at each menstrual cycle 15 to 20 primary follicles enter to the growing pool. They progress through meiosis I and become arrested at the metaphase stage of meiosis II. Meiosis II is completed after fertilization.

Oocytogenesis is the process in which oogonia is transformed into primordial oocyte. This stage is completed before birth, with approximately 6 million immature oocytes being produced by the 20th week of gestation. Most of these primordial oocytes undergo apoptosis, leaving approximately 2 million follicles at the time of birth. By the time of puberty approximately 400,000 primordial oocytes will be left in the ovary [7, 8].

Ootidogenesis is the development of a primordial oocyte into a primary oocyte. It involves dictyate, a prolonged resting phase in oogenesis until puberty. The process starts at prenatal age. The primary oocyte remains in this state until menarche, first menstrual cycle, when a few of these oocytes are recruited into the growing pool and become secondary oocyte. This stage involves completion of the first meiosis by the expulsion of the first polar body, disappearance of the nucleus, immediate entry in to a second round of meiosis and arrest at the metaphase of meiosis II until fertilization, and extrusion of the second polar body.

There are two mechanisms of oocyte maturation; the long cytoplasmic maturation and the short nuclear maturation. Both mechanisms are crucial to acquire a mature and competent oocyte.

2.2.1 Cytoplasmic Maturation

Cytoplasmic maturation is made up of several events that are necessary to prepare the oocyte for fertilization and later the embryonic development. This process includes cytoplasmic reorganization, accumulation of mRNAs and proteins, and epigenetic modifications.

During oocyte maturation, a great amount of RNA is stored in the cytoplasm. The total RNA amount in human germinal vesicle is 2.0ng [9]. This makes-up nearly 8% of the total amount of RNA, which is more than in a somatic cell [10]. Large amount of mRNA gets degraded during maturation, leaving the mRNA content of MII oocyte at 40% less than the germinal vesicle (GV) [11].

During the oocyte growth, large amounts of cytoplasmic organelles are formed and reorganized. Since cytoplasmic inheritance to the zygote comes almost exclusively from the egg, DNA replication of mitochondria must be done with great accuracy. During the growth phase, mitochondria become more vacuolated, which is a sign for low activity, and also the number of mitochondria and ribosomes increases which is a sign for the accumulation of mRNA proteins [12]. Oocyte stores granules, lipids and proteins that are required for the formation of new membranes in cytoplasm post fertilization [13, 14].

As the oocyte matures, zona pellucida is formed. ZP is a 15µm thick layer which is consisted of three proteins (ZP-I, ZP-II, ZP-III) and surrounds the oocyte [15]. ZP-III acts as a primary sperm receptor by binding the sperms head and starting acrosome reaction [16]. Afterwards, ZP-II aids the sperm penetration by acting as a secondary receptor. These two dimmers are non-covalently linked by ZP-I dimmers [17].

2.2.2 Nuclear Maturation

Germ cells (oocytes and sperm) are produced through meiotic cell divisions where genetic information contained in each daughter cell is divided equally. Although two nuclear divisions are completed, only replication of a single nuclear DNA takes place.

Prior to first meiotic division, the oocyte is called germinal vesicle oocyte and the nucleus it contains is called germinal vesicle. Nuclear maturation is defined by the germinal vesicle break down, in which germinal vesicle oocyte turns into metaphase I oocyte. Subsequently, first meiotic division happens; the polar body disintegration which leads to the formation of the mature metaphase II oocyte.

At GV, the cytoplasm reaches maturity but the nucleus is still immature. The nuclear maturation is obtained through the finalization of the meiosis I, which is moderated by the activation of M-phase promoting factor (MPF). The MPF activity is low in GV oocyte but increases during the first meiosis with its peak at MI. Consequently, it decreases during the first meiotic division and increases and remains high during metaphase II arrest [18, 19].

The final maturation of the oocyte, including the second polar body extrusion, occurs immediately after fertilization. The entire process is called nuclear maturation.

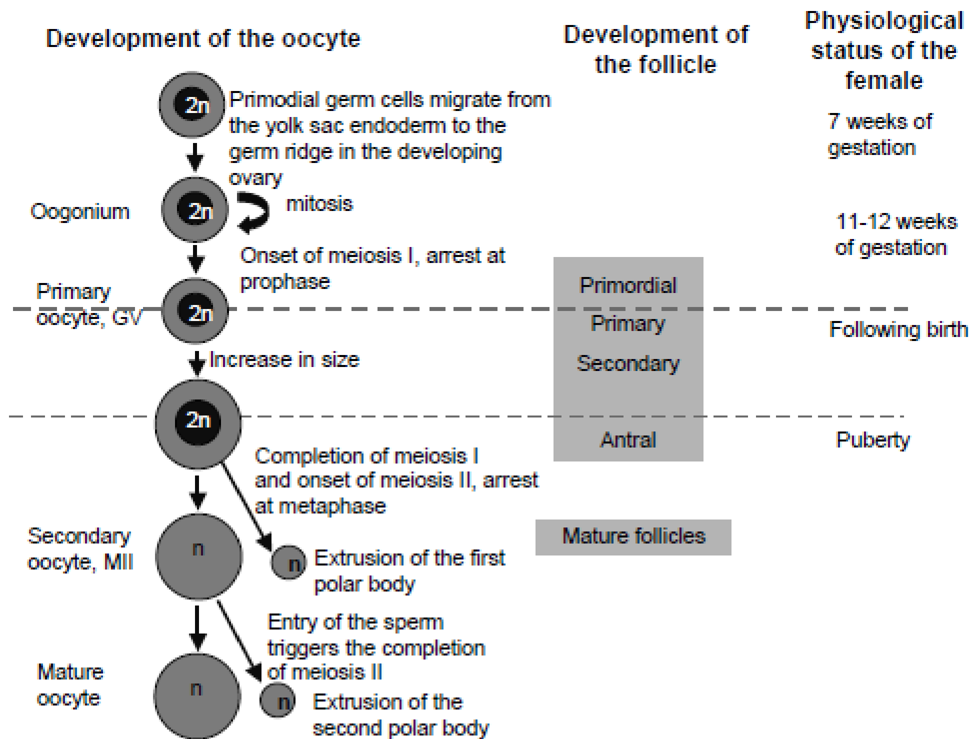


Table 1: The relationship between the developing follicles and oocytes in relation to physiological status of the female.

2.2.3 Ovarian Reserve

The female reproductive system contains a fixed number of non-renewable germ cells that are established prior to birth. Although this dogma has been challenged by Johnson, where it is suggested that there are germ stem cells originating from the circulation and bone marrow cells, this hypothesis has not been confirmed by others [20, 21].

According to the traditional standpoint, the oocyte number reaches its peak number around 20 weeks of gestation with approximately six million follicles. The oocyte reserve starts decreasing at fetal life until the women enters menopause. At the time of birth, number of follicles is decreased to around two million and at the time of

puberty only 300,000 follicles will be left. When the women enters menopause at an average age of 50 only 1000 follicles will remain in the ovary [7]. During the life span of a woman, approximately 450 oocytes will be ovulated. The progressive decline in follicle number with age is due to a natural breakdown process called follicular atresia which affects 99% of follicles at all stages.

2.3 Folliculogenesis

The process of ovarian follicle development is called folliculogenesis and involves two major processes: recruitment of the follicle into the growing pool and the proliferation and differentiation of the theca and granulosa cells. The recruitment of follicles is regulated by autocrine and paracrine signals, which are produced by the ovary itself, while the proliferation and differentiation of theca and granulosa cells is controlled by endocrine signals from outside the ovary in addition to the internal autocrine/paracrine signaling.

In order to become a mature oocyte, an ovarian follicle must pass through the primordial (resting), primary, secondary (pre-antral), tertiary (antral) and pre-ovulatory (Graafian) stages. Although at the antral stage most follicles go through atresia, under the influence of gonadotropins few of them reach the Graafian stage. Following the gonadotropin surge, only the dominant follicle will survive and release the mature oocyte. This follicle will be ready for fertilization [22].

The folliculogenesis begins with the primordial follicle, which is an oocyte surrounded by a single layer of squamous granulosa cells surrounded by a thin basal lamina. Once primordial follicle gets recruited into the growing phase, it grows into a primary follicle. At this stage zona pellucida is formed while granulosa cells change shape and become cuboidal.

Secondary follicle is surrounded by several layers of granulosa cells. With the production of proliferating cell nuclear antigen (PCNA) granulosa cell proliferation has started. As the granulosa cells continue to proliferate, theca interna and theca externa are formed with capillaries in-between the two layers. With the vessels, the follicle starts to gain blood supply and thereby it is exposed to any factor circulating in the bloodstream [23].

During the follicular development, proliferating granulosa cells provide necessary nutrients and molecular signals to the oocyte. While the oocyte increase in size, the proliferation and differentiation of the granulosa cells result in the formation of the fluid filled cavity, antrum. At the antral (Graafian) stage, under the influence of gonadotropins, pre-ovulatory follicle is formed. The communication between the surrounding granulosa cells and the oocyte gives result to a mature fertilizable oocyte.

2.3.1 Stages of Follicular Development

Primordial follicles contain an immature oocyte surrounded by single layer of flat, squamous granulosa cells. The follicle is separated from the surrounding somatic cells by a basal membrane [10]. A primordial follicle has a diameter of approximately 30µm. The process by which primordial follicles wake up is known as initial recruitment. The initial recruitment is mediated by various locally produced growth factors and stimulatory and inhibitory hormones [24].

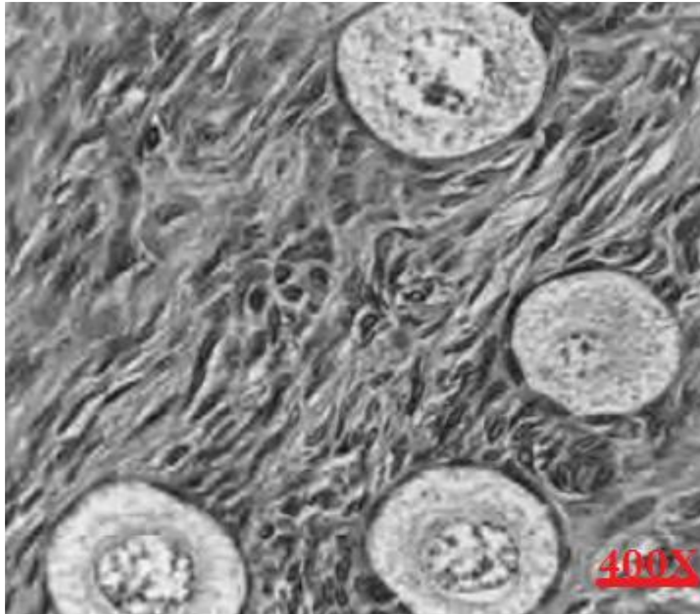


Figure 3: Primordial follicles under light microscope.

