

Development of an *in vitro* microenvironment for maturing oocytes

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PAR

Jong Eun IHM

acceptée sur proposition du jury:

Prof. N. Stergiopulos, président du jury

Prof. J. A. Hubbell, directeur de thèse

Prof. Y. Barrandon, rapporteur

Prof. J. M. Lim, rapporteur

Prof. N. Mermod, rapporteur



ÉCOLE POLYTECHNIQUE
FÉDÉRALE DE LAUSANNE

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Abstract

The development of *in vitro* culture systems, comparable to the *in vivo* microenvironment in terms of effect on the oocyte growth and development could provide a valuable experimental tool for studying the mechanisms governing folliculogenesis. This tool might serve as well for practical clinical, agricultural, zoological, or biotechnological applications.

This thesis reports on the importance of the microenvironment for the ovarian folliculogenesis process. The complexity of such a microenvironment was approached with a strategy based on functionalized PEG-hydrogels. The PEG matrix not only served as a scaffold, but it was also used as a “reservoir” of immobilized cues. Tethered integrin-binding peptides in combination with other signaling factors aimed at better understanding the interactions of the oocyte and its surrounding granulosa cells that, most probably, determine the efficiency of the actual and the future *in vitro* mature oocyte production strategies. This work led also to investigating the lymph angiogenesis and the lymphatic transport in the context of oocyte maturation and their impact on mice fertility.

In a first step, the mechanical properties of PEG-hydrogel were optimized for culturing secondary follicles. This report shows that the growth of the follicles was highly dependent on the mechanical properties of the surrounding environment. The optimal elastic modulus was found to be close to 900 Pa. In a second step, the effect of Arg-Gly-Asp (RGD) peptide, the minimal integrin-binding sequence, was studied. RGD presence did not influence the follicle growth rate but it significantly improved the quality of the produced oocytes. These

findings demonstrated that approaching, biochemically and mechanically, the complexity of the ovarian extracellular matrix could be a winning strategy.

The effect of key soluble factors was also investigated in order 1- to confirm their compatibility with the established 3-D culture system and 2- to further improve qualitatively and quantitatively the produced mature oocytes. Various combinations of gonadotropins such as the follicle-stimulating hormone (FSH) or the luteinizing hormone (LH) were tested. Interestingly, the effects of the gonadotropins in the 3-D PEG system were close to their known effects *in vivo*. Here the aberrant effects of these hormones in the used 2-D systems appeared clearly.

c-Kit ligand (KL) is suspected to be one of the most important factors for the activation of primordial follicles and thus for controlling the exit from the resting pool. Previously, studying the effects of the two forms of KL (soluble and membrane-attached) had to cope with the lack of biologically-relevant immobilization strategies. For overcoming this problem, KL constructs were designed to include a substrate sequence for Factor XIIIa (NSEQVSPL or NSEQVSPLRCG). Thus the produced recombinant KL proteins could be enzymatically crosslinked to the PEG matrix. The different constructs of KL, including a wild type extracellular domain, were successfully cloned and produced from mammalian HEK-293 cells. The identity and the activity of the produced proteins were confirmed. Ovarian tissues from four days-old mice were cultured in PEG-hydrogels functionalized with KL. The results showed that the primordial follicles grew and were activated in the PEG-hydrogels where KL was immobilized but not when the soluble form of KL was preset in the medium. This experiment showed that the membrane-attached and the soluble form of KL play a different role in the rodent folliculogenesis.

In parallel it was observed that blocking vascular endothelial growth factor receptor-3 (VEGFR-3) signaling had a critical but still unsuspected role in reproduction. This study demonstrates that variation in lymphangiogenesis is a regular, non-pathological event during folliculogenesis in the ovary; blocking lymphangiogenesis, might have an effect on hormone transport and thus on pregnancy. The reported results demonstrates that the blockade of lymphangiogenesis decreases the progesterone and estradioal levels during pregnancy and *in fine* results in failed fetal development.

In conclusion, this study demonstrates the efficiency and the flexibility of a novel 3-D culture system. Circumventing problems inherent to the “on-plastic” standard culture, such as the loss of the granulosa-oocyte interactions, allowed the emergence of a culture system tailored for investigating fundamental folliculogenesis-related questions. Furthermore, the reported culture system might serve as a platform for developing clinical and biotechnology applications.

Keywords: folliculogenesis, follicle, primordial follicle, PEG-hydrogel, FactorXIIIa substrate, extracellular matrix, c-Kit, c-Kit ligand, lymphangiogenesis, VEGFR-3

Résumé

Le développement d'un système de culture *in vitro* dans lequel le développement des oocytes s'approcherait de ce qui est observable *in vivo* constituerait un outil expérimental décisif pour la compréhension des mécanismes gouvernants à la folliculogénèse. Il est aussi envisageable qu'un tel outil puisse, tout aussi bien, être employé au profit d'applications cliniques, zoologiques ou encore biotechnologiques. Le présent document apporte une nouvelle preuve de l'importance du microenvironnement ovarien pour la folliculogénèse. La complexité d'un tel microenvironnement a été abordée en utilisant un système de culture en hydrogels à base de PEG. Ce substrat artificiel n'a pas seulement servi en temps que structure de maintiens tridimensionnelle mais aussi en temps que « réservoir à signaux ». En effet l'immobilisation de peptides contenant la séquence minimale d'attachement aux intégrines (RGD), ainsi que d'autres signaux, a permis d'étudier les interactions oocytes-granulosa qui déterminent certainement la quantité et la qualité des oocytes matures produits par les systèmes de culture *in vitro* actuels et à venir. Par ailleurs, ce travail a aussi abordé les transports lymphatiques et la lymphangiogénèse ainsi que leurs influences sur la fertilité.

Dans un premier temps, les propriétés mécaniques des hydrogels employés ont été optimisées pour la culture de follicules secondaires. Ce travail établit que le développement des follicules secondaires dépend largement des propriétés mécaniques du milieu environnant et que l'élasticité optimale semble être située autour de 900 Pa. De même, ce travail démontre que la présence de RGD à des concentrations variables ne semble pas influencer sur la croissance des follicules secondaires mais que par ailleurs, la présence de ce peptide améliore significativement la qualité des oocytes produits. Ces résultats

démontrent que mimer biochimiquement et mécaniquement le microenvironnement ovarien peut constituer une stratégie efficace.

Dan un deuxième temps, l'effet de certain éléments solubles a aussi été étudié 1- pour confirmer leur activité dans le système hydrogel et 2- pour améliorer qualitativement et quantitativement les oocytes matures produits. Diverses combinaisons de gonadotrophines (FSH et LH) ont été testées. Les effets observés de ces hormones dans le système hydrogel sont relativement proches des effets observés *in vivo*. Ceci souligne ici l'effet aberrant que ces hormones peuvent avoir dans les systèmes de culture bidimensionnels.

Les ligands des récepteurs c-Kit (KL) semblent jouer un rôle important dans l'activation des follicules primordiaux et donc de leur sortie de la phase de dormance. Les études précédentes voulant étudier l'effet des deux formes de KL (soluble ou encrée à la membrane) on dues composer avec l'absence de stratégies d'immobilisation biocompatible. Pour résoudre ce problème, des protéines fusionnées à une séquence-substrat du facteur XIIIa (coagulation) ont été produites. Ces dernières on put ainsi être immobilisées enzymatiquement sur hydrogel. Les différentes protéines recombinantes on put être produites par des cellules HEK-293 (mammifère). La conformité de leurs séquences ainsi que leur bioactivité ont été confirmés. Des tissu ovarien récoltés sur des animaux âgés de quatre jours on été mis en culture dans des hydrogels où différentes formes et concentrations de KL furent préalablement immobilisées. Il a ainsi put être démontré que les oocytes primordiaux ont été activés où seule la forme ancrée de KL était présente.

Parallèlement, il a été observé que le blocage de la voie de signalisation passant par VEGFR-3 a un effet drastique mais encore non documenté sur la reproduction. Ce travail démontre que la régulation de la lymphangiogenèse dans l'ovaire est un phénomène naturel non pathologique et qui est concomitant à la folliculogenèse. Le blocage de la formation de

vaisseaux lymphatiques peut avoir un effet sur le transport des hormones et donc sur la fertilité. Les résultats présentés démontrent qu'en absence de lymphangiogenèse les taux de progestérone et d'œstradiol décroissent pendant la grossesse aboutissant *in fine* à l'avortement.

Les résultats de ce travail démontrent l'efficacité et la flexibilité du système de culture en hydrogel établis. Le contournement des problèmes inhérents à la culture standard en boîte de Petri, tel que la perte du contact oocyte-granulosa, a permis l'émergence d'un système de culture à même de servir à l'étude des mécanismes sous-tendant la folliculogenèse. De plus ce nouveau système de culture pourrait servir d'ébauche pour le développement de plateformes à usage clinique ou biotechnologique.

Mots-clés: folliculogenèse, follicule, follicule primordial, PEG-hydrogel, Factor XIIIa, matrice extracellulaire, c-Kit, ligand c-Kit, Lymphangiogenèse, VEGFR-3

Abbreviations

cAMP Cyclic adenosin monophosphate

BMP-15 Bone morphogenetic protein-15

ECM Extra cellular matrix

EGF Epidermal growth factor human

FBS Fetal bovine serum

FSH Follicle-stimulating hormone

GDF-9 Growth differentiation factor 9

GV Germinal vesicle

GVBD Germinal vesicle breaks down

HBSS Hank's buffered salt solution

HCG Human chorionic gonadatropin

ITS The mixuture of 5 µg/mL insulin, 5 µg/mL transferrin, and 5 ng/mL selenite

KL c-Kit ligand

LECs Lymphatic endothelial cells

LH Luteinizing hormone

LHR Luteinizing hormone receptor

LIF Leukemia inhibitory factor

L15 Leibovitz medium

α -MEM α -Minimal essential medium

MII Methaphase II

MGF Mast cell growth factor

MMPs Matrix metalloproteinases

PEG Polyethylene glycol

PEG-VS PEG vinyl sulfone

P/S 100 IU/mL penicillin and 100 μ g/mL streptomycin

RGD Arg-Gly-Asp

SAMS Swiss academy of medical science

SCF Stem cells factor

SCNAT Swiss academy of sciences

SF Steel factor

VEGF Vascular endothelial growth factor

VEGFR-3 Vascular endothelial growth factor receptor-3

VS Vinyl sulfone

Chapter I:

Overview and general introduction

1.1. Overview

Primordial follicles are the earliest form of ovarian follicle and consist of primary oocytes surrounded by a single layer of flattened pre-granulosa cells. There are thousands of primordial follicles present in the neonatal mammal's ovaries [1, 2]. Primordial follicles can be considered as the storage form of the ovarian follicles and constitute a potentially valuable source of oocytes that could be used for clinical, agricultural, and zoological purposes [3-5]. To be fully functional, an oocyte has to accomplish the following: A nuclear maturation, including completion of the meiotic division. A cytoplasmic maturation must be accomplished for the accumulation of maternal factor gene products essential for supporting fertilization and early embryogenesis. An epigenetic maturation must be accomplished, including genomic imprinting [5]. Thus, it is a considerable challenge to achieve full oocyte development *in vitro*.

During last decade, several culture systems have been developed allowing the growth of rodent oocytes *in vitro* from secondary follicle [6-9]. Oocytes recovered from these culture systems can be fertilized, and live young can be obtained [8]. However, approaches for recovery and reproducible *in vitro* development of primordial follicles to maturity, in which oocytes acquire complete competence to undergo maturation, fertilization, and embryonic development, are still needed. More recently, two groups reported the possibility to produce a matured mouse oocyte from a primordial follicle [3-5]. These achievements used a complex and poorly characterized two-step procedure involving organ culture *in vitro* [5] or *in vivo* grafting [3, 4] for primordial follicles to begin growing, followed by isolation of the growing follicles.

The medical needs of methods for follicular maturation are substantial. Ovaries are especially sensitive to cytotoxic treatments, alkylating agents and ionizing radiation, generally resulting in loss of both endocrine and reproductive function [10]. While not all

chemotherapy causes women patients to become infertile, the effects vary with drugs, doses, and individual sensitivities; many regimens do result in loss of fertility. Fertilized eggs survive freezing and thawing better than unfertilized ones, consequently the best chances of success come from *in vitro* fertilization (IVF). Generally, a woman undergoing this procedure before having chemotherapy is treated with hormones in order to superovulate. The oocytes are subsequently fertilized with sperm from the partner or a donor and conserved frozen. In many cases, eggs cannot be harvested because the patient might be too young, the type of cancer might exclude a hormonal treatment causing superovulation, or the patient might need to start treatment immediately because of the aggressiveness of the cancer [5, 10]. For patients under those circumstances, cryopreservation of ovarian tissue might be a possible alternative. Transplantation of ovarian tissue is a possibility, but rises the question of re-transferring the cancer cells to the patient [5, 10]. A single report appeared in 2004 [10] describing a cancer survivor (32-year-old) who became infertile after chemotherapy and gave birth after ovarian tissue removal, freezing and reimplantation. This report mentioned that the loss of primordial follicles in cryopreserved ovarian tissue after transplantation is estimated to be more than 50 % due to hypoxia. Moreover, it is difficult to say that only primordial follicle of the grafted tissue contributed for giving a birth, because the biopsy samples indicated not only survival of primordial follicles in grafted tissue but also follicular maturation [10].

The development of an efficient cell culture system could also have a profound effect on the treatment options of infertile couples by avoiding the need for intensive drug treatment and repeated egg collection, as several hundred follicles at early stages could potentially be recovered in a small ovarian biopsy, even from young ovaries. Additionally, *in vitro* growth of follicles together with *in vitro* maturation of fully-grown oocytes could be used to generate sufficient oocytes for the needs of patients and provide spare oocytes for donation

or research purposes. The combined technologies of ovarian tissue cryopreservation as well as growth and maturation of oocytes *in vitro* could also help young cancer patients to preserve their fertility through the storage of their own germ cells before aggressive chemo- or radio-therapy [11].

In the last decade, the studies of *in vitro* maturation of oocytes aimed at better understanding the underlying mechanisms of oocyte growth and maturation [1, 2, 8, 9, 12]. One of the difficulties in the *in vitro* cultivation of these very early follicles is the prevention of pre-granulosa cells from migrating away from the oocyte and in so doing breaking the metabolic link between the two cellular compartments causing apoptosis of the oocytes [6]. In nature, when follicles leave the resting pool they undergo a primordial-to-primary follicle transition and the surrounding squamous pre-granulosa cells become cubical granulosa cells and begin to proliferate. Several growth factors have been identified to act locally within the ovary and to be involved in the regulation of primordial-to-primary follicle transition such as bone morphogenetic protein-15 (BMP-15, produced by the oocyte and stimulating the granulosa cells) [13, 14], leukemia inhibitory factor (LIF) [15], and c-Kit ligand (KL, produced by the granulosa cells and stimulating the oocyte) [14-17]. KL was shown to participate in a feed-back loop, where it enhances the expression of KL by BMP-15, which as consequence stimulates granulosa cells proliferation [14]. As follicles continue to develop through the primary, secondary and pre-antral stages they gain successive layers of granulosa cells, the oocyte increases in size and theca cells surround the follicle.

1.2. Objectives

Our overall objective is to develop a culture system that is more robust and that takes over some of the figures of the regulatory cycle described above. Our aim is to explore the effect of a three-dimensional and functionalized hydrogel network (made of polyethylene glycol) on the *in vitro* follicle development.

The study will aim at answering the following questions:

- 1- Can we mimic the biomechanical effects of the ovarian tissues and mature oocytes in an artificial 3-D environment?
- 2- Can we elucidate the gonadotropin-mediated (FSH and LH) regulation during follicle growth in an artificial microenvironment (hydrogel)?
- 3- Is KL sufficient or are more signals necessary for activating primordial follicles (in the PEG-hydrogel culture system)?
- 4- Can we bring more insights into the lymphatic transports-fertility relation?

The current *in vitro* culture systems are now using pre-antral follicles for initiating cultures [3-5]. These isolated secondary follicles are matured in a drop culture system. This 2D culture system obliges the follicle to attach on the bottom of the culture dish. This attachment induces the remodeling of the original follicle structure [9]. This remodeling leads to confuse hormonal or growth factor effects due to the loss of cell-cell interactions. The microenvironment system that we intend to develop will help to better understand maturation process by mimicking the ovarian extra cellular matrix (ECM). The used crosslinked hydrogel network is composed of hydrophilic polymer chains and as the extra cellular matrix (ECM) it is sensitive to proteolytic degradation [18, 19]. The molecular structure and composition of the hydrogel allow the transport of macromolecules, which may enhance the three dimensional organization of the oocyte. In addition to that, it is

known that KL plays an important role in the “critical” transition from the primordial to the primary stage of the follicle [8, 14, 15, 17, 20]. In this regard, the production and binding to the hydrogel of recombinant KL will allow the recapitulation of a microenvironment combining the advantages of 3-D culture and KL signaling.

1.3. Folliculogenesis

The follicle structure is important to sustain oocyte growth and maturation as a morphological and functional unit in the ovary of mammalian species. The follicle is basically composed of an oocyte in the center surrounded by granulosa cells and/or theca cells.

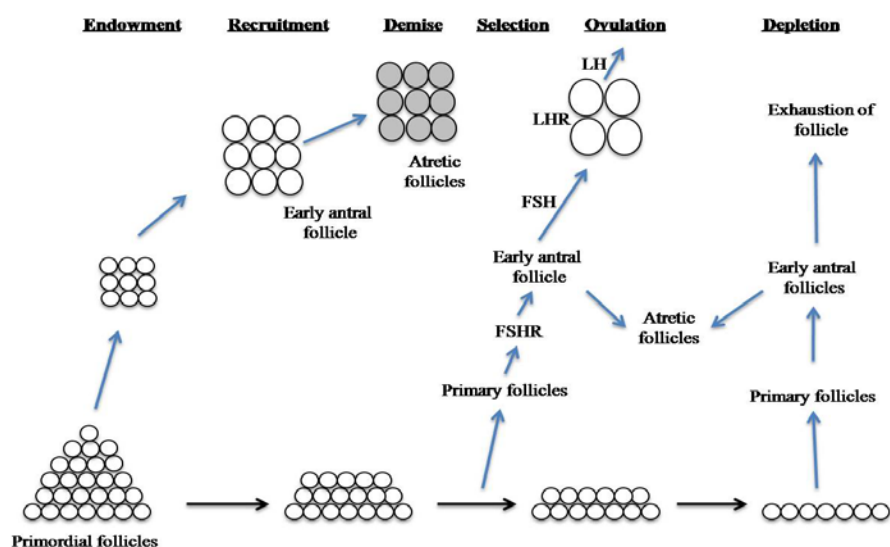


Fig. 1.1. Small fractions of the original stockpile of primordial follicles are recruited throughout the reproductive life, whereas most of primordial follicles remain arrested at the initial stage of development. This figure is adapted from Kapia A. & Hsueh AJW, *Annu. Rev. Physiol.* **59**: 349-363, 1997

The initial primordial follicles populate the ovarian cortex and serve as a pool of oocytes during the entire reproductive life span [21]. They are quiescent or non-growing or resting until their growth is actively initiated. It was observed that the flattened granulosa cells of

primordial follicles can occasionally enter the cell cycle [22]. The follicles that entered the growth phase do not always continue to grow and the majority will undergo apoptotic demise (Fig.1.1) [23].

Folliculogenesis is the growth process that brings the primordial follicle to a fully-grown matured secondary oocyte that is able to be fertilized (Fig.1.2). A large number of primordial follicles exist in the ovary, but only few follicles enter the developing phase (Table. 1.1). The beginning of the growth phase is marked by changes in morphology of the granulosa cells that become cuboidal. Follicle with more than two layers of granulosa cells, complete zona pellucida and theca cells are called preantral or secondary follicle. When these follicles reach 200 μm in the size, fluids are accumulated between the granulosa cells layers and form a small single antral cavity. This stage is called antral follicle and is characterized by the expression of luteinizing hormone (LH)-receptors in the theca cells. The last stage is the preovulatory or graffian follicle which has a big antrum and that is ready to ovulate a fully mature oocyte [2, 21]. The different steps of folliculogenesis are regulated by various endocrine and paracrine factors such as cytokines, growth factors and neuropeptidergic substances.

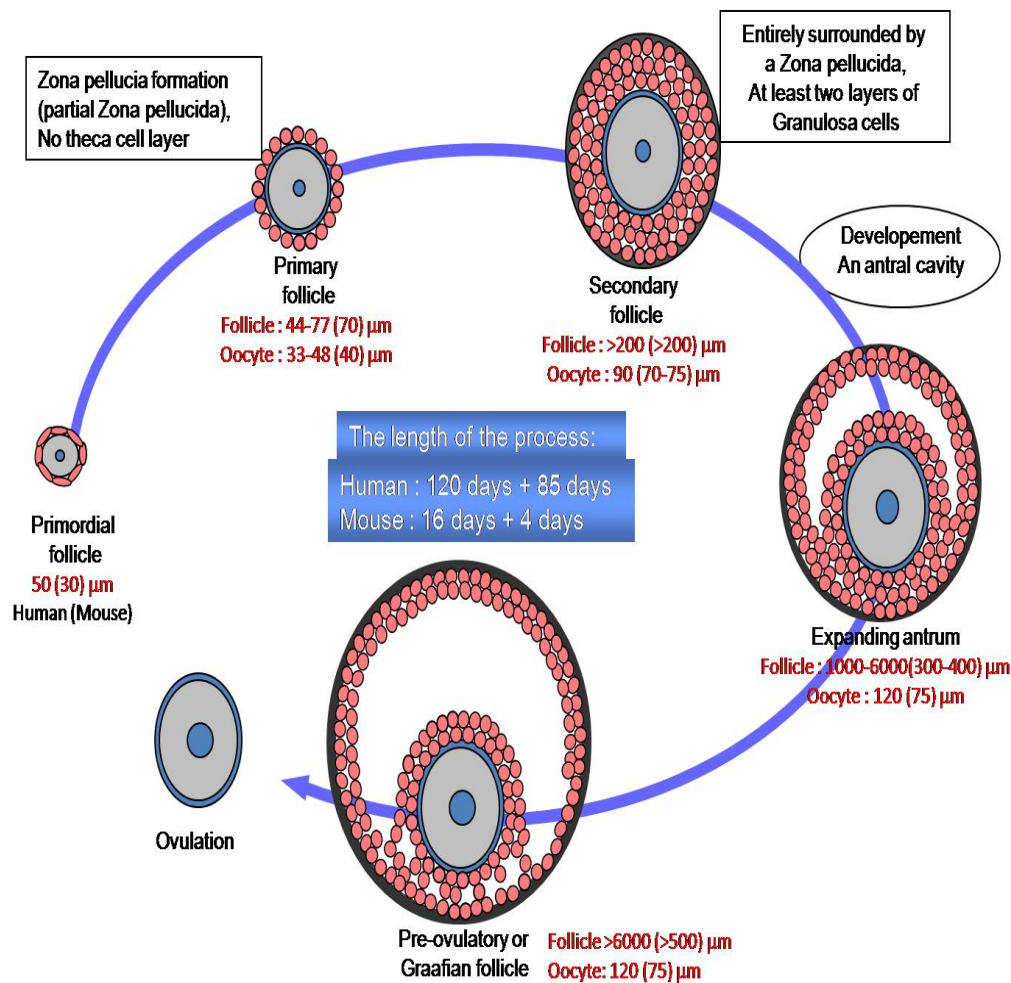


Fig. 1.2. Schematic folliculogenesis of mouse and human. The growth process necessary to bring the enclosed small immature primary oocyte to a fully-grown mature secondary oocyte, ready to be fertilized, is dependent on the growth and development of the follicular structure. This process is called folliculogenesis.

Table 1.1. Number of ovarian follicles in mammals. The ovary contains a huge number of primordial follicles at the birth, however only few of them reach to developing follicle.

Species	No. of primordial follicle*	No. of developing follicles*
Mouse	4,270	676
Sheep	105,450	475
Cow	120,000	300
Pig	420,000	84,000
Human	302,000	12,090

* Mean number of pair of ovaries. Miyano, *Reprod. Dev.*, **51**: 169-176, 2005

1.4. Oocyte maturation

Oocyte maturation is essential for fertilization and support of the early embryo development. Maturation can be nuclear or cytoplasmic. Nuclear maturation is referred to as the reinitiation of the first meiotic division and the progression to metaphase II (MII) [24]. Fig. 1.3 represents the progression of the nuclear maturation. Germinal vesicle (GV) characterizes oocytes arrested at prophase I and having a nucleus visible in light microscopy. Once the meiosis is resumed, the nuclear envelope is dislocated; it is the germinal vesicle breaks down stage (GVBD). The dispersion of nuclear contents in the cytoplasm is followed by the chromatin condensation into discrete bivalents that align on the meiotic spindle at metaphase I (MI). The bivalents separation during anaphase and telophase I is followed by the metaphase II (MII). MII is recognized by the emission of polar bodies. The MII stage is arrested until fertilization happens [25]. Cytoplasmic maturation is concomitant to nuclear maturation. This maturation is referred to as the processes modifying the oocyte cytoplasm and it involves the production and the presence of specific factors, the relocation of cytoplasmic organelles and the post-transcriptional modification of mRNAs that have accumulated during oogenesis [24]. Both nuclear and cytoplasmic regulations are important to support the entire oocyte maturation process.