As the primordial follicles are recruited to the growing pool, the GCs grow larger and become cuboidal while they continue to surround oocyte as a single layer. This change marks the beginning of primary follicle. As the primordial follicle grows into a primary follicle the oocyte genome gets activated and genes become transcribed. Paracrine signaling pathways that are necessary for communication between the follicle and the oocyte are formed.

Although at this time follicles are gonadotropin independent, primary follicles develop receptors for follicle stimulating hormone. However, research has shown that the presence of FSH contributes follicle growth in vitro.

With the primary follicular stage, zona pellucida is formed around the oocyte. It separates the oocyte from surrounding granulosa cells. The ZP, which stays with the oocyte after ovulation, binds spermatozoa and is required for acrosome reaction.

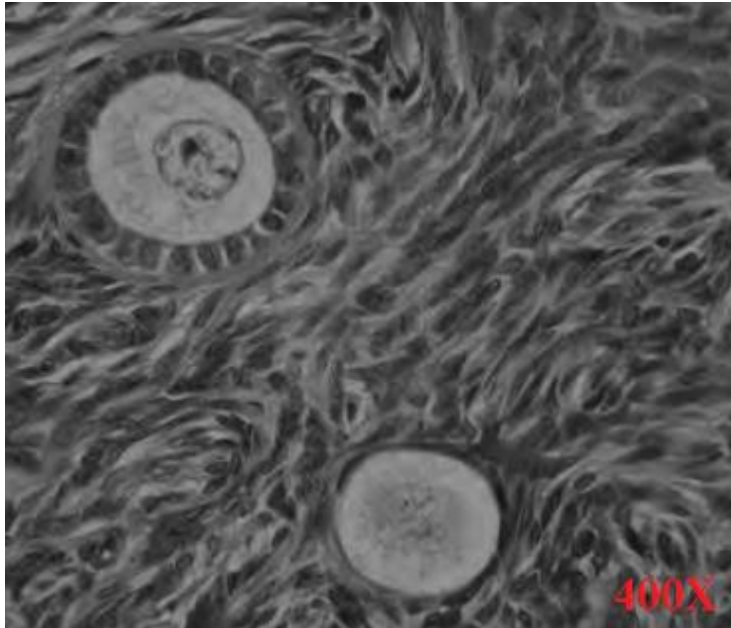


Figure 4: Primary (upper) and primordial (lower) follicle under light microscope.

As the primary follicle grows into the secondary and pre-antral follicle, the granulosa cells proliferate and form multiple layers around the oocyte. At this stage, theca cells are recruited by oocyte secreted signals. Theca cells surround the basal lamina and undergo cytodifferentiation to become theca interna and theca externa. The differentiation of theca cells into theca interna and theca externa is thought to be promoted by LH. A network of capillaries is formed between the two layers resulting in blood circulation from and to the follicle. The diameter of the follicle is now approximately 100-200µm.

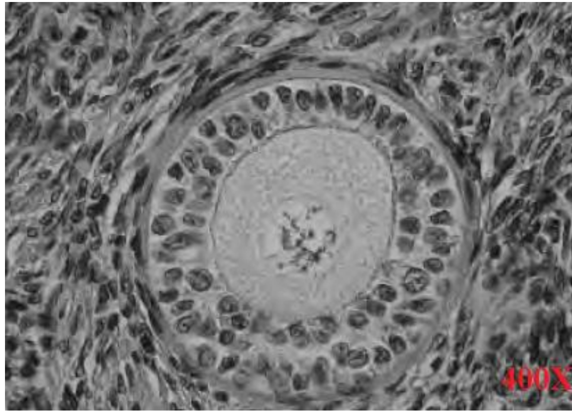


Figure 5: Secondary follicle under light microscope.

The formation of antrum, fluid filled cavity adjacent to the oocyte, designates the follicle as antral or Graffian follicle. The large antrum is a characteristic feature of the mature follicle. These follicles contain two types of GCs; mural granulosa cells and cumulus granulosa cells. Mural granulosa cells are the ones that form a thin layer along the periphery of the follicle while cumulus granulosa cells are the ones surrounding the oocyte. The basement membrane separates these GCs from theca interna and theca externa. At this stage the diameter of the follicle is approximately 20mm.

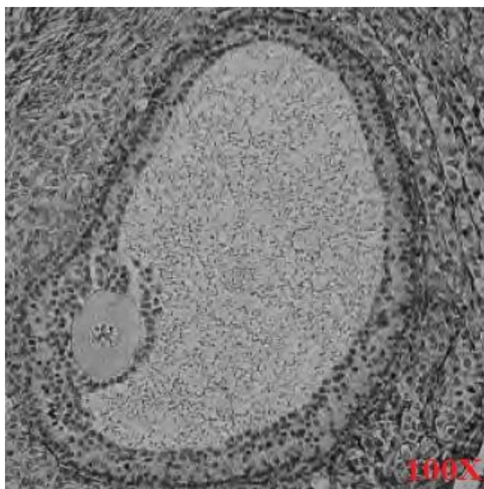


Figure 6: Graffian follicle under light microscope.

2.4 Gonadotropins and other Hormones

2.4.1 FSH & LH

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are glycoproteins that are produced by anterior pituitary. They are released after stimulation of gonadotropin releasing hormone (GnRH) produced by hypothalamus.

Until late secondary to early antral stage, follicle development is FSH independent; starting from secondary stage follicle depends on gonadotropins for further development. In humans, follicle stimulating hormone receptor (FSH-R) is expressed in 33% of the primary and secondary follicles [25]. In ovaries with FSH-R mutation, follicles beyond primary stage are barely seen [26].

LH is produced by gonadotrope cells in anterior pituitary gland. An acute rise of LH triggers the ovulation and development of the corpus luteum. In human females, LH supports theca cells that provide androgens and hormonal pre-cursors for production of estradiol. Binding sites for LH was discovered on rat theca cells using labeled hCG, since hCG binds to the same receptor as LH [27]. During menstruation, FSH initiates follicular growth. With the rise of estrogen level, LH-R is also expressed on the mature follicle, which leads it to produce more estradiol. When the follicle is fully mature, a spike in hydroxyprogesterone by the follicle arrests the production of estrogen which leads to a decrease in negative feedback of GnRH, which than stimulates the release of LH from anterior pituitary. The increase of LH production lasts for 24-48 hours and triggers ovulation. This increase is necessary for the luteal function during the first two weeks of menstrual cycle.

Starting from preantral stage, follicles start to depend on FSH secretion from anterior pituary. During the growth phase, which takes approximately 85 days for a follicle to grow from preantral to ovulatory stage, the follicles depend on gonadotropin

fluctuations happening during menstrual cycles. According to two-cell two-gonadotropin theory, both FSH and LH are essential for the production of steroid hormones.

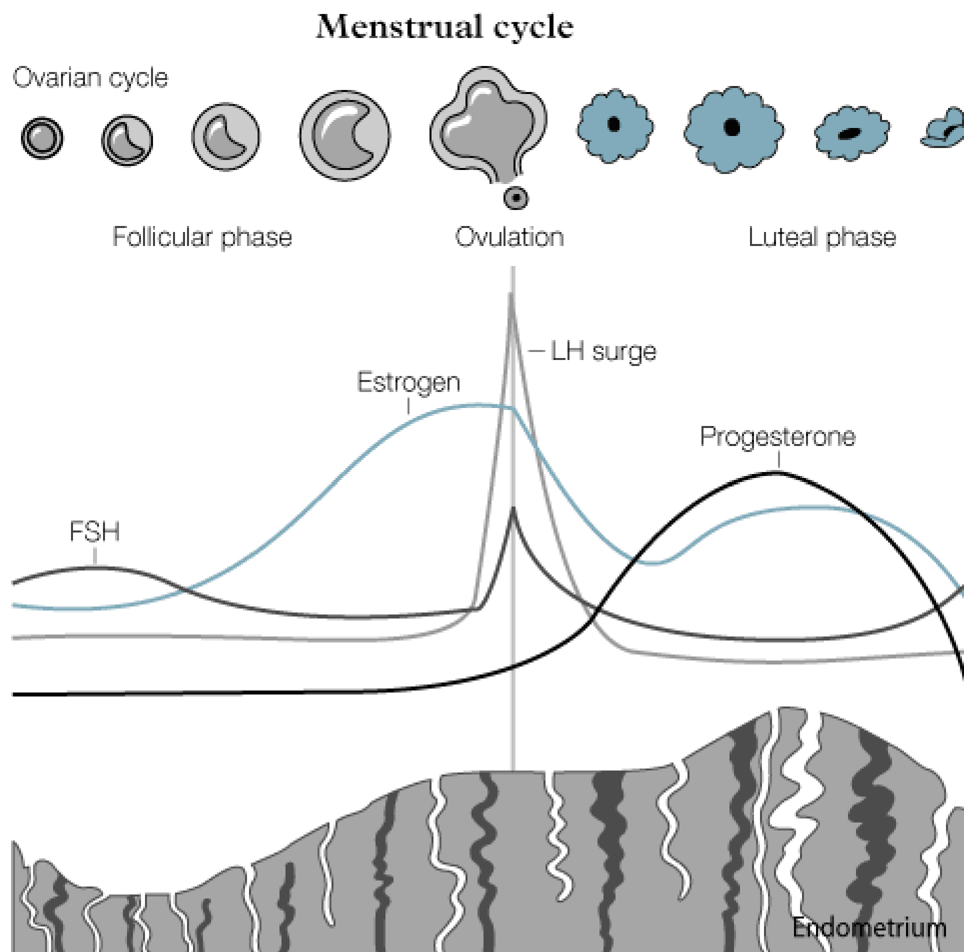


Figure 7: FSH, LH, estrogen and progesterone levels during menstrual cycle.

2.4.2 Estrogen

The production of estrogen, mainly E2 (estradiol-17β), starts to increase when gonadotropins act on the granulosa and theca cells. The production reaches its maximum level in the preovulatory follicle [28]. One of the main actions of estrogen is the negative feedback mechanism over the hypothalamus-pituitary-ovarian axis. This mechanism works by down regulating the release of gonadotropin releasing hormone from the hypothalamus which causes the plasma gonadotropin levels to decrease.

Estrogen, in combination with IGF-1 and FSH, increases differentiation and growth of granulosa cells [29, 30]. Furthermore, estrogen, in combination with FSH, decreases apoptosis of GCs, induces LH-R on GCs and inhibits androgen synthesis in theca cells [31, 32, 33].

Estrogen signals through two different estrogen receptors: ERα and ERβ. Both receptors are expressed in the ovary. Knockout ERα mice are infertile and have hyperemic ovaries without corpus luteum [34]. The ERβ knockout mice have small ovaries that are partially arrested in follicular development with increased numbers of primordial follicles [35]. Therefore, it is hypothesized that the proliferative actions of E2 is dependent on ERα while the anti-proliferative effects and differentiation is mediated by ERβ [36].

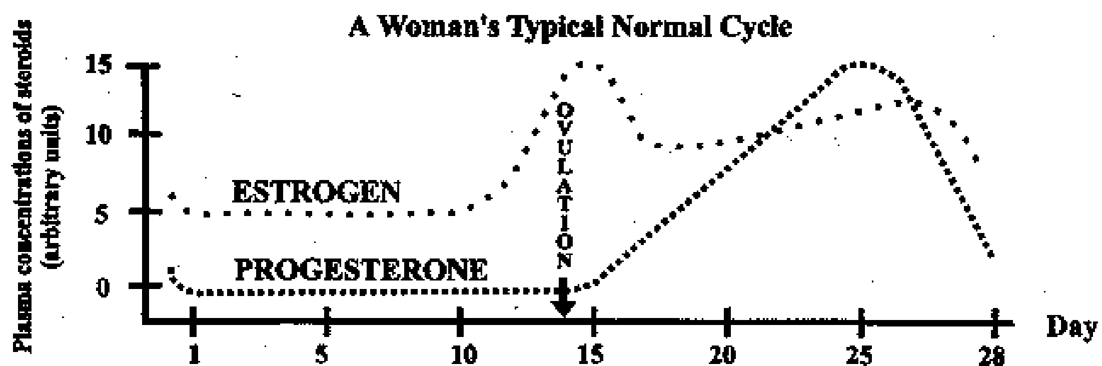


Figure 8: Estrogen and progesterone levels during menstrual cycle.

2.4.3 Progesterone

Progesterone, P4, is a C-21 steroid hormone which is important for ovulation, implantation and maintenance of the pregnancy. Progesterone production takes place in granulosa cells. It starts just before the gonadotropin surge and increases with the corpus luteum. This production is suggested to be crucial for ovulation [37]. It increases the production of proteolytic enzymes [38].

There are two different progesterone receptors: PR-A and PR-B. PR-A knockout mice fail to ovulate and is infertile, while PR-B knockout mice is ovulatory and produces viable offspring [39, 40]. These receptors are expressed in large follicles and expression is increased by the LH surge [41]. Progesterone amplifies the effect of FSH on GCs by increasing cAMP and it inhibits FSH induced estradiol production [42, 43, 44]. It, also, inhibits transition from primordial to primary follicles in newborn rats and inhibits insulin dependent granulosa cell mitosis [45, 46, 47].

2.4.4 Androgen

Androgen receptors are localized on granulosa cells, theca cells and stromal cells of the human ovary [48]. The principal androgens are androstenedione and testosterone. The productions of these hormones are in the theca cells and induced by luteinizing hormone. During the different stages of folliculogenesis, androgen receptor (AR) expression goes up and down. This is assumed to be under control of oocyte derived factors [49, 50]. Activated ARs proliferates GCs by enhancing the growth promoting action of GDF-9 [51]. In contrast to growth-promoting actions, it is also shown that androgens can cause follicular atresia in immature mice primed with PMSG (pregnant mare serum gonadotropin) [52]. It has also shown that apoptosis of rat GCs was induced by androgen [53].

2.5 Growth Factors

2.5.1 Transforming Growth Factor β Superfamily

Many of the growth factors influencing the growth and development of follicles are members of the transforming growth factor β superfamily [54]. The TGF-β superfamily is a large group of proteins that exhibit similar structural motifs, including 7 cysteine residues [55]. These proteins are widely distributed in the body and function as extracellular ligands involved in several physiological processes during pre and post-natal life [56]. Many of these proteins are produced in the ovary and are important local regulators for follicular development and oocyte maturation. Members of this superfamily can be classified into several subfamilies: TGF-β subfamily (TGF-β1, TGF-β2, TGF-β3), the activin/inhibin subfamily (including activins A, AB, B, inhibins A, B), an extensive bone morphogenetic protein subfamily (BMP) with approximately 20 members and several additional members such as anti-Müllerian hormone (AMH) and nodal.

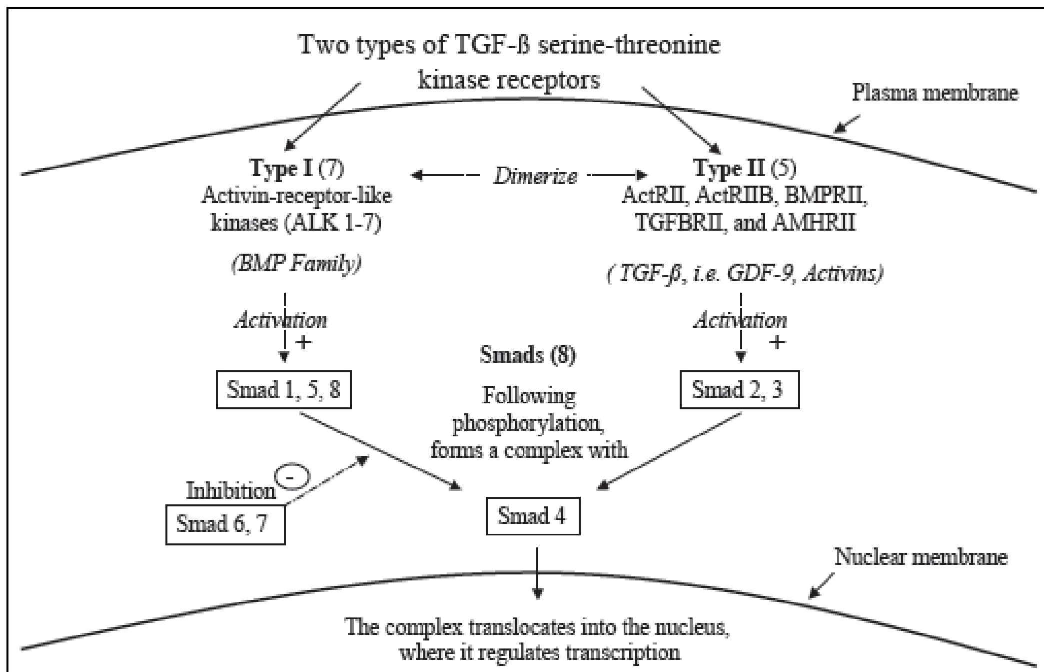


Table 2: The TGF-β activated signaling pathway.

2.5.1.1 Growth Differentiation Factor-9(GDF-9) and GDF-9B/BMP15

Growth differentiation factor 9 was discovered in 1993 [57]. The expression was first localized to oocyte but later it was found in testis, pituitary, hypothalamus, uterus and bone marrow [58]. In humans this factor plays essential role during folliculogenesis [59]. GDF-9 enhances the survival rate, growth rate and recruitment of follicles [60]. Mutations of the GDF-9 gene might be associated with pre-mature ovarian failure [61]. GDF-9 mRNA and its corresponding protein are present in oocytes located in primary follicles [62].

Growth differentiation factor 9B (GDF-9B) is also known as BMP15. It is expressed progressively in oocytes from primary stage onwards throughout folliculogenesis and is a regulator for follicular growth.

Both GDF-9 and GDF-9B have mitogenic effects on theca and granulosa cells [63, 64, 65, 66, 67, 68]. Also, they have the ability to inhibit gonadotropin stimulated progesterone production from GCs [69, 70, 71, 72]. Moreover, recent studies show that GDF-9 and GDF-9B have a positive effect on oocyte quality in in vitro cultured oocytes. In bovine oocytes, these factors resulted in increased blastocyst formation whereas in mice addition of GDF-9 to the culture resulted in double rate of survival [73, 74].

2.5.1.2 Activins and Inhibins

Activins and inhibins are two members of the TGF-β family which takes part in the oocyte maturation and follicular development. Inhibins, produced by GCs in the ovary, take its name due to its negative effects on FSH secretion. They are heterodimers that contain one α- and one β-subunit. Inhibin B secretion is high during the follicular phase and drops after the LH surge [75]. Inhibin can be used as a marker for ovarian reserve as reproductive age results in lower amounts of circulating inhibin and thereby increased FSH levels [76].

Activins are homo- and heterodimers that are consisted of two different inhibin β-subunits; covalently linked βA and βB [77]. Activin A the most common form of the activins. Its production takes place in GCs. Receptors Act-RI and Act-RII and the Smads necessary for signaling are present in GCs and oocytes in humans and rodents [78]. This shows the paracrine signaling between the granulosa cells and oocyte. Activin induces proliferation in human granulosa lutein cells [79].

2.5.1.3 Anti Müllerian Hormone

In contrast to stimulatory actions of the most other members of the TGF-β superfamily, anti-Müllerian hormone (AMH) is suggested to inhibit the follicular recruitment and progression of early stages of follicular development [80]. Originally AMH is named Müllerian inhibitory substance (MIS) due to its role in male fetal sex differentiation during embryonic development [81].

It is produced by GCs of human females from after birth until menopause. AMH expression is first observed at the primary stage of follicle development and persists until the antral stage and later the expression disappears in the dominant follicle [82, 83]. Immunohistochemical analysis of the adult human ovary consisting monoclonal antibody against human AMH has showed no staining for primordial follicles while GCs of 74% of the primary follicles presented expression for this protein [84]. Pre-antral follicles were the most intensely stained while follicles bigger than 5 mm had almost no staining. On the basis of these findings it has been suggested that growing follicles produce AMH as negative regulator for follicle recruitment in a paracrine way. AMH knockout mice showed decrease in primordial follicles and increase in growing follicles, this revealed that AMH plays a role in inhibiting follicular development [80].

2.5.2 Growth Hormone

Growth hormone plays important role in folliculogenesis and maturation of oocytes. Although it is primarily synthesized in the pituitary gland, growth hormone mRNA in bovine GCs and oocytes has been indicated that this hormone is also synthesized locally in the ovary and act in autocrine and paracrine way [85]. Growth hormone receptors have been identified in rat ovaries, in bovine GCs, cumulus cells and oocytes, and in human GCs of antral follicles and corpus luteum which indicates a direct GH effect in the ovary [86, 87, 88, 89].

The GH-R knockout mice showed delayed sexual maturation and gave birth to abnormally small litters with high mortality [30]. These effects appear to be due to the defect of the ovary, rather than the pituitary gland. Furthermore, the IGF-I expression in the ovary of the knockout mice was not impaired, showing that its expression in the ovary is GH independent [90].

In vitro studies showed that GH stimulates growth and follicular cell proliferation of murine preantral follicles. It shows a direct inhibitory effect on apoptosis in both rat and bovine follicles at early stages of their development [91, 92]. Furthermore, over-expression of bovine GH on a transgenic mouse strain decreases apoptosis in the mouse ovarian follicles [93]. In addition, in vitro studies show that GH has direct inhibitory affect on the apoptosis of early bovine and rat follicles [94, 95, 96]. In rats, GH deficiency is associated with low LH responsiveness [97]. Since binding sites for GH are missing on the atretic follicles in pigs and GH-R deficient bovine have mal-development of the dominant follicle it can be disputed that growth hormone might also be involved in the selection of the dominant follicle [98, 99].