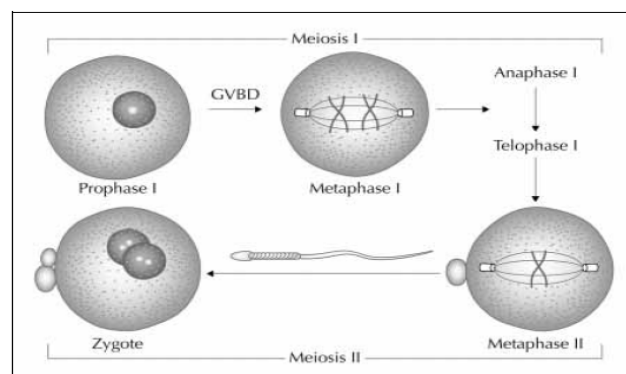


Fig. 1.3. Schematic diagram representing the progression of nuclear maturation. The figure is from Smith, G.D., *Current Women's Health Reports*, 1:143-151, 2001

1.5. *In vitro* follicle culture systems

During last few decades, *in vitro* follicle culture was intensively studied in different animals and Human. The mouse is the most studied animal and serves as a model. This is mainly due to their short life cycle. Within the first 2-3 weeks after birth, the mice oocytes grow from a diameter of 15-20 μm to a final size of 70-75 μm [26].

Generally the secondary follicles already in the growth phase are chosen for research. The secondary follicles can be retrieved from the two week-old mice by enzymatic digestion or by mechanical dissection. It has been demonstrated that the proteolytic digestion of the interstitial matrix destroys the extracellular matrix between the follicle and the surrounding cell layer [27, 28]. Thus, the mechanical isolation method is preferred for the follicle culture. In this method, the ovary is dissected with a thin gage syringe and the secondary follicles (100-130 μm size) are retrieved without destroying the extracellular matrix. The standard follicle culture system consists of single droplets culture. Single follicle are seeded in each droplet of medium and covered with mineral oil for preventing evaporation [29]. Although, this system gives satisfying results including birth of offspring [30-32], it is difficult to study the ovarian physiology in this *in vitro* system because of the induced remodeling and because of the loss of oocyte-granulosa contact. This situation produced discrepancies in the literature addressing the *in vitro* follicular physiology. There were efforts to preserve the spherical organization of the follicle by culturing them on collagen treated/hydrophobic membranes or in individual V-shape 96-well plates. Here, attachment was avoided by daily transferring the follicle to a new well [33-35]. Recently, Pangas, et al. [36] demonstrated that the three dimensional (3-D) culture of follicle using alginate bead allowed the follicles to grow and developed and to maintain its original structure [37]. However, this method was limited by the limited control over the follicle number per bead.

Daily microscopic observation and growth factor immobilization were also found challenging in this system.

1.6. Synthetic PEG-based ECM analogs

The reported synthetic hydrogel system is based on PEG. This system combines self-selective cross-linking chemistry and rapid gelation in aqueous solution at physiological pH and temperature [38, 39]. It is perfectly suitable for maturing oocytes in contrast with other synthetic hydrogels that need cross-linking by large doses of γ -radiation, ultraviolet light, or high temperatures that may lead to damage the contained material [38]. This system exploits novel synthetic schemes, based on Michael-type conjugate addition reaction, for the preparation of the tailored interactive network architecture based on PEG [18, 19, 38-40]. The PEG-hydrogel forms after the reaction of the end-functionalized PEG vinyl sulfone (PEG-SV) with bis-cystine peptides containing protease-sensitive sequences under physiological conditions [18, 39, 40]. Due to the used Michael-type addition, the reaction is based on thiol structure-reactivity relationship. This system allows to easily functionalizing the network with adhesion sites or with receptor or ligand signal site by their thiols.

1.7. c-Kit ligand

Mutation in the murine locus for c-kit ligand (KL) and its receptor c-Kit induce defects in gametogenesis, melanogenesis, or hematopoiesis [41]. KL is referred to as the mast cell growth factor (MGF), stem cells factor (SCF) or steel factor (SF). KL signaling is triggered upon recognition of the ligand and after activating the type III tyrosine kinase receptor: c-Kit (Fig.1.4) [42].

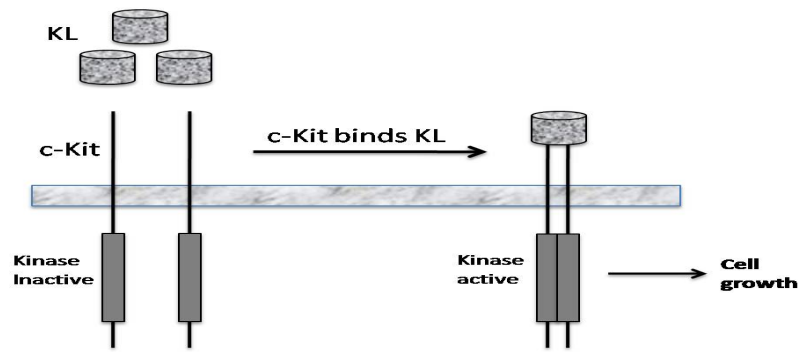


Fig. 1.4. Binding of KL on c-Kit and consecutive activation.

The ovarian expression pattern of KL and c-Kit mRNA and protein was established in primates and humans [43-45], sheep [46], and rodents [47, 48]. KL is expressed in the granulosa cells and in the ovarian epithelial cells while c-Kit is expressed in primordial germ cells, theca cells and oocytes [49]. The interaction of KL and c-Kit was reported to be important for primordial follicle activation, thus inducing the exit from the resting pool and the entrance in the growth phase [50, 51]. It was confirmed that KL could promote granulosa cell mitogenesis. However, another unknown signals from the oocyte are necessary to mediate the proliferation [14]. Growth differentiation factor 9 (GDF-9) [52] and bone morphogenetic factor 15 (BMP-15) [13] were suggested as candidates. KL was suggested to be involved in promoting the survival of both primordial [53] and secondary follicle [50]. Often contradictory results are published regarding the effect of KL on primordial follicle survival.

1.8. Lymphangiogenesis

Lymphangiogenesis is the growth of new lymphatic vessels. It is modulated by many of the lymphatic molecules. The molecules involved in developmental lymphangiogenesis are different from those considered in lymphangiogenesis in the adult associated or those associated with wound healing or tumor growth and metastasis [54]. The most widely

studied growth factors driving lymphangiogenesis are members of the vascular endothelial growth factor (VEGF) family. VEGF-C and -D were identified and characterized by investigating the lymphatics in tumor progression and by the search for potential lymphangiogenic therapies in lymphedema [55]. Growth factors are essential for the proliferation and migration of lymphatic endothelial cells (LECs) [56]. When the primary receptor, VEGFR-3 is blocked it inhibits lymphangiogenesis in healing wounds [56, 57], inflammation [58, 59], and prevents tumor metastasis [60].

1.9. Guiding thread

Along the four chapters of this thesis we tried to establish a new 3-D *in vitro* culture system for maturing oocytes. The developed system was based on PEG-hydrogel. The main idea was to approach and reconstruct a part of the complexity of the natural microenvironment in which maturation of the oocyte occurs: the ovarian tissue and its ECM. This microenvironment was assumed to have four major type of influence on folliculogenesis: I. biomechanical, II. soluble compounds and hormonal signalling, III. tethered signalling proteins and IV. lymphatic related. Each chapter of the thesis tried to go deeper in the understanding of these mechanisms.

In vivo, the first role of the ECM is purely mechanical. It determines the tissue rigidly and/or elastically. Moreover, ECM components such as fibronectin are not only playing a structural role; they can trigger biological responses such as cell attachment [61]. In this regard we tried, in the first chapter, to find the most relevant rheological properties of the culture system. The biochemical roles of ECM were investigated by studying the effects of the minimal peptide that mediates cell attachment (RGD).

The ECM can also be seen as a filter that interacts with different compounds in solution within the ovarian tissue. It was reported as playing a major role for the hormonal balance

in vivo. However when it comes to 2-D culture systems, an abundant and controversial literature exists. This controversy is often linked to the lack of ECM in these studies. This aspect of the ECM was investigated in chapter II. There we tried to investigate the complex relation hormones-ECM. There, the effects of two soluble factors (FSH & LH) on secondary follicles development and oocyte maturation were studied.

In the third chapter the efforts to reconstruct the ovarian tissue environment continued with investigating the effect a tethered signaling factor that is important for folliculogenesis: c-Kit ligand (KL). KL was a very good model system as its bioactivity (for folliculogenesis) is closely linked to its immobilization. There, recombinant KL with different immobilization strategies (compatible with our hydrogel system) was produced. Immobilization of rKL was achieved and preliminary experiments were processed with mice ovarian tissue.

Chapter four investigated lymphangiogenesis in the ovary as, ultimately, the reconstruction of an efficient artificial ovarian tissue needs to embed the features of a vascular system. Numerous studies dealt with angiogenesis and reproduction. However, little is known about lymphangiogenesis in the ovarian tissue. In this chapter we were the first to describe the abundant lymphatic vasculatures in the ovary and the importance of lymphangiogenesis during follicles growth. This work demonstrated that the success of future artificial ovarian tissues will largely depend on their ability to include complex transport mechanisms such as lymphangiogenesis.

Chapter II

Optimization of the biophysical properties of
PEG-hydrogels for murine ovarian follicle culture

2.1. Introduction

The extracellular matrix (ECM) has many different functions; 1-Mechanically, it gives the tissues rigidly and/or elastically. 2-Chemically, it contains many cues that modulate the cellular activities. These properties are essential to the basic cellular activities that are, differentiation, migration, proliferation, apoptosis, attachment, etc [62, 63]. ECM is also playing an important role in the regulation of the interstitial flow by generating osmotic forces, or by filtering materials from solutions [62]. From the ECM point of view, the mammalian ovary is a special organ because it undergoes cyclical ECM remodeling. This remodeling is caused by the follicular development, atresia, and the breakdown of the follicle wall at the time of ovulation, the luteal formation and subsequent regression [64].

For the ovulation of a matured oocyte to occur, primordial follicles as an initial stage must develop into large antral follicle (called graffian follicle), which is ready to ovulate. During this process, the size of the oocyte increases about 100-folds [26]. The ovarian ECM supports the follicle structure and generates osmotic forces for follicular fluid formation and filtering soluble materials through the follicular basal lamina, transfers the hormone and nutrients, and cyclic remodeling [63]. It has been reported that the remodeling of the ovarian ECM is related to matrix metalloproteinases (MMPs) activity [64-66] and to integrin-binding activity of adhesive factors, such as fibronectin, (or synthetic peptide containing Arg-Gly-Asp sequences). This remodeling has been shown to favor both proliferation and differentiation of granulosa cell [67].

Two-dimensional culture systems routinely used for follicle culture induce remodeling of the follicles structure as the granulosa cells are migrating away from oocyte. This remodeling is necessary for getting better oxygenation, nutrients, and access of hormone

support to the inner most follicle cells [68]. An abundant literature dealing with *in vitro* follicle culture has been produced. The effects of the most important growth factors and hormones on the development of ovarian follicles were described for *in vitro* systems. However, the underlying mechanisms by which the ovarian ECM controls of the follicular growth and development remains poorly understood.

So it is necessary to develop *in vitro* culture systems to mimic the *in vivo* follicle environment. The development of biomimetic follicle's microenvironments will be useful for understanding the ovarian ECM, identifying the mechanical mechanisms underlying ovarian disorders such as polycystic ovary syndrome, and preservation reproductive potential for infertility couple and cancer patients.

Mimicking the ovarian ECM requires several conditions; first, maintaining the three-dimensional structure of the follicles is essential. Second, the matrix should be cell responsive, as it has to be remodeled for allowing the follicles to grow. Third, the artificial ECM must be easily functionalized with signaling elements such as integrin binding proteins or growth factors. Finally, artificial ECM must allow single follicle culture and must be compatible with daily microscopical observation during follicle growth.

The 3D culture system was first introduced to follicle culture by using alginate gel [36]. In this system, the follicles grew and developed without losing the typical morphology of *in vivo* grown-follicles. However, this system was limited by the difficulty to control single cell culture and to observe the growth process because of the beads shape. Immobilizing signaling factors was also limited in the alginate bead culture system.

The goal of this study is to demonstrate the importance of the interactions between the ECM and the follicle in the maturation process of the oocyte. The used PEG based hydrogel system was chosen for several reasons:

1- It adds the 3rd dimension that is missing to the culture on plastic systems. 2- It has comparable rheological properties (elastic modulus) when compared to ovarian tissues. 3- It is cell-responsive, as it allows the cells to remodel their 3-D environment by MMP secretion 4- Key signaling factors can be efficiently tethered to activate specific pathways and thus mediate the desired biological function [40].

2.2. Materials and Methods

2.2.1. Animals

Female F1 hybrid (C57BL/6 x CBA/caj) mice bred and housed in temperature-, lighting-, and humidity-controlled room and given food and water *ad libidum*. All animal experimental procedures were approved by the veterinary authorities of the canton de Vaud in accordance with the Swiss Academy of Medical Science (SAMS) and the Swiss Academy of Sciences (SCNAT) guidelines.

2.2.2. Synthesis of PEG-VS and peptide precursors

PEG vinyl sulfone (PEG-VS) was synthesized by adapting a previously published protocol [18]. Briefly, 15 g of 4-arm PEG-OH (Mw = 20 kDa, Shearwater polymer, Huntsville, AL) was dried by azeotropic distillation in toluene (VWR, Nyon, Switzerland) for 4 hr using a Dean-Stark. The dried PEG-OH was dissolved in 500 mL of dichloromethane (Fisher Scientific, Wohlen, Switzerland) and sodium hydride (Sigma-Aldrich, Buchs, Switzerland) was added at 20-fold molar excess over OH-group of PEG. Then, divinyl sulfone (Fluka, Buchs, Switzerland) was added at a 50-fold molar excess over the OH groups. This reaction was carried out at room temperature under argon with constant stirring. After 3 days, the excess sodium hydride was neutralized by acetic acid (Fluka, Buchs, Switzerland). The mixture was filtered over a filter cell cake and concentrated by rotary evaporation. The concentrated polymer was precipitated in ice-cold diethylether (Brunschwig, Basel,

Switzerland) and filtered. This precipitation was repeated 3 times. Finally, the product was dried under vacuum at room temperature for three days and the yield was 82 %. ¹H NMR showed characteristic vinyl sulfone peaks at 6.1, 6.4, and 6.8 ppm. The degree of end-group conversion was found to be 95 %. The polymer was stored under argon at -20 °C until used. The procedure was reviewed in detailed by Kraehenbuehl et al. (2008) [69].

RGDSP peptide (Ac-GCGWGRGDSPG-NH₂) was synthesized by solid phase peptide synthesis using an automated peptide synthesizer (Chemspeed, Augst, Switzerland). NovaSyn TGR resin (Merck Biosciences, Laeufelfingen, Switzerland) with a standard Fmoc chemistry was used. Purification was performed by mass-directed reverse phase-C₁₈ HPLC using a Water Autopurification System. Separation and collection of the samples were performed upon UV with broad wavelength detection (210-400 nm) (Water PDA 996 UV photodiode array) and mass directed software (Waters Masslynx software). Peptide sequences were confirmed by MALDI-TOF in the proteomics core facility of EPFL.

The cross-linking peptide that contains a sequence sensitive to MMP cleavage (Ac-GCREGPQG↓IWGQERCG-NH₂) was obtained from Neosystem S.A. (Strasbourg, France).

2.2.3. Formation and characterization of the PEG-hydrogels

The PEG-hydrogels were formed by Michael-type addition of thiol-containing peptides onto VS-functionalized PEG. First, the PEG-VS necessary for forming hydrogels was functionalized with arginine-glycine-aspartic acid (RGD) peptides (also by Michael-type addition). Thirty-nine μL of 10 % PEG hydrogel (in TBS pH 8) solution were mixed with 6 μL of a 400 μM RGDSP peptide solution (in H₂O). The addition proceeded at room temperature for 30 min. Second, 15 μL of medium containing or not cells, was added into the RGD reacted with PEG-VS solution. Finally the gel polymerization was initiated by adding 1.24 mg of the cross-linker peptide dissolved in 24 μL of triethanolamine buffer

(TEOA, 0.3 M, pH8.0; Fluka, Buchs, Switzerland). Before curing, drops of 28 μL of hydrogel solution were placed between two hydrophobic glass slides separated by a 1 mm spacer and pretreated with SigmaCote (Sigma-Aldrich, Buchs, Switzerland). The slides were fixed with clamps and the whole system was put in an incubator (37°C, 5% CO_2) for 18 min to polymerize. For characterization, the prepared gels were incubated overnight at 37 °C in bidistilled water. The swelling of the hydrogels was investigated by weighing the gels after the overnight incubation in water. The swelling ratio Q was determined as the swollen gel mass divided by the gel's dry mass.

The swollen gels were also used to measure storage and loss moduli (G' and G'' respectively) by small-strain oscillatory shear rheometry using a Bohlin CVO 120 high-resolution rheometer (Marvern Instruments, Worcestershire, UK). Measurements were performed at 25 °C. Storage and loss moduli, as well as phase angle, were measured as a function of frequency (from 0.1 to 10 Hz) in a constant strain mode (0.05 Hz). The swollen hydrogel disks of 1-1.4 mm thickness were placed between the two plates of the rheometer. Compression of 70 to 75 % was applied to avoid slippage.

2. 2.4. Follicle isolation, seeding and culture

Ovaries were collected from two-week old F_1 female mice (C57BL/6 x CBA) after euthanasia by cervical dislocation. The collected ovaries were washed with HBSS (Hank's buffered salt solution, Gibco-Invitrogen, Switzerland) and placed in L15 (Leibovitz medium, Gibco-Invitrogen, Switzerland) supplemented with 10 % heat-inactivated FBS (fetal bovine serum, Hyclone), 100 IU/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin (P/S). The ovaries were mechanically dissected using insulin syringe. Only follicles with two layers of granulosa cells (100-120 μm) were collected using a mouth-operated micropipette

[70]. The follicles were washed and kept in L15 washing droplets covered by paraffin oil (Vitrolife, Sweden).

The follicle were seeded in the above described hydrogel system. Six μL hydrogel drops, containing a unique follicle were formed. After curing, each of these gels was placed in a well of a 96 wells-plate, 150 μL of culture medium was added.

The culture medium consisted of α -minimal essential medium (α -MEM with glutamax, Gibco) supplemented with 5 % FBS, ITS (5 $\mu\text{g}/\text{mL}$ insulin, 5 $\mu\text{g}/\text{mL}$ transferrin, 5 ng/mL selenite, Sigma, Buchs, Switzerland), 1 % of P/S, 100 mIU/mL of recombinant follicle stimulating hormone (rFSH, Organon, Switzerland). Next day, the medium was changed completely with fresh medium. Every second day, half of the medium was replaced with fresh one.

2.2.5. Oocyte maturation and quality assessment

The oocytes meiotic competences were assessed by induced maturation after 11 days of culture. The grown follicles were retrieved from gels by incubating the gels in a 5 mg/mL collagenase I solution (Sigma, Buchs, Switzerland). The follicles were moved to culture medium with 5 ng/mL of human epidermal growth factor (EGF, Roche, Basel, Switzerland) and 2.5 IU/mL of human chorionic gonadatropin (HCG, Organon, Switzerland) without rFSH and incubated at 37 °C for 18-20 hrs to induce the ovulation. After the induction of the ovulation, oocytes were removed from the cumulus oocyte complex or the follicle by using a mouth-operated micropipette. The collected oocytes were washed in M2 medium (Sigma, Buchs, Switzerland) droplets and their state was assessed under stereomicroscope and characterized as DG (degenerated), GV (intact germinal vesicle), or GVBD (germinal vesicle breakdown) based on the presence or the absence of a germinal vesicle and MII (metaphase II) due to the presence of polar body.

Parthenogenesis was induced by 10 mM strontiumchloride in Ca^{2+} free KOSM medium with 1 $\mu\text{g/mL}$ of cytochalasin D. After 4 hr, the oocytes were moved to M16 medium and two-cell embryos were observed one or two days later.

2.2.6. Statistic analysis

The statistical analysis of the data was performed with the program OriginPro 8.0 (Originlab corporation, MA, USA). Significances at $p < 0.001$, $p < 0.01$ and $p < 0.05$ (***, ** and * respectively) of the differences were assessed with a T test.

2.3. Results and discussion

2.3.1. PEG-hydrogel physical properties

A wide variability in term of mechanical properties can be measured across the different mammalian tissues. For example, the softest tissue: the brain has an elastic modulus (G') of 200-400 Pa [71]. G' of the bones (the hardest tissue) is more than 17 GPa [72]. The first step of this study was to investigate the effect of different stiffness on the follicle development. Thus, the mechanical properties and the swelling ratios of the PEG-hydrogel were modulated by controlling the crosslinks density. The hydrogel networks were formed with 4-arm-PEG-VS, (20 kDa) and bis-Cys crosslinking peptide with an MMP cleavage site. The effect on G' of four different stoichiometric ratios (r equals the molar ratio of SH and VS groups) were investigated (Fig. 2.1). A large variation in stiffness was obtained and a linear relatio between r and G' was observed. At the lowest stoichiometric ration the hydrogels had a mean G' of 291 ± 87.64 Pa and at the highest, G' was 2.277 ± 0.47 kPa. Swelling was also influenced by the stoichiometric ratios. The highest swelling ratios ($Q = 75.57 \pm 7.02$) was obtained for the lowest $r = 0.8$. A clear negative correlation between swelling and elastic modulus was observed. These measured mechanical and swelling

properties are in accordance with previous reports [18, 19] that characterized the PEG-Hydrogel systems.

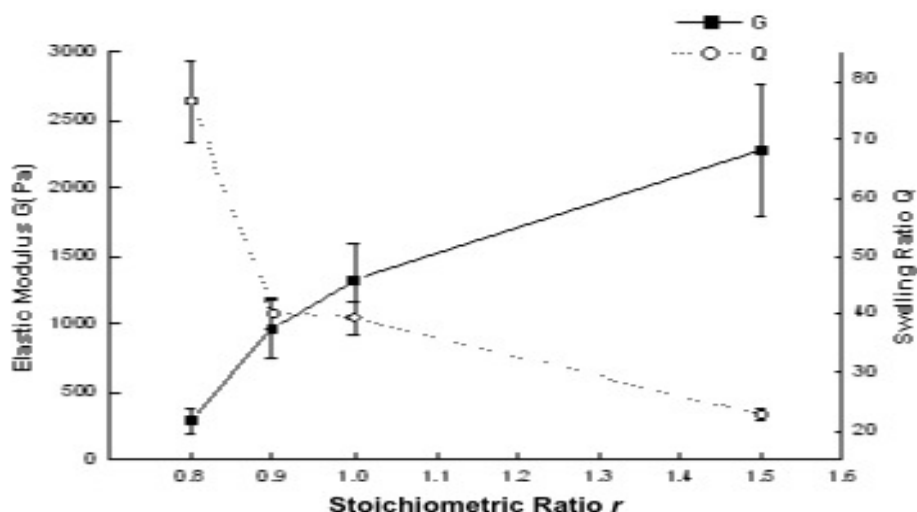


Fig. 2.1. Elastic modulus and swelling properties of the PEG-hydrogel as a function of the stoichiometric ratio. The density of the crosslink was modulated by four different stoichiometric ratios (SH/VS). Elastic modulus G' (left axis) and Swelling ratio Q (right axis) were measured. Mean \pm SD is shown. The swelling ratio and elastic modulus were negatively correlated.