It is showed that GH stimulates the differentiation and proliferation of luteinized GCs and production of estrogen in humans [100, 101]. The expression of steroid hormones is modulated by GH by increased up-regulation of enzymes involved in steroid synthesis [102]. As a result, GH acts in synergy with the gonadotropins to increase the steroid production [103, 104].

2.5.3 Kit Ligand/ Stem Cell Factor

Kit ligand (KL), also known as stem cell factor (SCF) or steel factor, is a ligand for the c-Kit protooncogene receptor tyrosine kinase. It is a pluripotent growth factor which is in most species involved in the growth and differentiation of hematopoetic

stem cells, melanoblasts and neuroblasts [105]. Its receptor is expressed in theca cells, primordial germ cells (PGC) and oocytes while KL is expressed by GCs [106].

In human oocytes kit ligand receptor is weekly expressed in the primordial oocytes in fetal ovaries [107]. The expression continues to increase through primary, secondary and pre-antral follicles and it drops at antrum formation [108, 109]. In fetal human ovaries KL is expressed at GCs of primordial, pre-antral and early antral follicles [107]. In adult human ovaries, KL is expressed in GCs of primary follicles [110].

In rodent ovary KL is expressed in GCs and its receptor is detected in theca interna cells and oocytes [111, 112, 113, 114, 115, 116]. Blocking the kit ligand receptor in mouse impairs follicular recruitment [117, 118].

Chapter 3

Mouse Preantral Growth in 2D vs. 3D In Vitro Culture Model

3.1 Aim of the Study

In vitro ovarian follicle culture is a new frontier in assisted reproductive technology with tremendous potential, especially for fertility preservation. Folliculogenesis within the ovary is a complex process requiring interaction between somatic cell components and the oocyte. Conventional two-dimensional culture of ovarian follicles disrupts spherical growth and spatial arrangements between oocyte and surrounding granulosa cells. Granulosa cell attachment and migration can leave the oocyte naked and unable to complete the maturation process. Recognition of the importance of these arrangements between cells has lead to research in to three-dimensional culture system. In the present work preantral follicles isolated from 14-day-old immature mouse ovaries were cultured either 3-dimensionally using matrigel or using 2D conventional culture. The result suggests that matrigel culture provides better milieu for growth and maturation of mouse preantral follicles.

3.2 Materials and Methods

3.2.1 Reagents

Alpha-minimum essential media (α-MEM) and Dulbecco's modified eagle's medium–F12 (DMEM-F12) were purchased from Gibco (Invitrogen, Carlsbad, CA).

Bovine serum albumin (BSA) and insulin transferin selenite (ITS+3), collagenase (from clostridium histolyticum type IA; cat no. 9891), laminin (purified from Engelbreth-Holm-Swarm murine sarcoma; cat no. L2020), poly-L-lysine (cat no. P4707), Dnase-I (from bovine pancreas; cat no. D4263), Hoechst 33342 (cat no. B2261), and benzly benzoate (cat no. B6630) were purchased from Sigma (St Louis, MO). Recombinant FSH was obtained from Serono (Rockland, MA). Growth factor– reduced (GFR) matrigel (a solubilized basement membrane preparation extracted from Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in ECM proteins, whose major component is laminin, followed by collagen 4, heparan sulfate proteoglycans, and entactin; cat no. 356230) and fibronectin (from human plasma; cat no. 354008) were purchased from BD Bioscience (San Jose, CA). Collagen type 4 (purified from Engelbreth-Holm-Swarm murine sarcoma; cat no. 3410-010-01) was from Trevigen (Gaithersburg, MD). Human recombinant activin-A (cat no. 114700) was from Calbiochem (EMD Biosciences, San Diego, CA).

3.2.2 Animals and Follicle Isolations

Immature 14-day-old SV129/B6 mice were used in all experiments. The ovaries were removed after euthanasia and minced into 2 or 3 pieces in pre-equilibrated and HEPES-buffered DMEM-F12 culture medium. Then the pieces were digested with collagenase type IA, Dnase-I in DMEM-F12 supplemented with 5% BSA for 30 minutes at 37° C as described previously. Preantral follicles were mechanically isolated using 28- to 30-gauge needles under stereomicroscope (Olympus SZX12), and those with intact basal membrane and theca cells were chosen for culture.

3.2.3 Culture Medium

A defined serum-free culture medium was prepared using α-MEM supplemented with 100 mIU/mL recombinant FSH, 3 mg/mL BSA, ITS+3 (insulin 10 µg/mL, transferin 5.5 µg/mL, selenite 5 ng/mL) and 100 U/mL penicillin-G, 100 µg/mL streptomycin, 0.25 µg/mL amphotericin-B at 37°C, and 5% CO2 in air. Half of the culture media was replaced every other day.

3.2.4 Follicle Culture on ECM, Polylysine, and Standard Plates

Follicles were cultured on 12mm glass cover slips coated overnight at 4°C with fibronectin (15 µg/mL), collagen 4 (15 µg/mL), laminin (15 µg/mL), or polylysine (15 µg/mL) covered with mineral oil. All matrix proteins and polylysine were dissolved in phosphate-buffered saline and filtered through 20 µm filters before use. The ECM components and polylysine were refreshed by adding the same concentrations in the media every other day. Follicles that were cultured in standard plates were placed in microdroplets of 30 µL culture media under mineral oil in 6cm culture plates. Conventional tissue culture plastic has a net negative surface charge produced by plasma treatment of the polystyrene, a nonbiological plasma. Polylysine surface treatment improves adhesive properties by altering the charge on the vessel surface from negative to positive. Therefore, both treatments facilitate cell attachment by ionic forces rather than ECM-integrin coupling.

3.2.5 Culture in 3-Dimensional Basement Membrane Matrix (Matrigel)

First, GFR matrigel was allowed to liquefy at 4°C and was then diluted with the culture medium in a 1:1 ratio (100 µL from each) and placed in 8-well-format chamber slides. Following the transfer of isolated follicles into them, the chamber slides were put

in the incubator to allow the matrigel to polymerize for 30 minutes; subsequently, 100 μL of culture media was added on the top of the solidified matrigel.

3.2.6 Follicle Measurements and Evaluation of Survival

Follicles were imaged every other day using Olympus IX 70 inverted microscope with digital imager under 300X and 150X magnifications. Follicle diameter was measured by calculating the mean of 2 perpendicular measurements including the theca layer. The percentage follicular growth was calculated according to the formula [(follicle diameter on day 7 – diameter day 0) X 100/follicle diameter on day 0]. Follicles with disrupted basal lamina or somatic cell layers; extruded, damaged, or misshapen oocytes; and those that did not attach to culture plates in the first 24 hours of the culture period were excluded from the analysis.

3.2.7 Three-Dimensional Imaging of Follicles Using Confocal Microscopy

Isolated preantral follicles cultured in matrigel and Standard plates were treated with fluorochrome DNA stain Hoechst 33342 (10 μM) for an hour and fixed in situ with 4% paraformaldehyde for 1 hour. Then, the follicles were dehydrated using a series of graded ethanol (70%, 85%, 95%, and 100% every 15 minutes) and cleared with a 1:2 benzyl alcohol/benzyl benzoate mixture for 30 minutes at room temperature before scanning. The follicles were then serially scanned at 0.5-μm thickness, and images were obtained using a Leica 5410 inverted confocal microscope.

3.2.8 Statistical Analysis

Follicular growth was expressed as the percentage of the mean growth ± standard error of mean (SEM) of 3 independent experimental replicates. For comparison of growth, *t* test, ANOVA, or the Kruskal-Wallis test were used where appropriate. If a significant *P* value was reached, then the groups were compared with post hoc tests. The survival of each group for the indicated time points was created using the Kaplan-Meier method. Survival rates and curves were compared using X^2 and long-rank tests, respectively. $P<0.05$ was considered significant.

3.3 Results and Discussion

In this study it is shown for the first time that immature murine preantral follicles cultured in matrigel with FSH had the highest growth and survival rates compared to other tested culture conditions. Furthermore, by analyzing 3-dimensional confocal images it is determined that follicles preserved their structure better in matrigel than conventional 2-dimensional culture environment.

Previous works has showed that follicles cultured in 2-dimensional conventional cultures become flattened structures which are characterized by the disruption of their basal lamina. The paracrine communication between oocyte and somatic cells gets impaired [119]. In an attempt to keep the 3-dimensional follicular structure, other researchers have used different in vivo culture conditions such as collagen gels or collagen alginate capsules [120, 121, 122]. In other studies, follicles were cultured in membrane inserts or inside treated microwells to prevent follicle attachment and flattening [123,124]. Some other researchers have tried daily transfer of follicles in to new wells [125]. However, no quantitative evidence for a better maintenance of follicular structure was provided.

The early stages of the mammalian folliculogenesis are less understood than the antral stages of the follicle development. The significance of integrins and ECM in follicular growth initiation and development has been discussed in other studies. For example, prepubertal mouse ovaries cultured with collagen and laminin have significantly higher number of follicles initiating growth than the ovaries cultured on polylysine [126].

It is observed that FSH enhanced growth and survival of follicles during 7 days of culture in matrigel. Follicles cultured in matrigel without FSH had better growth and survival rates than those cultured in 2-dimensional culture plate. These findings suggest that even without the positive effects of growth hormones, matrigel provides a better milieu for preantral follicles than 2-dimensional culture conditions. At this study with Matrigel environment we had a mature MII oocyte with polar body.

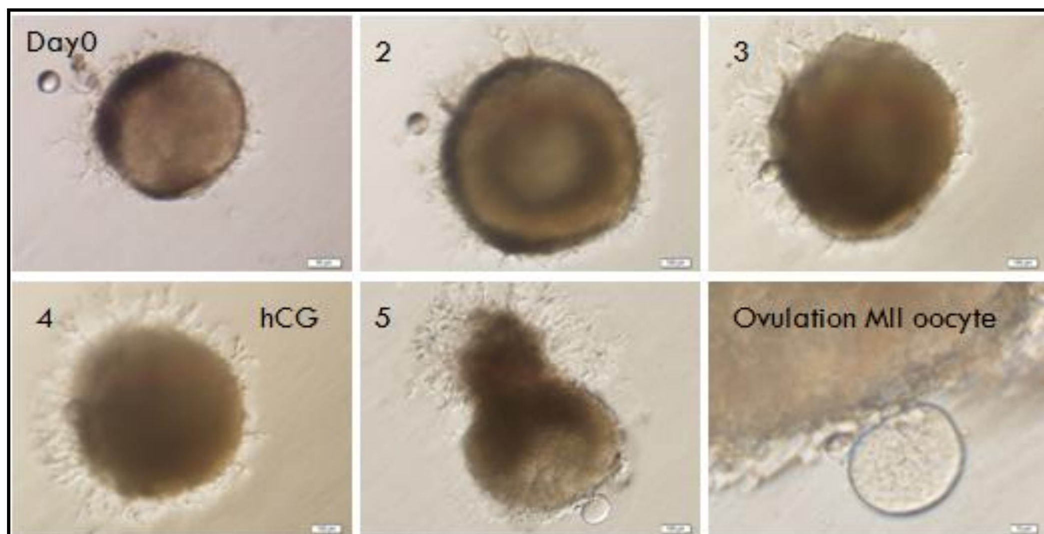


Figure 9: Preantral mouse follicle cultured in matrigel to MII stage.

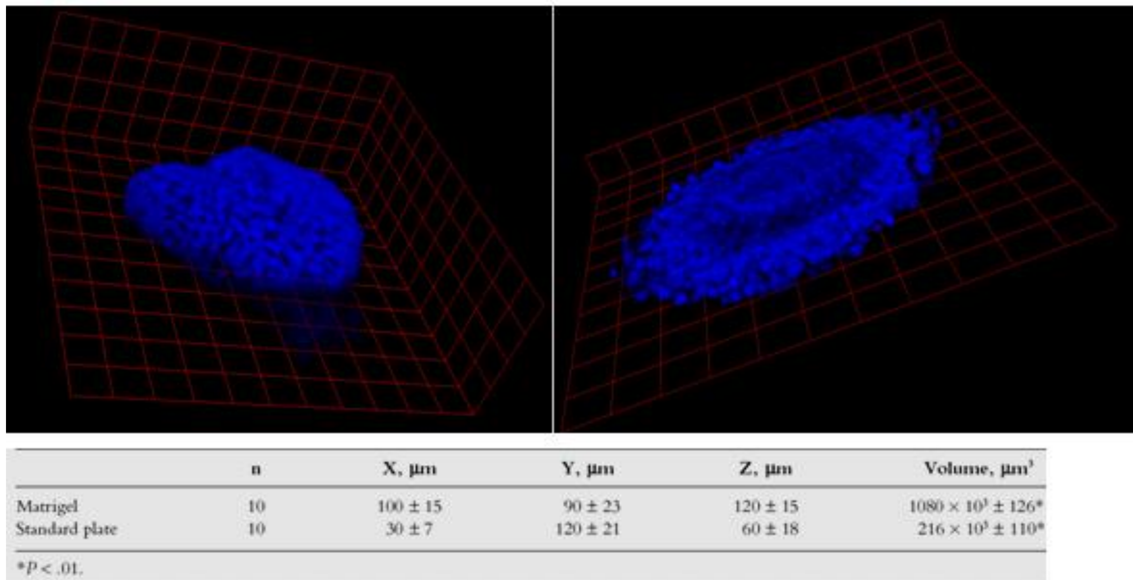
Group	n	Mean ± SEM Oocyte Diameter at Day 0, µm	Percentage of Damaged Follicles on Day 1	Mean ± SEM Follicle Diameter at Day 0, µm	Mean ± SEM Follicle Diameter at Day 7, µm	Percentage ± SEM Growth	Percentage ± SEM Survival
Standard culture plate	20	65.5 ± 4.2	16	140.0 ± 7.6	391.6 ± 31.3	142.6 ± 18.9	47.3 ± 11.4
Matrigel	33	63.9 ± 2.5	15	126.5 ± 5.6	292.4 ± 21.8	113.2 ± 12.2*	54.5 ± 8.6

$P < .05$, by contingency table analysis.

Table 3: Follicle growth and survival after 7 day culture.

Another important finding of this study is that preantral follicles from immature mice could be grown to antral stage for up to 8 days in a serum-free defined medium. Even though growth and antrum formation have been reported for bovine preantral follicles using a serum free medium supplemented with insulin-like growth factor, in rodent models isolated rat preantral follicles became atretic, as evidenced by a substantial increase in internucleosomal DNA fragmentation after 24 hours in serum free culture [119, 127]. Other studies could not grow murine preantral follicles in vitro [128].

In conclusion, it is shown that 3-dimensional matrigel culture conditions provide a better in vitro environment for growth and survival of preantral follicles in immature mice. The maintenance of the 3-dimansional structure of the follicle can be explained in part by the resemblance of matrigel to follicle basement membrane.



	n	X, µm	Y, µm	Z, µm	Volume, µm^3
Matrigel	10	100 ± 15	90 ± 23	120 ± 15	1080 × 10^3 ± 126*
Standard plate	10	30 ± 7	120 ± 21	60 ± 18	216 × 10^3 ± 110*

*$P < .01$.

Figure 10: Confocal microscopy of preantral murine follicles grown in matrigel and standard plate conditions.

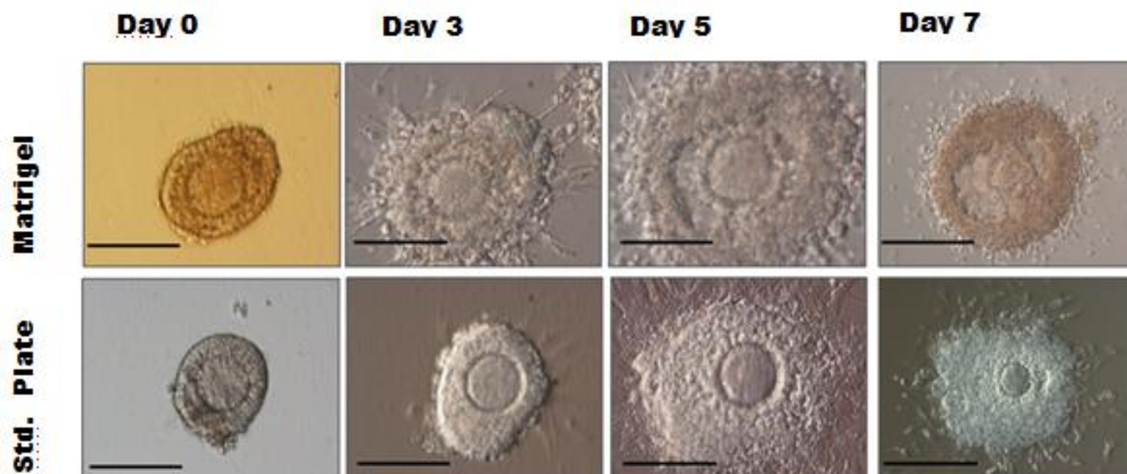


Figure 11: Preantral follicle culture in matrigel and standard plate conditions for 7 days.

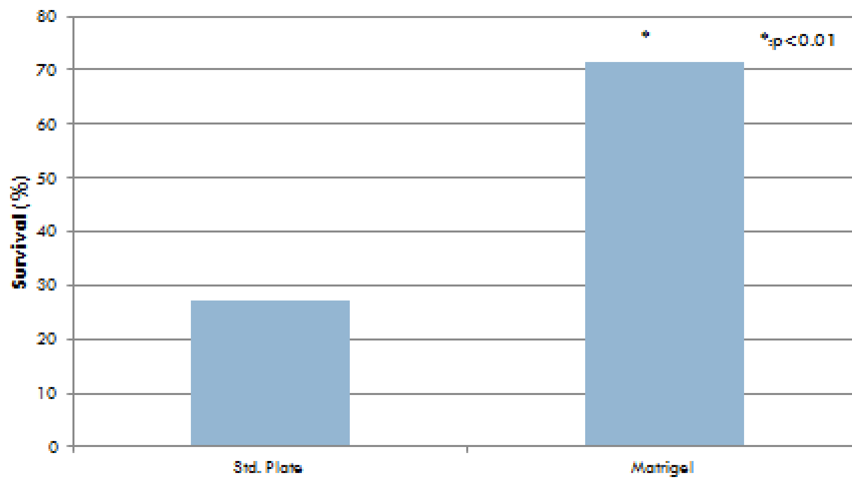


Table 4: Survival rates of preantral murine follicles cultured in standard 2-dimensional plate vs. matrigel conditions.

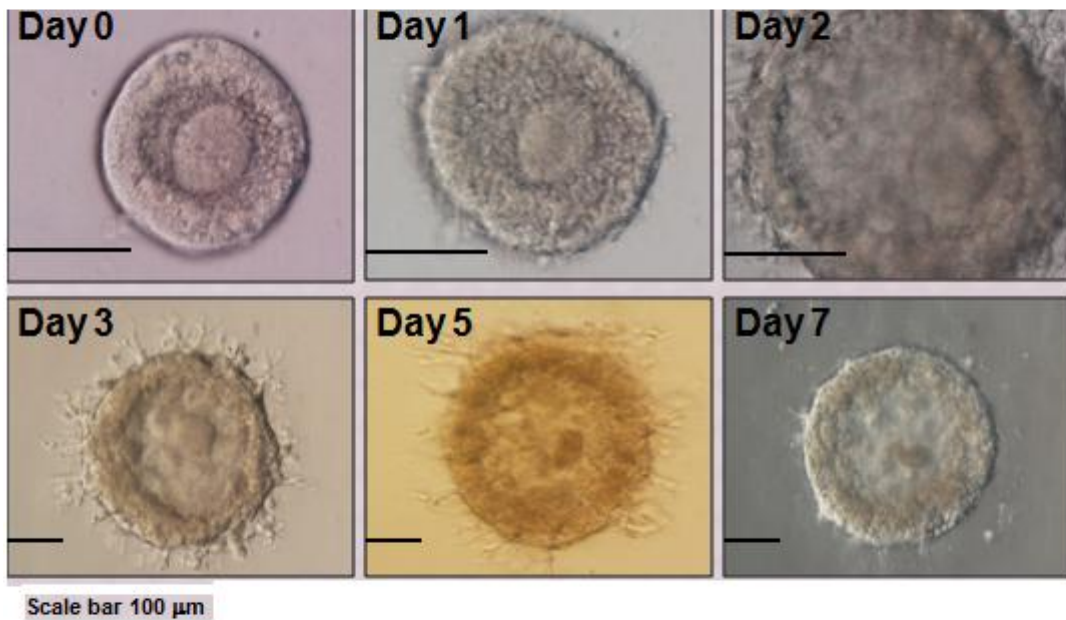


Figure 12: Preliminary experiences in human preantral follicle culture.

3.4 Future Perspectives

The matrigel model enables us to determine the effects of different drugs on preantral follicle growth in vitro.

Human primordial and primary follicle culture that will yield a healthy MII oocyte and live birth in future may revolutionize infertility treatment.

BIBLIOGRAPHY

[1] S.V. Nicosia, M. Matus-Ridley, A.T. Meadows, Gonadal Effects of Cancer Therapy in Girls. Cancer, (1985), 2364-2372.

[2] W.H. Wallace, S.M. Shalet, J.H. Hendry, P.H. Morris-Jones, H.R. Gattamaneni, Ovarian Failure Fallowing Abdominal Irradiation in Childhood: The Radiosensitivity of the Human Oocyte. Br J Radiol, 62 (1989), 995-998.

[3] J. Byrne, T.R. Fears, M.H. Gail, D. Pee, R.R. Conelly, D.F. Austin, G.F. Holmes, F.F. Holmes, H.B. Latourette, J.W. Meigs, Early Menopause in Long-Term Survivors of Cancer During Adolescence. Am J Obstet Gynocol, 166 (1992), 788-793.