2.3.2. Follicle growth in PEG-hydrogels and oocytes quality

Two-layered secondary follicles at 100-120 μm sizes were collected and seeded individually into each PEG-hydrogel functionalized with RGD peptides. Overall, more than 400 secondary follicles were cultured for 11 days in all the different PEG-hydrogel systems. Fig. 2.2 shows that the size of the follicles was greatly increased in the gel with less than 1 kPa of elastic modulus ($r = 0.8$ and 0.9) after 11 days of culture. In the stiffest gels ($r=1.5$) follicle growth was limited to 200 μm . Surprisingly, at identical swelling ratios but different G' ($r = 0.9$ or 1) the follicle size was significantly different. It appeared that above a barrier of approximately 1 kPa, the follicle growth was greatly inhibited. Fig. 2.3 shows the follicle morphology at the end of the culture period in gels with different stiffness. The

follicles in the soft gels ($r=0.8$ and 0.9) had similar morphologies to *in vivo* graffian follicles; A big antrum cavity was observable. In the stiffer gels ($r=1.0$ and 1.2), no antrum formation but only granulosa cell layers was detectable. These results are in agreement with previous studies that demonstrated that the stiffness of the matrix effects the antrum formation during follicle growth [37, 73]. *In vivo*, when the size of the follicle reaches $200\text{ }\mu\text{m}$, fluid accumulates between the granulosa cell layers and starts to form the antrum cavity [61]. The follicle size is then multiplied by 3 to 5 when compared to two-layered secondary follicle [68]. Our observations are fitting with this model where flexibility of the microenvironment is required for the follicle to undergo fluid accumulation and antrum formation. A limit of 1 kPa can be proposed as the maximal elastic modulus that allows antrum formation.

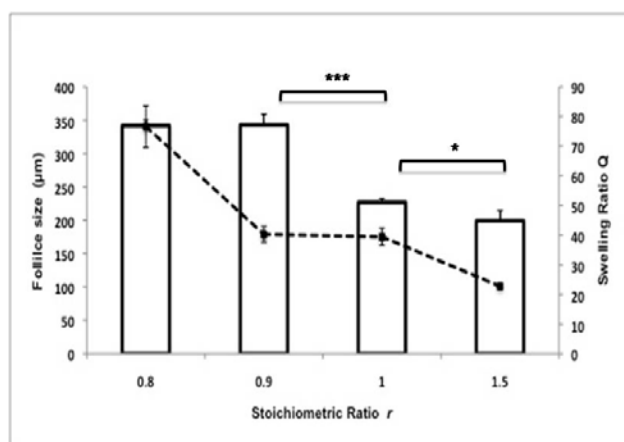


Fig. 2.2. The comparison of follicle size (bar) and swelling ratio (dash line) by changing stoichiometric ratio. The secondary follicles ($100\text{--}120\mu\text{m}$) were cultured in the PEG-hydrogel with different mechanical properties for 11 days. Average \pm SD is shown. (** $P < 0.001$ and $*P < 0.05$). The size of follicles were significantly different between $r=0.9$ and 1 with identical swelling ratios.

Whether the limited growth in stiff gels is due to smaller mesh size (thus to lower nutrient and signaling factor transport [72, 74] or to the mechanical constraints is still in question. However the different follicle sizes observed in gels with identical swelling but different G' ($r = 0.9$ and $r = 1$) tend to prove that the limitation is due to the mechanical constraints.

The quality of the oocytes was assessed by the ability to resume meiosis. The gels were digested by collagenase I for retrieving the follicles. After, the induction of the ovulation, cumulus-oocyte complexes were observed as it can be observed *in vivo*. The cumulus cells are made of granulosa cells adjacent to the oocyte following it after ovulation. This process is called cumulus expansion or mucification [68].

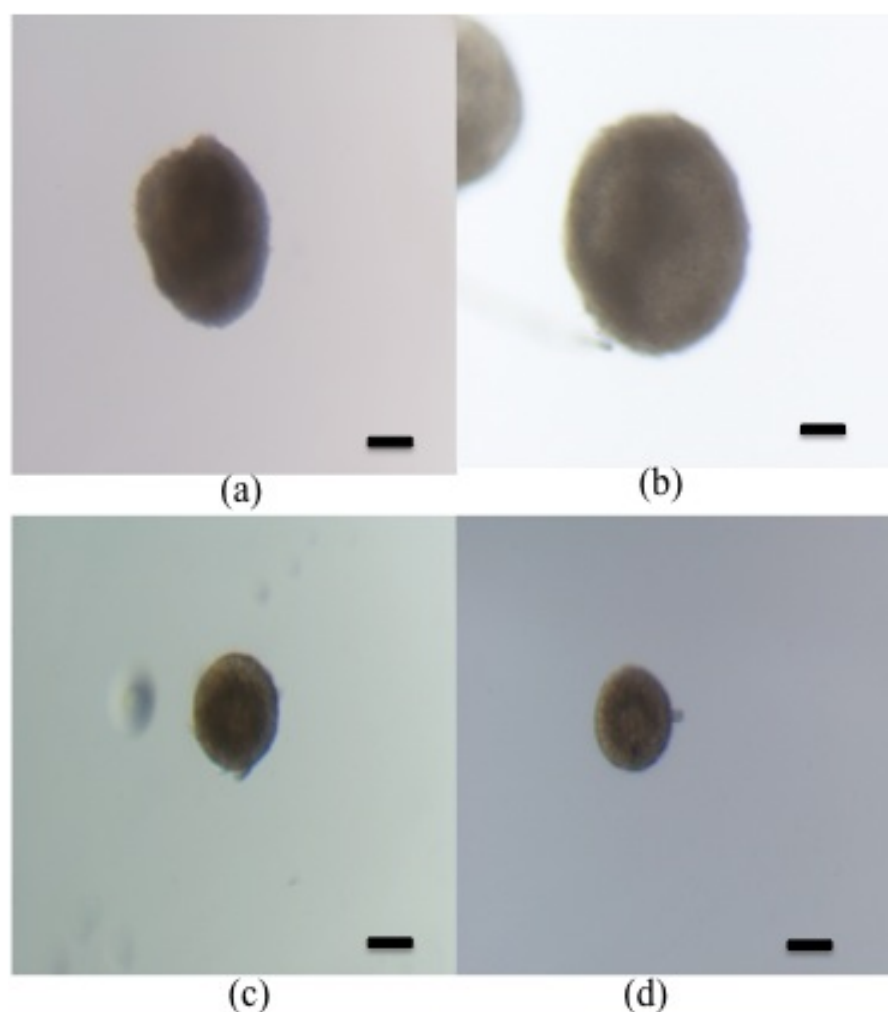


Fig. 2.3. Morphological differences between the follicles cultured in the PEG-hydrogel of different stoichiometric ratios after 11 days. The r ratios are (a) 0.8 , (b) 0.9, (c) 1.0, and (d) 1.5(Scale bar, 100 μm)

When follicles with diameters higher than 250 μm were ovulated, the collected oocytes had well expended cumulus cells (Data not shown). Their survival rate was assessed as well as their maturation quality. The retrieved oocytes from $r = 0.9$ hydrogels showed the highest rate of matured oocyte (MII = 56 %). In stiff gels ($G' > 1 \text{ kPa}$), no MII oocyte ($r = 1$) or only 10 % ($r = 1.5$) MII oocytes were found (Fig. 2.4). The few oocytes collected from stiff gels had a squashed shape. The softest gel ($r=0.8$) produced only 20 % of MII oocytes even though the follicle size were large enough and formed antrum. There, the softest matrix seemed to allow the follicle to grow faster producing already degenerated oocyte after 11 days of culture. When survival rates were compared, only 50 % of the oocytes from the very soft gels survived where the survival in the other condition was between 85 to 95%. The MMP degradation of the already very soft gels played probably a role in the reduced quality of the produced oocytes as well.

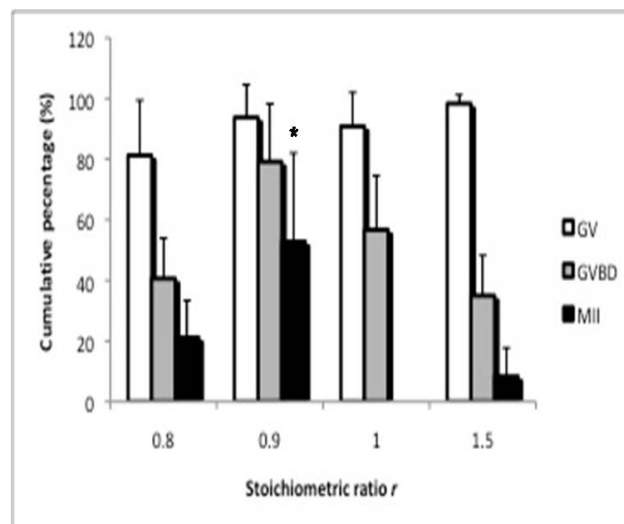


Fig. 2.4. Mature oocytes grown in the follicle cultured in different stiffness PEG-hydrogel. The significant different (* $P < 0.05$) was demonstrated in MII oocytes in the PEG-hydrogel at 0.9 of r ratio. Average \pm SD is shown. Elastic modulus influences the nuclear maturation.

In this study, the stiffness and swelling ratios of the used microenvironments had a measurable affect on the follicle growth and on the subsequent oocyte quality. Around 900 Pa gels ($r = 0.9$) seemed to be the most relevant for follicle culture. These results confirm that the physical characteristics of the follicle microenvironment are potent regulators of the granulosa cells proliferation and thus of the oocyte maturation.

2.3.3. RGD effects on the follicle development

For studying the effect of RGD, the secondary follicles were cultured in PEG-hydrogel with or without RGD. Optimal elastic moduli for the PEG-hydrogel were used ($r = 0.9$). The stoichiometric ratios were adjusted to compensate for the RGD presence or absence. No significant differences in size of the follicles were observed between the studied groups indicating that the elastic moduli were not influenced by the presence of RGD (Data not shown). Fig. 2.5 shows the morphologies of the follicles during growth. At day 1, follicles cultured with RGD showed theca cells attached to the artificial ECM. At day 3, theca cells proliferated and surrounded the base membrane. The group without RGD did not show attachment however; the theca cells proliferated and grew normally. The final morphologies were not different between the two groups. Ovulation was induced and the retrieved oocytes were assessed for their ability to resume meiosis. Follicles from the hydrogel with RGD produced 53.2 % matured oocytes in average where the follicles cultured without RGD produced only 39.5 % (Fig. 2.6.a). The impact on quality of RGD was monitored by inducing parthenogenesis and measuring the rate of the obtained two-cell embryos. Fig. 2.6.b shows the development rate of the obtained two-cell embryos from the follicles culture with or without RGD. The oocytes collected from the hydrogel with RGD reached the 2-cellembryo stage with higher frequency than the oocytes from non-RGD hydrogel (31 % vs 8.5 %, the difference is significant at $p < 0.01$). These results demonstrated that RGD-mediated cell attachment had a positive effect on oocyte quality.

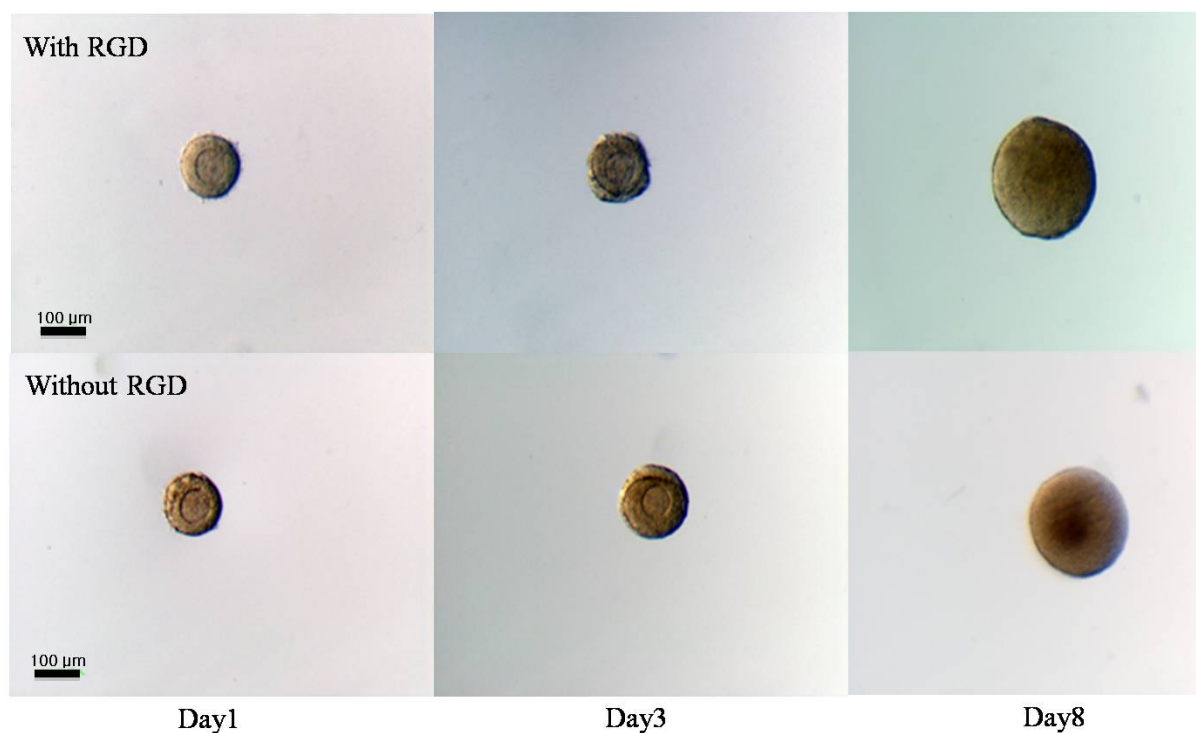


Fig. 2.5. Morphological details of follicles cultured in PEG-hydrogels with or without RGD.