[4] J.G. McVie, Cancer Treatment: The Last 25 Years. Cancer Treat Rev, 25 (1999), 323-331.

[5] C. Polage, Low-Temperature Storage of Mammalian Spermatozoa. Royal Society of London, Series B, Biological Sciences, 147 (1957), 498-508.

[6] R.G. Gosden, M. Bownes, Cellular and Molecular Aspects of Oocyte Development. Hum Reprod, 8 (1995), 23-53.

[7] M.J. Faddy, R.G. Gosden, A. Gougeon, S.J. Richardson, J.F. Nelson, Accelerated Disappearance of Ovarian Follicles in Mid-Life: Implications for Forecasting Manopause. Hum Reprod, 7 (1992), 1342-1346.

[8] M.J. Faddy, Follicle Dynamics During Ovarian Ageing. Mol Cell Endocrinol, 163 (2000), 43-48.

[9] L. Neilson, A. Andalibi, D. Kang, C. Coutifaris, J.F. Strauss, D.P. Green, Molecular Phenotype of the Human Oocyte by PCR-SAGE, Genomics, 63 (2000), 13-24.

[10] R.G. Gosden, J. Mullan, H.M. Picton, S.L. Tan, Current Perspective on Primordial Follicle Cryopreservation and Culture for Reproductive Medicine, Hum Reprod, 8 (2002), 105-110.

[11] A.T. Dobson, R. Raja, M.J. Abeyta, T. Taylor, S. Shen, C. Haqq, R.A. Pera, The Unique Transcriptome Through Day 3 of Human Preimplantation Development, Hum Mol Genet, 13 (2004), 1461-1470.

[12] P.M. Wassarman, W.J. Josefowicz, Oocyte Development in the Mouse: An Ultrastructural Comparison of Oocytes Isolated at Various Stages of Growth and Meiotic Competence, J Morphol, 156 (1978), 209-235.

[13] H. Picton, D. Briggs, R. Gosden, The Molecular Basis of Oocyte Growth and Development, Mol Cell Endocrinol, 145 (1998), 27-37.

[14] Y. Obata, T. Kono, Maternal Primary Imprinting is Established at a Specific Time for Each Gene Throughout Oocyte Growth, J Biol Chem, 227 (2002), 5285-5289.

[15] J. Cohen, M. Alikani, J. Trowbridge, Z. Rosenwaks, Implantation Enhancement by Selective Assisted Hetching Using Zona Drilling of Human Embryos with Poor Prognosis, Hum Reprod, 7 (1992), 685-691.

[16] P.M. Wassarman, Zona Pellucida Glycoproteins, Annu Rev Biochem, 57 (1988), 415-442.

[17] P.M. Wassarman, C. Liu, E.S. Litscher, Constructing the Mammalian Egg Zona Pellucida: Some New Pieces of an Old Puzzle, J Cell Sci, 109 (1996), 2001-2004.

[18] J. Fulka, T. Jung, R.M. Moor, The Fall of Biological Maturation Promoting Factor (MPF) and Histone H1 Kinase Activity During Anaphase and Telophase in Mouse Oocytes, Mol Reprod Dev, 32 (1992), 378-382.

[19] B. Wu, G. Ignotz, W.B. Currie, X. Yang, Dynamics of Maturation-Promoting Factor and its Constituent Proteins During in Vitro Maturation of Bovine Oocytes, Biol Reprod, 56 (1997), 253-259.

[20] J. Johnson, J. Canning, T. Kaneko, J. K. Pru, J. L. Tilly, Germline Stem Cells and Follicular Renewal in the Postnatal Mammalian Ovary, Nature, 428 (2004), 145-150.

[21] J. Johnson, J. Bagley, M. Skaznik-Wikiel, H. J. Lee, G. B. Adams, Y. Niikura, K. S. Tschudy, J. C. Tilly, M. L Cortes, R. Forkert, Oocyte Generation in Adult Mammalian Ovaries by Putative Germ Cells in Bone Marrow and Peripheral Blood, Cell 122 (2005), 303-315.

[22] E. A. McGee, A. J. Hsueh, Initial and Cyclic Recruitment of Ovarian Follicles, Endocr Rev 21 (2000), 200-214.

[23] L. P. Reynolds, S. D. Killilea, D. A. Redmer, Angiogenesis in the Female Reproductive System, Faseb J 6 (1992), 886-892.

[24] J. E. Fortune, R. A. Cushman, C. M. Wahl, S. Kito, The Primordial to Primary Follicle Transition, Mol Cell Endocrinol 163 (2000), 53-60.

[25] K. Oktay, D. Briggs, R. G. Gosden, Ontogeny of Follicle-Stimulating Hormone Receptor Gene Expression in Isolated Human Ovarian Follicles, J Clin Endocrinol Metab 82 (1997), 3748-3751.

[26] K. Aittomaki, R. Herva, U. H. Stenman, K. Juntunen, P. Ylostalo, O. Hovatta, A. de la Chapelle, Clinical Features of Primary Ovarian Failure Caused by a Point Mutation in the Follicle-Stimulating Hormone Receptor Gene, J Clin Endocrinol Metab 81 (1996), 3722-3726.

[27] A.J. Zeleznik, A.R. Midgley, L.E. Reichert, Granulosa Cell Maturation in the Rat: Increased Binding of Human Chorionic Gonadotropin Following Treatment with Follicle-Stimulating Hormone in Vivo, Endocrinology, 95 (1974), 818-825.

[28] G.F. Erickson, S. Shimasaki, The Physiology of Folliculogenesis: The Role of Novel Growth Factors, Fertil Steril, 76 (2001), 943-949.

[29] E.Y. Adashi, C.E. Resnick, A.J. D'ercole, M.E. Svoboda, J.J. Van Wyk, Insulin-like Growth Factors as Intraovarian Regulators of Granulosa Cell Growth and Function, Endocr Rev, 6 (1985), 400-420.

[30] J. Zhou, T.R. Kumar, M.M. Matzuk, C. Bondy, Insulin-like Growth Factor I Regulates Gonadotropin Responsiveness in the Murine Ovary, Mol Endocrinol, 11 (1997), 1924-1933.

[31] X.N. Wang, G.S. Greenwald, Synergistic Effects of Steroids with FSH on Folliculogenesis, steroidogenesis and FSH- and hCG-Receptors in Hypophysectomized mice, J Reprod Fertil, 99 (1993), 403-413.

[32] A. Kaipia, A.J. Hsueh, Regulation of Ovarian Follicle Atresia, Annu Rev Physiol, 59 (1997), 349-363.

[33] P.C. Leung, D.T. Armstrong, Estrogen Treatment of Immature Rats Inhibits Ovarian Androgen Production in Vitro, Endocrinology, 104 (1979), 1411-1417.

[34] J.F. Couse, K.S. Korach, Reproductive Phenotypes in the Estrogen Receptor-Alpha Knockout Mouse, Ann Endocrinol, 60 (1999), 143-148.

[35] J.H. Krege, J.B. Hodgin, J.F. Couse, E. Enmark, M. Warner, J.F. Mahler, M. Sar, K.S. Korach, J.A. Gustafsson, O. Smithies, Generation and Reproductive Phenotypes of Mice Lacking Estrogen Receptor Beta, Proc Natl Acad Sci, 95 (1998), 15677-15682.

[36] K.L. Britt, J.K. Findley, Estrogen Actions in the Ovary Revisited, J Endocrinol, 175 (2002), 269-276.

[37] S. Zalanyi, Progesterone and Ovulation, Eur J Obstet Gynecol Reprod Biol, 98 (2001), 152-159.

[38] J. Iwamasa, S. Shibata, N. Tanaka, K. Matsuura, H. Okamura, The Relationship Between Ovarian Progesterone and Proteolytic Enzyme Activity During Ovulation in the Gonadotropin-treated Immature Rat, Biol Reprod, 46 (1992), 309-313.

[39] J.P. Lydon, F.J. DeMayo, C.R. Funk, S.K. Mani, A.R. Hughes, C.A. Montgomery, G. Shyamala, O.M. Conneely, B.W. O'Malley, Mice Lacking Progesterone Receptor Exhibit Pleiotropic Reproductive Anormalities, Genes Dev, 9 (1995), 2266-2278.

[40] B. Mulac-Jericevic, R.A. Mullinax, F.J. DeMayo, J.P. Lydon, O.M. Conneely, Subgroup of Reproductive Functions of Progesterone Mediated by Progesterone-Receptor B Isoform, Science, 289 (2000), 1751-1754.

[41] A.E. Drummond, The Role of Steroids in Follicular Growth, Reprod Biol Endocrinol, 4 (2006), 16.

[42] A.K. Goff, P.C. Leung, D.T. Armstrong, Stimulatory Action of Follicle-Stimulating Hormone and Androgens on the Responsiveness of Rat Granulosa Cells to Gonadotropins in Vitro, 104 (1979), 1124-1129.

[43] J.E. Fortune, S.E. Vincent, Progesterone Inhibits the Induction of Aromatase Activity in Rat Granulosa Cells in Vitro, Biol Reprod, 28 (1983), 1078-1089.

[44] J.R. Schreiber, K. Nakamura, G.F. Erickson, Progestins Inhibit FSH-Stimulated Granulosa Estrogen Production at a Post-cAMP Site, Mol Cell Endocrinol, 21 (1981), 161-170.

[45] P. Kezele, M. K. Skinner, Regulation of Ovarian Primordial Follicle Assembly and Development by Estrogen and Progesterone: Endocrine Model of Follicle Assembly, Endocrinology 144 (2003), 3329-3337.

[46] A. M. Luciano, J. J. Peluso, Effect of in Vivo Gonadotropin treatment on the Ability of Progesterone, Estrogen, and Cyclic Adenosine 5'- Monophosphate to Inhibit Insulin-dependent Granulosa Cell Mitosis in Vitro, Biol Reprod 53 (1995), 664-669.

[47] J. J. Peluso, A. M. Luciano, A. Pappalardo, B. A. White, Cellular and Molecular Mechanisms that Mediate Insulin-dependent Rat Granulosa Cell Mitosis, Biol Reprod 52 (1995), 124-130.

[48] K. Horie, K. Takatura, H. Fujiwara, H Suginami, S. Liao, T. Mori, Immunohistochemical Localization of Androgen Receptor in the Human Ovary Throughout the Mentrual Cycle in Relation to Oestrogen and Progesterone Receptor Expression, Hum Reprod 7 (1992), 184-190.

[49] A. E Drummond, The Role of Steroids in Follicular Growth, Reprod Biol Endocrinol 4 (2006), 16.

[50] M. Tetsuka, P. F. Whitelaw, W. J. Bremner, M. R Millar, C. D Smyth, S. G. Hillier, Developmental Regulation of Androgen Receptor in Rat Ovary, J Endocrinol 145 (1995), 535-545.