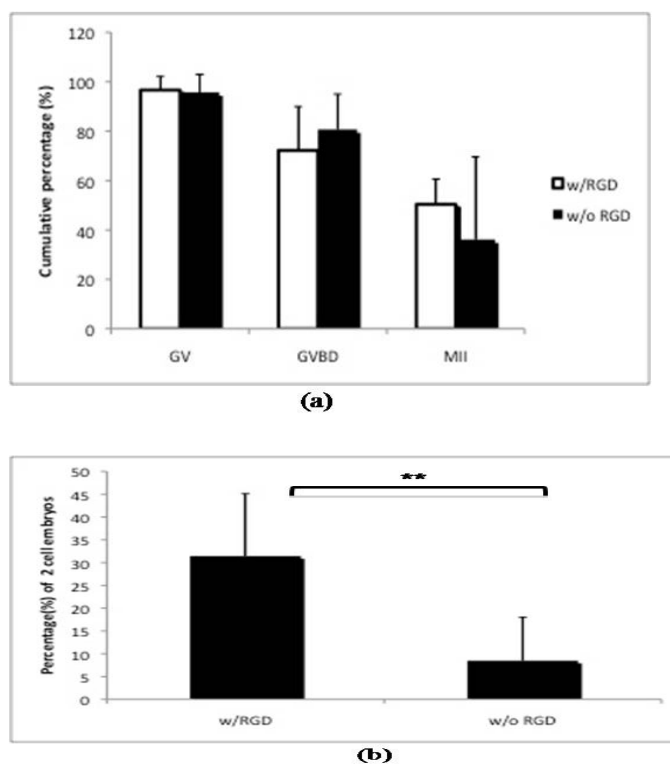


Fig. 2.6. Maturation rates of oocytes (a) and 2 cell embryos development (b) from the follicle cultured in PEG-hydrogel with or without RGD (** $P < 0.01$). Average \pm SD is shown. The RGD effect on development of the oocytes to 2 cell embryos stages.

Ovarian follicles are surrounded by basal lamina. Recent studies reported that this basal lamina regulates granulosa cell functions [75-77]. Laminine, type IV collagen, fibronectin, heparin sulphate proteoglycans is contained in the ECM components of basal lamina [62]. Huet et al. (2001) [77] demonstrated that the presence of an RGD peptide increased the proliferation and attachment of granulosa cells and mimicked the effect of fibronectin and laminin when only granulosa cells were cultured on plates. Previous studies demonstrated that peptide containing RGD sequences improved the expression of LH receptors in porcine granulosa cells stimulated by FSH [78] and increased progesterone and estradiol secretions of murine granulosa cells [79]. In this study, the follicles cultured in the PEG-hydrogel with or without RGD were not different in terms of morphology and size. However, the oocytes maturation and quality showed interesting differences highlighting the beneficial effects of the RGD presence. When the granulosa cells cultured on the RGD coated plastic, they first attachment and then secreted their own ECM attachment proteins [67]. The no difference in follicle growth could be partially explained by the endogenous production of ECM protein. However, the differences in quality could be explained by the stimulating effect of RGD on estrogen and progesterone secretion of the granulosa cells as well as the LH receptors production. These results demonstrated that mimicking physically and chemically the ovarian ECM improved the quality and the quantity of the produced mature oocytes.

2.4. Conclusion

In this study, the PEG-hydrogel system was efficiently tailored to mimic the ovarian ECM for follicle culture. The development of the follicles was largely influenced by the stiffness of the microenvironment. Less than 1 kPa gels were identified as optimal. The RGD presence in the system was also associated with improved quality and higher two-cell embryos rates obtained from the cultured follicles.

Chapter III

Effects of FSH and LH on 3-D cultured murine ovarian follicles

3.1. Introduction

The development of *in vitro* ovarian follicle culture systems provides not only the knowledge of ovarian physiology but also benefits to clinical applications such as infertility treatments and fertility preservation in cases such as oocyte-devastating cancer treatments. The follicle as a morphological and functional unit in the mammalian ovary is composed of the oocyte and its surrounding somatic cells (granulosa and theca cells). The oocyte growth and maturation is depended on the growth and development of follicle structure. The process of follicle development is called folliculogenesis. The earliest stage of the follicle is called primordial follicle. It has the oocyte in the center surrounded by a single layer of flattened granulosa cells. Once the follicle enters the growth phase, the granulosa cells shape change to cuboidal, at the stage called primary follicle. The follicles with granulosa cells more than two layers are called secondary follicle or preantral follicle. The theca cells differentiate and the zona pellucida is completely formed at this stage. When the follicles reach 200 μm in size, a fluid-filled space is formed between the granulosa cells layers. This stage is known as the early antral follicle. Then the antrum increases in size by the ovulation and is finally called a graffian follicle or a late antral follicle [2, 21, 26]. The granulosa cells proliferate and differentiate by maintaining cellular communication with the oocyte during the folliculogenesis. It is demonstrated that metabolic coupling occurs between the oocyte and the granulosa cells. The transfer of small molecules into the oocyte through gap junctions during the *in vitro* growth period is as a key factor for the support of the oocyte's activities [80, 81]. Heterologous gap junctions are present between oocytes and granulosa cells before forming the zona pellucida [82]. After the zona pellucida formation, the contact between the oocyte and the granulosa cells is maintained though the emission of cytoplasmic processes that penetrate the zona pellucida and reach the oocyte [2]. Thus, the maintenance of communication paths is essential for the follicle development.

Another important factor for the follicle development is the presence of physiological concentration of gonadotropins [83]. The main gonadotropins are the follicle-stimulating hormone (FSH) and the luteinizing hormone (LH). FSH stimulates the antral follicle for further growth and differentiation [84]. It promotes the granulosa cell proliferation in secondary follicles and prevents atresia. FSH is also essential for steroid hormones production as it stimulates the aromatase enzyme activity (P450 aromatase) and promotes the synthesis of luteinizing hormone receptors (LHR) leading to the follicle antrum formation [85-87]. LH plays an essential role in the stimulation of the enzyme cascade responsible for androgen biosynthesis in the theca-interstitial cells. Thus LH is determinant for achieving the final differentiation of the granulosa cell that leads to the resumption of the meiotic maturation and to the ovulation [88].

In the established *in vitro* follicle culture, the use of these two gonadotropins is still controversial. FSH plays an important role for the acquisition of the oocyte's developmental competences. FSH is usually added to the secondary follicle culture medium in mice and in large mammals [5, 33, 89]. It has been demonstrated that FSH is required at the minimal concentration of 10 mIU/mL [90] or 100 mIU/mL [33]. Mitchell et al., [91] reported that the populations of ovulation were significantly decreased at 1000 mIU/mL when compared to conditions using 100 mIU/mL for secondary follicle *in vitro* culture. The LH is not an essential factor for the oocyte growth and maturation *in vivo* [92]. Thus, the usage of LH *in vitro* remains controversial. Cortvrindt et al., [93] demonstrated that a low amount of LH enhances the percentage of healthy follicle and impacts positively the oocyte production while Lee and colleagues (2007) [94] reported no effect of LH on follicle growth and oocyte maturation.

These contradictory results could be explained by the used culture system which is different from the *in vivo* situation. Follicles attached on plastic dishes lose their three-dimensional

(3-D) structure during the culture. The cell-cell interactions between the oocyte and the granulosa cells or between the granulosa cells and other the granulosa cells (or theca cells) are highly disturbed in 2-D systems. This “disconnection” may lead to the different results observed in the literature because these interactions can interfere with the hormone balance or the steroid production[93]. This justifies the necessity of studying the effects of hormone or growth factor in 3-D culture systems, which are closer to the *in vivo* ovarian microenvironment.

3.2. Materials and Method

3.2.1. Animals

Female F1 hybrid (C57BL/6 x CBA/caj) mice bred and housed in temperature-, lighting-, and humidity-controlled room and given food and water *ad libidum*. All animal experimental procedures were approved by the veterinary authorities of the canton de Vaud in accordance with the Swiss Academy of Medical Science (SAMS) and the Swiss Academy of Sciences (SCNAT) guidelines.

3.2.2. Synthesis of PEG-VS and peptide precursors

PEG vinyl sulfone (PEG-VS) was synthesized by adapting the previously published protocol [18]. Briefly, 15 g of 4-arm PEG-OH (Mw = 20 kDa, Shearwater polymer, Huntsville, AL) was dried by azeotropic distillation in toluene (VWR, Nyon, Switzerland) for 4 hr using a Dean-Stark. The dried PEG-OH was dissolved in 500 mL of dichloromethane (Fisher Scientific, Wohlen, Switzerland) and sodium hydride (Sigma-Aldrich, Buchs, Switzerland) was added at 20-fold molar excess over OH-group of PEG. Then, divinyl sulfone (Fluka, Buchs, Switzerland) was added at a 50-fold molar excess over the OH groups. This reaction was carried out at room temperature under argon with constant stirring. After 3 days, the excess sodium hydride was neutralized by acetic acid

(Fluka, Buchs, Switzerland). The mixture was filtered over a filter cell cake and concentrated by rotary evaporation. The concentrated polymer was precipitated in ice-cold diethylether (Brunschwig, Basel, Switzerland) and filtered. This precipitation was repeated 3 times. Finally, the product was dried under vacuum at room temperature for 3 days and the yield was 82 %. ¹H NMR showed characteristic vinyl sulfone peaks at 6.1, 6.4, and 6.8 ppm. The degree of end-group conversion was found to be 95 %. The polymer was stored under argon at -20 °C until used. The procedure was reviewed in detailed by Kraehenbuehl et al. [69].

RGDSP peptide (Ac-GCGWGRGDSPG-NH₂) was synthesized by solid phase peptide synthesis using an automated peptide synthesizer (Chemspeed, Augst, Switzerland). NovaSyn TGR resin (Merck Biosciences, Laeufelfingen, Switzerland) with a standard Fmoc chemistry were used. Purification was performed by mass-directed reverse phase-C₁₈ HPLC using a Water Autopurification System. Separation and collection of the samples were performed upon UV with broad wavelength detection (210-400 nm) (Water PDA 996 UV photodiode array) and mass directed software (Waters Masslynx software). Peptide sequences were confirmed by MALDI-TOF in the proteomics core facility of EPFL.

The cross-linking peptide that contains a sequence sensitive to for MMP cleavage (Ac-GCREGPQG ↓IWGQERCG-NH₂) was obtained from Neosystem S.A. (Strasbourg, France).

3.2.3. Preparation the aliquots of PEG-hydrogel

The PEG hydrogels were formed by Michael-type addition of thiol-containing peptides onto VS-functionalized PEG. First, the PEG-VS necessary for forming hydrogels was functionalized with arginine-glycine-aspartic acid (RGD) peptides (also by Michael-type addition). Thirty-nine μL of 10 % PEG hydrogel (in TBS pH 8) solution were mixed with

6 μL of a 400 μM RGDSP peptide solution (in H_2O). The addition proceeded at room temperature for 30 min. The PEG functionalized with RGD solutions were stored in aliquots of 22.5 μL at -20°C .

3.2.4. Follicle isolation, seeding and culture

Ovaries were collected from two-week old F_1 female mice (C57BL/6 x CBA) after euthanasia by cervical dislocation. The collected ovaries were washed with HBSS (Hank's buffered salt solution, Gibco-Invitrogen, Switzerland) and placed in L15 (Leibovitz medium, Gibco-Invitrogen, Switzerland) supplemented with 10 % heat-inactivated FBS (fetal bovine serum, Hyclone), 100 IU/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (P/S). The ovaries were mechanically dissected using insulin syringe. Only follicles with two layers of granulosa cells (100-120 μm) were collected using a mouth-operated micropipette [70]. The follicles were washed and kept in L15 washing droplets covered by paraffin oil (Vitrolife, Sweden).

For the PEG-hydrogel culture system, 7.5 μL of culture medium was added to the PEG solution and then mixed with 12 μL of crosslinker. The crosslinker was diluted in 0.3 M of TEOA and the amounts were adjusted to meet a 0.9 stoichiometric ratio that produces an elastic modulus of 967.67 ± 202.74 Pa and a swelling value of 40.25 ± 2.72 . The follicle were seeded in the above described hydrogel system. Six μL hydrogel drops, containing a unique follicle were formed. After curing, each of these gels was placed in a well of a 96 wells-plate, 150 μL of culture medium was added. The follicles were cultured for 11 days.

The culture medium consisted of α -minimal essential medium (α -MEM with glutamax, Gibco) supplemented with 5 % FBS, ITS (5 $\mu\text{g}/\text{mL}$ insulin, 5 $\mu\text{g}/\text{mL}$ transferrin, 5 ng/mL selenite, Sigma, Buchs, Switzerland), 1 % of P/S, 100 mIU/mL of recombinant follicle stimulating hormone (rFSH, Organon, Switzerland). Next day, the medium was changed

completely with fresh medium. Every second day, half of the medium was replaced with fresh one.

In the drop culture 2-D system, droplets of 10 μ L of culture medium were placed on a 60 mm culture dish and covered with 7 mL of paraffin oil (Vitrolife, Sweden). The retrieved secondary follicles were seeded into each droplet and the next day, 10 μ L of medium was added into the each droplet. Every 2 days, the half of the medium was changed. The follicles were cultured for 9 days as found ideal by Lee et al. (2007) [28].

3.2.5. The effect of FSH and LH

Two main gonadotropins were studied for follicle culture in PEG-hydrogel. Generally, murine follicles in 2-D culture systems are cultured in droplets of medium as single cells. In this drop culture system, the droplets were covered with mineral oil for maintaining a spherical shape and preventing evaporation. The concentration of FSH is usually set to 100 mIU/mL [95]. However, as the follicle cultured in the presented PEG-hydrogel system could require different concentration of FSH, several concentrations were tested (1, 10, 100 mIU/mL) and compared with the drop culture system.

The follicles grown in the PEG-hydrogel system were cultured in three different conditions in terms of LH supply; (medium A) no addition of LH in the medium: no LH, (medium B) LH was added at the first day and till day 3. LH was added again only at day 8 and till the end of the culture. LH concentration was 10 mIU/mL, (medium C) LH (10 mIU/mL) was used during the entire culture period. All the conditions used the optimized follicle culture medium containing 100 mIU/mL of FSH.

3.2.6. Oocyte maturation and quality assessment

For the PEG-hydrogel system, the oocytes meiotic competences were assessed by induced maturation after 11 days of culture. The grown follicles were retrieved from gels by incubating the gels in a 5 mg/mL collagenase I solution (Sigma, Buchs, Switzerland). The maturation medium were prepared with the half of the obtained culture medium from last day of follicle culture and the half of fresh culture medium not containing FSH but 5 ng/mL of human epidermal growth factor (EGF, Roche, Basel, Switzerland) and 2.5 IU/mL of human chorionic gonadatropin (HCG, Organon, Switzerland). The retrieved grown follicles from the gel were incubated at 37 °C for 18-20 hrs to induce the ovulation. For the drop culture, 10 µL of cultured medium were removed and added to 10 µL of maturation medium (10 ng/mL of EGF and 5 IU/mL of hCG in culture medium absent FSH). The follicles were incubated for 16-18 hr. After the induction of the ovulation, oocytes were removed from the cumulus oocyte complex or from the follicle by using a mouth-operated micropipette. The collected oocytes were washed in M2 medium (Sigma, Buchs, Switzerland) droplets and their state was assessed under stereomicroscope and characterized as DG (degenerated), GV (intact germinal vesicle), or GVBD (germinal vesicle breakdown) based on the presence or the absence of a germinal vesicle and MII (metaphase II, matured oocyte) due to the presence of polar body.

3.2.7. Statistic analysis

The statistical analysis of the data was performed with the program OriginPro 8.0 (Originlab corporation, MA, USA). Significances at $p < 0.001$, $p < 0.01$ and $p < 0.05$ (***, ** and * respectively) of the differences were tested with a T test.

3.3. Results and discussion

3.3.1. Optimization of FSH concentration for PEG-hydrogel based culture systems

Two layered secondary follicles (100-120 μm) were cultured with different concentrations of FSH (1, 10, and 100 mIU/mL) either in PEG-hydrogel functionalized with RGD or in the droplet covered with mineral oil as a single cells culture. The survival rate of the follicles cultured in PEG-hydrogel was slightly decreased at 1 mIU/mL of FSH when compared to higher concentrations (Fig. 3.1a).

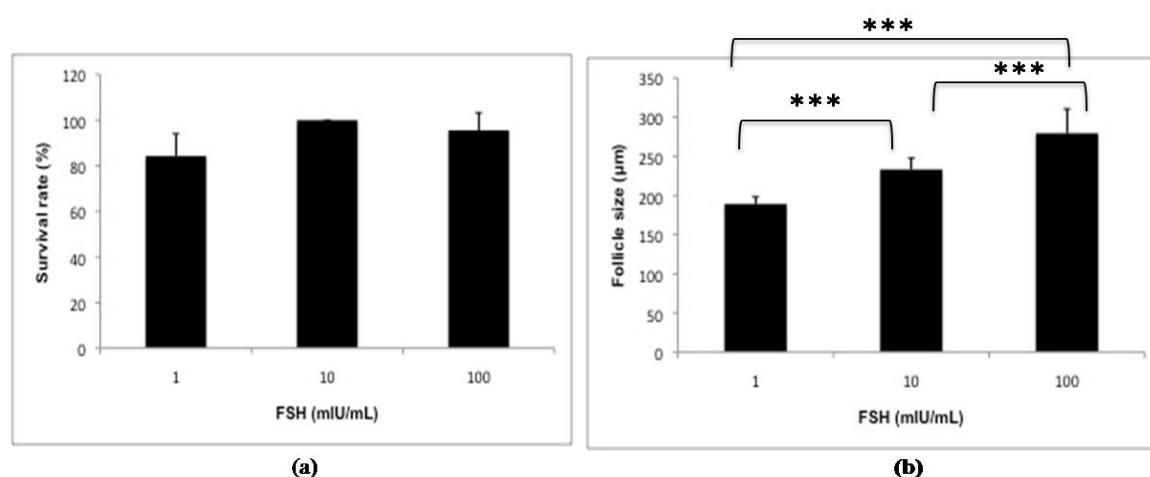


Fig.3.1. Effect of FSH concentration on (a) the survival rate and (b) on the size of the follicles cultured in PEG-hydrogel (***) $P < 0.001$). Average \pm SD is shown. Follicle size was increased by increasing the concentration of FSH.

The size of the follicles cultured in PEG-hydrogel was measured after 11 days in culture. This trait seemed to be a linear function of the concentration of FSH in the studied conditions. Interestingly, follicles cultured in the lower concentration of FSH (1 mIU/mL) could not reach 200 μm of diameter (Fig. 3.1b). The retrieved oocytes from the follicle cultured in PEG-hydrogel or from the droplets with the different FSH concentration were assessed for the resumption of meiosis (Fig. 3.2).

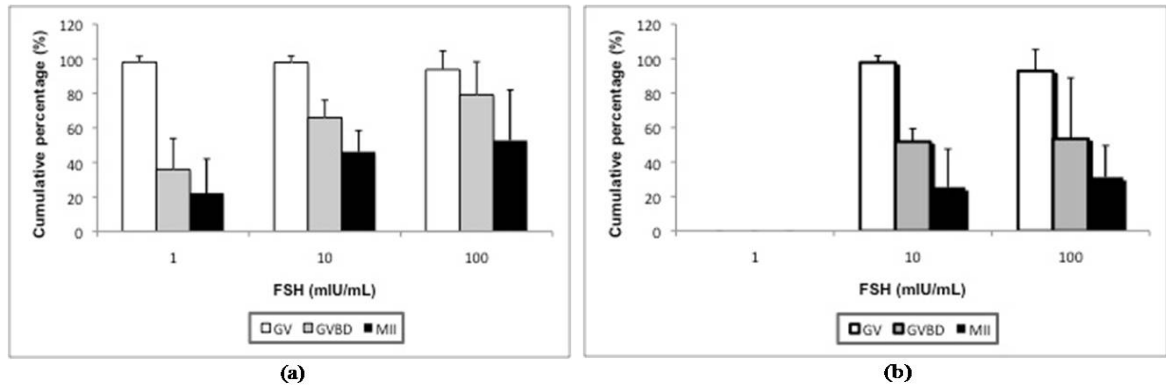


Fig.3.2. Effect of FSH concentration on the maturation of the oocytes and on the percentage of produced MII. (a) Follicle grown in PEG-hydrogel. (b) Follicle cultured in the 2-D drop culture system. Average \pm SD is shown. Average \pm SD is shown. The nuclear maturation is increased by increasing the concentration FSH.

Increasing the concentration of FSH increased the rate of MII oocytes in both of the culture systems. However, the oocytes from the drop culture with 1 mIU/mL of FSH did not have any matured oocyte and most of them were flatten and had squashed shapes. For some of the oocytes, the zona pellucida was also missing. Fig. 3.3 shows the morphological differences between the follicles grown PEG-hydrogels and in the drop culture system at different concentration of FSH. In the PEG-hydrogel system, the follicles cultured with the highest concentration of FSH (100 mIU/mL) formed large antrum cavities while the follicle grown with only 1 mIU/mL of FSH remained small in size and their granulosa cells started to migrate away and to degenerate. In the drop culture, it was observed that increasing the concentration of FSH increased the proliferation of the granulosa cells. *In vivo*, granulosa cells proliferate during the follicle diameter increase. These cells differentiate then under the control of FSH [67]. FSH plays a key role in proliferation and differentiation of granulosa cells and thus in antral follicle formation [33, 90]. The FSH receptors (FSH-R) are mediating the FSH physiological effect on the folliculogenesis. They are only expressed in granulosa cells of the secondary follicle, not in the granulosa cells of the primary follicle [96, 97]. The production of cyclic adenosin monophosphate (cAMP), lactate or estradiol

was found dependent on FSH-R synthesis and playing an important role in the development of the follicle [34]. The results demonstrated that decreasing the concentration of FSH is more dramatic for follicles grown in drop culture for follicles that developed in the PEG-hydrogel system. The granulosa cells in the drop culture system were significantly less proliferating and they migrated away from the oocytes. The lack of communication between the granulosa cells and the oocyte at lower amount of FSH (1mIU/ml) may prevent the oocyte maturation. Moreover, the oocytes grown in these conditions attached on the bottom and grew flat and had squashed shape because not enough granulosa cells were present for supporting them. The follicles in PEG-hydrogel showed a delay in the proliferation of granulosa cells when the FSH amount was low. It has been demonstrated that the FSH was involved in the production of the LH receptors [98]. FSH is also playing a role in the activity of aromatase, an enzyme of the P450 superfamily that aromatizes androgens, produces estrogens and thus prevents atresia [99]. The follicles cultured in PEG-hydrogels at the lowest concentration of FSH (1 mIU/mL) showed migration and degeneration of the granulosa cells. This phenomenon was probably related to the atresia due to the lack of FSH. During folliculogenesis, several follicles start to grow together and only one or few of them are ovulated. The non-ovulated follicles undergo atresia: a hormonally controlled apoptosis [23]. The atresia is caused by signaling cascade downstream of FSH during the follicle selection process [99]. It is suggested that sufficient FSH help the antral follicle to escape from atresia and reach the preovulatory follicle stage [23]. The presented results show that the lack of FSH induced the suppression of the proliferation of the granulosa cells and the follicular degeneration in the PEG-hydrogel culture system while in the 2-D culture system only the proliferation of the granulosa cells was impaired. However, in both systems, the optimal concentration of FSH for the follicle culture was found to be 100 mIU/mL.