[51] T.E. Hickey, D.L. Marrocco, F. Amato, L.J. Ritter, R.J. Norman, R.B. Gilchrist, D.T. Armstrong, Androgens Augment the Mitogenic Effects of Oocyte-Secreted Factors and Growth Differentiation Factor 9 on Porcine Granulosa Cells, Biol Reprod, 73 (2005), 825-832.

[52] B.A. Conway, V.B. Mahesh, T.M. Mills, Effect of Dihydrotestosterone on the Growth and Function of Ovarian Follicles in Intact Immature Female Rats Primed with PMSG, J Reprod Fertil, 90 (1990), 267-277.

[53] H. Billing, I Furuta, A.J. Hsueh, Estrogens Inhibit and Androgens Enhance Ovarian Granulosa Cell Apoptosis, Endocrinology, 133 (1993), 2204-2212.

[54] G.F. Erickson, S. Shimasaki, The Role of the Oocyte in Folliculogenesis, Endocrinol Metab, 11 (2000), 193-198.

[55] H. Chang, C.W. Brown, M.M. Matzuk, Genetic Analysis of the Mammalian Transforming Growth Factor-beta Superfamily, Endoc Rev, 23 (2002), 787-823.

[56] J. Massague, D. Wotton, Transcriptional Control by the TGF-β/Smad Signaling System, Embo J, 19 (2000), 1745-1754.

[57] A.C. McPherron, S.J. Lee, GDF-3 and GDF-9: Two New Members of the Transforming Growth Factor-beta Superfamily Containing a Novel Pattern of Cysteines, J Biol Chem, 268 (1993), 3444-3449.

[58] S.L. Fitzpatrick, D.M. Sindoni, P.J. Shughrue, M.V. Lane, I.J. Merchenthaler, D.E. Frail, Expression of Growth Differentiation Factor-9 Messenger Ribonucleic Acid in Ovarian and Nonovarian Rodent and Human Tissues, Endocrinology, 139 (1998), 2571-2578.

[59] S. Mazerbourg, A.J. Hsueh, Growth Differentiation Factor-9 Signaling in the Ovary, Mol Cell Endocrinol, 202 (2003), 31-36.

[60] J.G. Hreinsson, J.E. Scott, C. Rasmussen, M.L. Swahn, A.J. Hsueh, O. Hovatta, Growth Differentiation Factor-9 Promotes the Growth, Development and Survival of Human Ovarian Follicles in Organ Culture, J Clin Endocrinol Metab, 87 (2002), 316-321.

[61] E. Kovanci, J. Rohozinski, J.L. Simpson, M.J. Heard, C.E. Bishop, S.A. Carson, Growth Differentiating Factor-9 Mutations May be Associated with Premature Ovarian Failure, Fertil Steril, 87 (2007), 143-146.

[62] J. Aaltonen, M.P. Laitinen, K. Vuojolainen, R. Jaatinen, N. Horelli-Kuitunen, L. Seppa, H. Louhio, T. Tuuri, J. Sjoberg, R. Butzow, O. Hovata, L. Dale, O. Ritvos, Human Growth Differentiation Factor 9 (GDF-9) and its Novel Homolog GDF-9B are Expressed in Oocytes During Early Folliculogenesis, J Clin Endocrinol Metab, 84 (1999), 2744-2750.

[63] E. Di Pasquale, P. Beck-Peccoz, L. Persani, Hypergonadotropic Ovarian Failure Associated with an Inherited Mutation of Human Bone Morphogenetic Protein-15 (BMP-15) Gene, Am J Hum Genet, 75 (2004), 106-111.

[64] M. Hayashi, E.A. McGee, G. Min, C. Klein, U.M. Rose, M. van Duin, A.J. Hsueh, Recombinant Growth Differentiation Factor-9 (GDF-9) Enhances Growth and Differentiation of Cultured Early Ovarian Follicles. Endocrinology, 140 (1999), 1236-1244.

[65] J.G. Hreinsson, J.E. Scott, C. Rasmussen, M.L. Swahn, A.J. Hsueh, O. Hovatta, Growth Differentiation Factor-9 Promotes the Growth, Development, and Survival of Human Ovarian Follicles in Organ Culture, J Clin Endocrinol Metab, 87 (2002), 316-321.

[66] K.P. McNatty, S.M. Galloway, T. Wilson, P. Smith, N.L. Hudson, A. O'Connell, A.H. Bibby, D.A. Heath, G.H. Davis, J.P. Hanrahan, Physiological Effects of Major Genes Affecting Ovulation Rate in Sheep, Genet Sel Evol, 37 (2005), 25-38.

[67] K.P. McNatty, J.L. Juengel, K.L. Reader, S. Lun, S. Myllymaa, S.B. Lawrence, A. Western, M.F. Meerasahib, D.G. Mottershead, N.P. Groome, Bone Morphogenetic Protein 15 and Growth Differentiation Factor-9 Co-Operate to Regulate Granulosa Cell Function, Reproduction, 129 (2005), 473-480.

[68] E. E. Nilsson, M. K. Skinner, Growth and Differentiation Factor-9 Stimulates Progression of Early Primary but Not Primordial Rat Ovarian Follicle Development, Biol Reprod, 67 (2002), 1018-1024.

[69] F. Otsuka, Z. Yao, T. Lee, S. Yamamoto, G.F. Erickson, S. Shimasaki, Bone Morphogenetic Protein-15. Identification of Target Cells and Biological Functions, J Biol Chem, 275 (2000), 39523-39528.

[70] U.A. Vitt, M. Hayashi, C. Kle,n, A.J. Hsueh, Growth Differentiation Factor-9 Stimulates Proliferation but Suppresses the Follicle-Stimulating Hormone-Induced Differentiation of Cultured Granulosa Cells From Small Antral and Preovulatory Rat Follicles, Biol Reprod, 62 (2000), 370-377.

[71] U.A. Vitt, E.A. McGee, M. Hayashi, A.J. Hsueh, In vivo Treatment with GDF-9 Stimulates Primordial and Primary Follicle Progression and Theca Cell Marker CYP17 in Ovaries of İmmature Rats, Endocrinology, 141 (2000), 3814-3820.

[72] N. Yamamoto, L.K. Christenson, J.M. McAllister, J.F. Strauss, Growth Differentiation Factor-9 Inhibits 3’5’-adenosine monophosphate-stimulated Steroidogenesis in Human Granulosa and Theca Cells, J Clin Endocrinol Metab, 87 (2002), 2849-2856.

[73] T.S. Husseim, J.G. Thompson, R.B. Gilchrist, Oocyte-Secreted Factors Enhance Oocyte Developmental Competence, Dev Biol, 296 (2006), 514-521.

[74] C.X. Yeo, R. B. Gilchrist, J.G. Thompson, M. Lane, Exogenous Growth Differentiation Factor-9 in Oocyte Maturation Media Enhances Subsequent Embryo Development and Fetal Viability in Mice, Hum Reprod, 23 (2008), 67-73.

[75] N.P. Groome, P.J. Illingworth, M. O’Brien, R.Pai, F.E. Rodger, J.P. Mather, A.S. McNeilly, Measurement of Dimetric Inhibin B Throughout the Human Menstrual Cycle, J Clin Endocrinol Metab, 81 (1996), 1401-1405.

[76] C.K. Welt, D.J. McNicholl, A.E. Taylor, J.E. Hall, Female Reproductive Aging is Marked by Decreased Secretion of Dimeric Inhibin, J Clin Endocrinol Metab, 84 (1999), 105-111.

[77] D.M. deKretser, M.P. Hedger, .L. Loveland, D.J. Phillips, Inhibins, Activins and Follistatin in Reproduction, Hum Reprod, 8 (2002), 529-541.

[78] Y. Sidis, T. Fujiwara, L. Leykin, K. Isaacson, T. Toth, A.L. Schneyer, Characterization of Inhibin/Activin Subunit, Activin Receptor, and Follistatin Messenger Ribonucleic Acid in Human and Mouse Oocytes; Evidence For Activin’s Paracrine Signaling From Granulosa Cells to Oocytes, Biol Reprod, 59 (1998), 807-812.

[79] J. Rabinovici, S.J. Spencer, R.B. Jaffe, Recombinant Human Activin-A Promotes Proliferation of Human Luteinized Preovulatory Granulosa Cells in vitro, J Clin Endocrinol Metab, 71 (1990), 1396-1398.

[80] A.L. Durlinger, P. Kramer, B. Karels, F.H. de Jong, J.T. Uilenbroek, J.A. Grootegoed, A.P. Themmen, Control of Primordial Follicle Recruitment by anti-Mullerian Hormone in the Mouse Ovary, Endocrinology, 140 (1999), 5789-5796.

[81] N. Josso, I Lamarre, J.Y. Picard, P. Berta, N. Davies, N. Morichon, M. Peschanski, R. Jeny, Anti-Mullerian Hormone in Early Human Development, Early Hum Dev, 33 (1993), 91-99.

[82] W.M. BAarends, J.T. Uilenbrowk, P. Kramer, J. W. Hoogerbrugge, E.C. van Leeuwen, A.P. Themmen, J.A. Grootegoend, Anti-Mullerian Hormone and Anti-Mullerian Hormone Type II Receptor Messenger Ribonucleic Acid Expression in Rat Ovaries During Postnatal Development, The Estrous Cycle, and Gonadotropin-Induced Follicle Growth, Endocrinology, 136 (1995), 4951-4962.

[83] A.L. Durlinger, M.J. Gruijters, P. Kramer, B. Karels, H.A. Ingram, M. W. Nachtigal, J.T. Uilenbroek, J.A. Grootegoed, A.P. Themmen, Anti-Mullerian Hormone Inhibits Initiation of Primordial Follicle Growth in the Mouse Ovary, Endocrinology, 143 (2001), 1076-1084.

[84] C. Weenen, J.S. Laven, A.R.Von Bergh, M. Cranfield, N.P. Groome, J.A. Visser, P. Kramer, B.C. Frauser, A.P. Themmen, Anti-Mullerian Hormone Expression Pattern in the Human Ovary: Potential Implications for Initial and Cyclic Follicle Recruitment, Mol Hum Reprod, 10 (2004), 77-83.

[85] F. Izadyar, J. Zhao, H.T. Van Tol, B. Colenbrander, M.M. Bevers, Messenger RNA Expression and Protein Localization of Growth Hormone in Bovine Ovarian Tissue and in Cumulus Oocyte Complexed (COCs) During in Vitro Maturation, Mol Reprod Dev, 53 (1999), 398-406.

[86] P.E. Lobie, W. Breipohl, J.G. Aragon, M.J. Waters, Cellular Localization of the Growth Homone Receptor/Binding protein in the Male and Female Reproductive Systems, Endocrinology, 126 (1990), 2214-2221.

[87] T.S. Tiong, A.C. Herington. Tissue Distribution, Characterization, and Regulation of Messenger Ribonucleic Acid for Growth Hormone Receptor and Serum Binding Protein in the Rat, Endocrinology, 129 (1991), 1628-1634.

[88] F. Izadyar, H.T. Van Tol, B. Colenbrander, M.M. BEvers, Stimulatory Effect of Growth Hormone on in vitro Maturation of Bovine Oocytes is Exerted Through Cumulus Cells and Not Mediated by IGF-I, Mol Reprod Dev, 47 (1997), 175-180.

[89] F.I. Sharara, L.K. Neiman, Identification and Cellular Localization of growth Hormone Receptor Gene Expression in the Human Ovary, J Clin Endocrinol, 79 (1994), 670-672.

[90] A. Bachelot, P. Monget, P. Imbert-Bollore, K. Coshigano, J.J. Kopchick, P.A. Kelly, N. Binart, Growth Homone is Required for Ovarian Follicular Growth, Endocrinology, 143 (2002), 4104-4112.

[91] X. Liu, K. Andoh, H. Yokota, J. Kobayashi Y. Abe, K. Yamada, H. Mizunuma, Y. Ibuki, Effects of Growth Hormone, Activin and Follistatin on the Development of Preantral Follicle From Immature Female Mice, Endocrinology, 139 (1998), 2342-2347.

[92] J. Kobayashi, H. Mizzunuma, N. Kikuchi, X. Liu, K. Andoh, Y. Abe, H. Yokota, K. Yamada, Y. Ibuki, H. Hagiwara, Morphological Assessments of the Effect of Growth Hormone on Preantral Follicles from 11-day-old Mice in an in vitro Culture System, Biochem Biophys Res Commun, 268 (2000), 36-41.

[93] N.A. Danilovich, A. Bartke, T.A. Winters, Ovarian Follicle Apoptosis in Bovine Growth Hormone Transgenic Mice, Biol Reprod, 62 (2000), 103-107.

[94] A.V. Sirotkin, A.V. Makarevich, GH Regulates Secretory Activity and Apoptosis in Cultured Bovine Granulosa Cells Through the Activation of the cAMP/Protein Kinase A System, J Endocrinol, 163 (1999), 317-327.

[95] S.Y. Chun, A.J. Hsueh, Paracrine MAchanisms of Ovarian Follicle Apoptosis, J Reprod Immunol, 39 (1998), 63-75.

[96] K.M. Eisenhauer, S.Y. Chun, H. Billig, A.J. Hsueh, Growth Hormone Supperssion of Apoptosis in Preovulatory Rat Follicles and Pertial Neutralization by Insulin-Like Growth Factor Binding Protein, Biol Reprod, 53 (1995), 13-20.

[97] J.P. Advis, S.S. White, S.R. Ojeda, Activation of Growth Hormone Short Loop Negative Feedback Delays Puberty in Femlae Rat, Endocrinology, 108 (1981), 1343-1352.

[98] H. Quesnel, Localization of Binding Sites for IGF-I, Insulin and GH in the Sow Ovary, J Endocrinol, 163 (1999), 363-372.

[99] C.C. Chase, C.J. Kirby, A.C. Hammond, T.A. Olson, M.C. Lucy, Patterns of Ovarian Growth and Development in Cattle with a Growth Hormone Receptor Deficiency. J Anim Sci, 76 (1998), 212-219.

[100] P. Ovensen, H.J. Ingerslev, H. Orskov, T. Ledet, Effect of Growth Hormone on Steroidogenesis, Insulin-Like Growth Factor-I (IGF-I) and IGF-Binding Protein-I Production and DNA Sysnthesis in Cultured Human Luteinized Granulosa Cells, J Endocrinol, 140 (1994), 313-319.

[101] H.D. Mason, H. Martikainen, R.W. Beard, V. Anyaku, S. Franks, Direct Gonadortopic Effect of Growth Hormone on Oestradial Production by Human Granulosa Cells in Vitro, J Endocrinol, 126 (1990), R1-4.

[102] N. Doldi, M. Bassan, V. Bronzi, A. Ferrari, Effects of Growth Hormone and Growth Hormone-Releasing Hormone on Steroid Synthesis in Cultured Human Luteinizing Granulosa Cells, Gynecol Endocrinol, 10 (1996), 101-108.

[103] B. Carlsson, C. Bergh, J. Bentham, J.H. Olsson, M.R. Norman, H. Billig, P. Roos, T. Hillensjo, Expression of Functional Growth Hormone Receptors in Human Granulosa Cells, Hum Reprod, 7 (1992), 1205-1209.

[104] A. Lanzone, N. Di Simone, R. Castellani, A.M. Fulghesu, A. Caruso, S. Mancuso, Human Growth Hormone Enhances Progesterone Production by Human Luteal Cells in

Vitro: Evidence of a Synergistic Effect with Human Chorionic Gonadotropin, Fertil Steril 57 (1992), 92-96.

[105] S.J. Galli, K.M. Zsebo, E.N. Geissler, The Kit Ligand, Stem Cell Factor, Adv Immunol, 55 (1994), 1-96.

[106] K.J. Hutt, E.A. McLaughlin, M.K. Holland, Kit Ligand and c-Kit Diverse Roles During Mammalian Oogenesis and Folliculogenesis, Mol Hum Reprod, 12 (2006), 61-69.

[107] P.E. Hoyer, A.G. Byskov, K. Mollgard, Stem Cell Factor and c-Kit in Human Primordial Germ Cells and Fetal Ovaries, Mol Cell Endocrinol, 234 (2005), 1-10.

[108] L.L. Robinson, T.L. Gaskell, P.T. Saunders, R.A. Anderson, Germ Cell Specific Expression of c-Kit in the Human Fetal Gonad. Mol Hum Reprod, 7 (2001), 845-852.

[109] H. Stoop, F. Honecker, M. Cools, R. de Krijger, C. Bokemeyer, L.H. Looijenga, Differentiation and Development of Human Female Germ Cells During Prenatal Gonadogenesis: An Immunohistochemical study, Hum Reprod, 20 (2005), 1466-1476.

[110] I.B. Carlsson, M.P. Laitinen, J.E. Scott, H. Louhio, L. Velentzis, T. Tuuri, J. Aaltonen, O. Ritvos, R.M. Winston, O. Hovatta, Kit Ligand and c-Kit are Expressed During Early Human Ovarian Follicular Development and Their Interaction is Required for The Survival of Follicles in Long-Term Culture. Reproduction, 131 (2006) 641-649.

[111] K. Manova, K. Nocka, P. Besmer, R.F. Bachvarova, Gonadal Expression of c-Kit Encoded at the W Locus of the Mouse, Development, 110 (1990), 1057-1069.

[112] K. Horie, K. Takakura, S. Taii, K. Narimoto, Y. Noda, S: Nishikawa, H. Nakayama, J. Fujita, T. Mori, The Expression of c-Kit, Protein During Oogenesis and Early Embryonic Development, Biol Reprod, 45 (1991), 547-552.

[113] E. KEshet, S.D. Lyman, D.E. Williams, D. M. Anderson, N.A. Jenkins, N.G. Copeland, L.F. Parada, Embryonic RNA Expression Patterns of the c-Kit Receptor and Its Cognate Ligand Suggest Multiple Functional Roles in Mouse Development, EMBO J, 10 (1991), 2425-2435.

[114] B. Motro, D. van der Kooy, J. Rossant, A. Reith, A. Bernstein, Contiguous Patterns of c-Kit and Steel Expression: Analysis of Mutations at the W and SI Loci, Development, 113 (1991), 1207-1221.

[115] B. Motro, A. Bernstein, Dynamic Changes in Ovarian c-Kit and Steel Expression During The Estrous Reproductive Cycle, Dev Dyn, 197 (1993), 69-79.

[116] K. Manova, E.J. Huang, M. Angels, V. De Leon, S. Sanchez, S.M. Pronovost, P. Besmer, R.F. Bachvarova, The Expression Pattern of the c-Kit Ligand in Gonads of Mice Supports a Role for the c-Kit Receptor in Oocyte Growth and in Proliferation of Spermatogonia, Dev Biol, 157 (1993), 85-99.

[117] E.E. Nilsson, M.K. Skinner, Kit Ligand and Basic Fibroblast Growth Factor Interactions in the Induction of Ovarian Primordial to Primary Follicle Transition, Mol Cell Endocrinol, 214 (2004), 19-25.

[118] J.A. Parrott, M.K. Skinner, Kit-Ligand/Stem Cell Factor Induces Primordial Follicle Development and Inıtiates Folliculogenesis, Endocrinology, 140 (1999), 4262-4271.

[119] J.E. Smitz, R.G. Cortvrindt. The Earliest Stages of Folliculogenesis in vitro. Reproduction, 123 (2002), 185-202.

[120] J.E. Torrance, E. Telfer, R.G. Gosden. Quantitative Study of the Development of Isolated Mouse Preantral Follicles in Collagen Gel Culture. J Reprod Fertil, 87 (1989), 367-374.

[121] J.E. Gomes, S.C. Correia, A. Gouveia-Oliveria, A.J. Cidadao, C.E. Plancha. Three-Dimensional Environments Preserve Extracellular Matrix Compartments of Ovarian Follicles and Increase FSH-Dependent Growth, Mol Reprod Dev. 54 (1999), 163-172.

[122] P.K. Kreeger, N.N. Fernandes, T.K. Woodruff, L.D. Shea, Regulation of Mouse Follicle Development by Follicle Stimulating Hormone in a Tree-Dimensional in vitro Culture System is Dependent on Follicle Stage and Dose. Biol Reprod. 73 (2005), 942-950.

[123] P.L. Nayudu, S.M. Osborn, Factors Influencing the Rate of Preantral and Antral Growth of Mouse Ovarian Follicles in Vitro, J Reprod Fertil, 95 (1992), 349.

[124] A. Fehrenbach, N. Nüsse, P.L. Nayudu, Patterns of Growth, Estradiol and Progesterone Released by in vitro Cultured Mouse Ovarian Follicles Indicate Consecutive Selective Events During Follicle Maturation, J Reprod Fertil, 113 (1998), 287.

[125] U.A. Vitt, P.L. Nayudu, U.M. Rose, J. Mulders, H.J. Kloosterboer, Isoforms of Human Recombinant Follicle Stimulating Hormone: Comparison of Effects on Murine Follicle Development in vitro, Biol Reprod, 59 (1998), 854.

[126] K. Oktay, G. Karlıkaya, G.K. Ojakian, M. Oktay, Interaction of Extracellular Matrix and Activin-A in the Initiation of Follicle Growth in the Mouse Ovary, Biol Reprod, 63 (2000), 457-461.

[127] E. McGee, N. Spears, S. Minami, Preantral Ovarian Follicles in Serum-Free Culture: Supression of Apoptosis After Activation of the Cyclic Guanosine 3’5’-monophosphate Pathway and Stimulation of Growth and Differentiation by Follicle-Stimulating Hormone, Endocrinology, 138 (1997), 2417-2424.

[128] X. Liu, K. Andoh, Y. Abe, A Comparative Study on Transforming Growth Factor –β and Activin A for Preantral Follicles From Adult Immature and Diethylstilbestol-primed Immature Mice. Endocrinology, 140 (1999), 2480-2485.

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