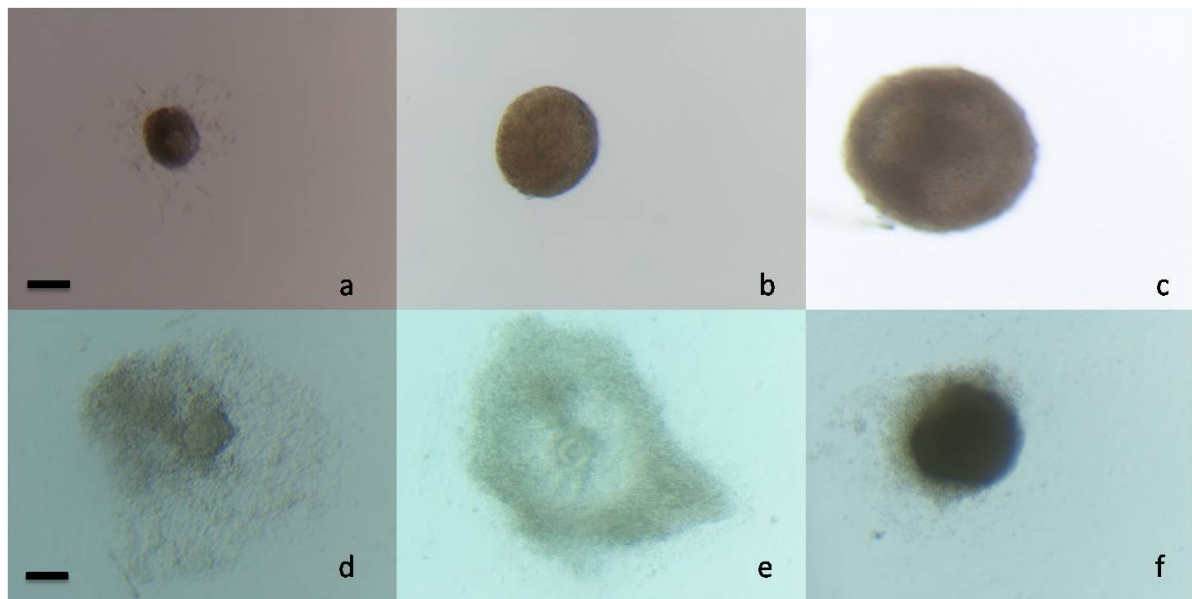


Fig.3.3. Morphological differences induced by the FSH concentration in both Hydrogel at 11 days of culture, (a) 1 mIU/ml b) 10 mIU/mL c) 100 mIU/mL of FSH) and drop culture at 9 days of culture (d) 1 mIU/ml e) 10 mIU/mL f) 100 mIU/mL of FSH). The scale bar is 100 μ m. FSH induced granulosa cells proliferation and atresia due to low level of FSH was observed only in hydrogel.

3.3.2. Effect of LH on the follicle culture in PEG-hydrogels

During extraction from the ovaries around 20 % of all retrieved follicles lost most of their associated theca cells. Fig. 3.4 shows the morphological differences induced by the addition of LH. The follicles with theca cells showed that these cells grew more in presence of LH than without LH at day 3. The growth of the follicles was not different between the group without LH (medium A) and the group with partial supply of LH (medium B). However, when the LH was added in the medium continuously, the size of the follicles was significantly decreased. A key role of LH is the stimulation of the enzyme responsible for androgen production and the initiation of the terminal differentiation of the granulosa cells [88, 93].

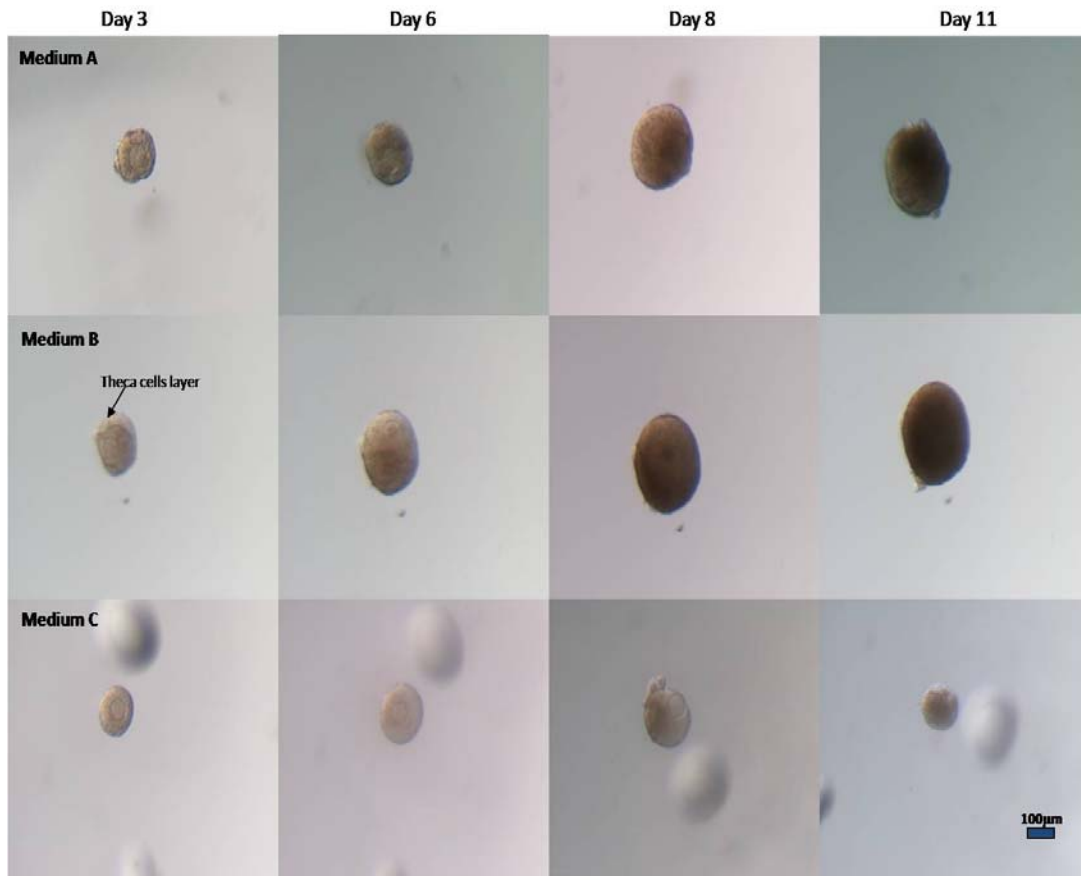


Fig.3.4. Morphological differences induced by LH supply strategies. Medium A was LH free. Medium B was supplemented with LH at day 1 to 3 and 8 to 11. Medium C had LH all along the culture period. The used concentration of LH was 10 mIU/ mL. The scale bar is 100 µm. LH terminated differentiation of granulosa cells in PEG-hydrogel culture system.

The results from the follicles grown in medium C support the above-described role of LH. It showed that the follicles stopped their growth and subsequently decreased the granulosa cells layers. The maturation rate was measured for all the oocytes grown in media A, B and C. The highest rate of MII oocytes was observed in the group cultured without LH. No MII oocytes were retrieved from the group where LH was added continuously (Fig. 3.5). When the follicles were cultured in the medium B, the percentage of matured oocytes (MII) was decreased as the concentration of LH was increased (Fig. 3.6). The group treated with low concentration of LH produced the highest MII oocytes ratio (52. 7 %). This low concentration of LH did not induce a significant difference in terms of MII rates when

compared with the group from which LH was absent. These results are different from the results of previous studies using 2-D *in vitro* systems. The necessity of LH during secondary follicle culture was always controversial. Cotvrindt, et al (1998) [93] demonstrated that the addition of low concentration of LH enhances the antral like-cavity formation and improves the oocyte's meiotic maturation during secondary follicle *in vitro* culture. On the other hand, Lee et al. (2007) [94] reported that there were no significant effects of LH in *in vitro* follicle culture systems. Our results support the latest; in the presented conditions LH supply did not have any beneficial effect on the rate of MII of oocytes cultured in PEG-hydrogels. Moreover, when LH was continuously supplied during the culture, the differentiation of the granulosa cells was terminated. The main difference between the studies is due to the loss of the follicle 3-D structure after six days of culture and to the absence of most intra-ovarian and 'extra-follicular' cues. This disconnection could potentially prevent the negative impact of LH. Conversely, the follicle culture in PEG-hydrogel (as a 3-D system) could maintain the follicle structure, thus the LH could be negatively effective on the follicle growth and the oocyte meiosis.

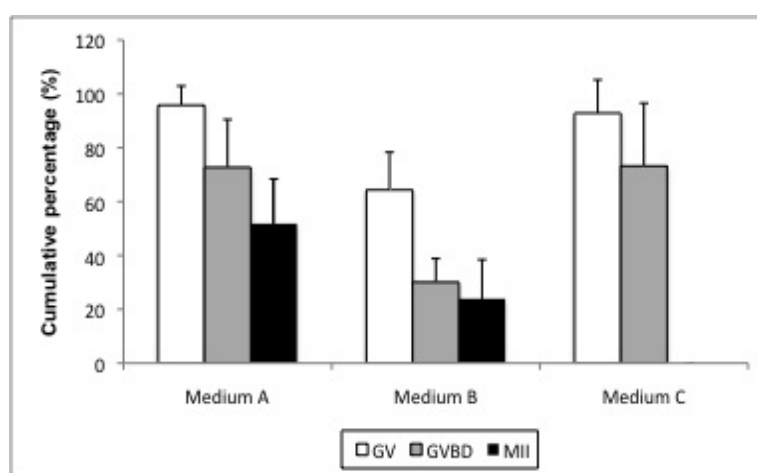


Fig.3.5. Comparison of the effects of LH supply on the oocyte maturation rate. Medium A was LH free. Medium B was supplemented with LH at day 1 to 3 and 8 to 11. Medium C had LH all along the culture period. The used concentration of LH was 10 mIU/ mL. Average \pm SD is shown. LH has negative effects for nuclear maturation.

3.4. Conclusion

The effects of FSH and LH on the follicle cultured in PEG-hydrogel were closer to the effects observed *in vivo* when compared to the effects observed in the standard 2-D culture system. The granulosa cells could proliferate and atresia could be prevented by increasing the concentration of FSH. The addition of LH along all the culture period led to the termination of granulosa cells differentiation. The present results show that the follicle culture in PEG-hydrogel is ideal for studying the effects of growth factors and hormone signaling on the ovarian microenvironment.

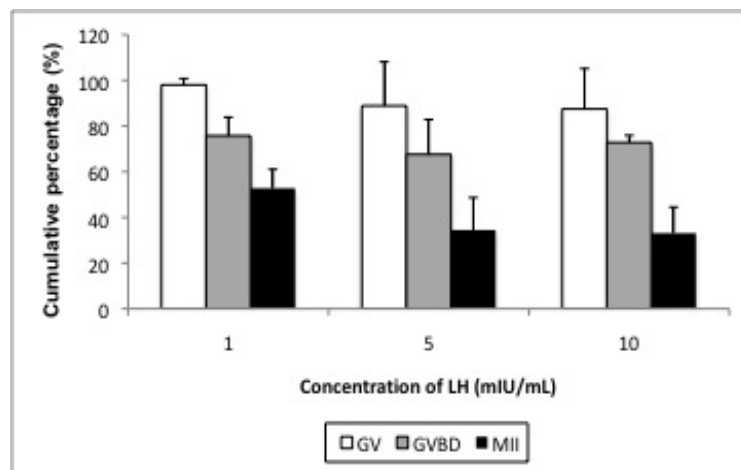


Fig.3.6. Effect of LH concentration on the maturation rate of the cultures oocytes. The follicles were cultured in PEG-hydrogel with different concentration of LH. The LH was added only at day 1 and then next day, the medium was refreshed with a half volume of culture medium free of LH till the end of culture period.

Chapter IV

Production of recombinant KL proteins and their effects on ovarian tissue culture.

4.1. Introduction

c-Kit ligand (KL) is a pleiotropic growth factor also known as steel factor, mast cell growth factor or stem cell factor [20]. KL binds a tyrosine kinase receptor on its target cells to trigger a further signaling cascade. It has been demonstrated that mutations in the c-kit gene (the white spotting locus, *W*) or in the Kit ligand gene (the steel locus, *Sl*) produced mice with defective fertility and hematopoiesis [41]. There are many studies that suggest that the c-kit/KL interaction plays a role in primordial germ cells (PGC) survival, migration and proliferation and in follicle development [49, 100, 101]. The mRNA and the protein of both the soluble (KL-1) and the membrane-bound (KL-2) forms of KL were found expressed in mouse ovaries. Their expressions were detected in the granulosa cells of follicles at all stages of development and in the cumulus cells of antral follicle [48, 102]. The KL-1 and KL-2 forms are known to be differentially regulated. They are reported to play a role within the ovary, in the germ cells or in the follicle development [49]. Thomas et al. (2005) [103] demonstrated that the KL-1/KL-2 mRNA ratio is important for controlling oocyte growth. They reported that, under FSH-stimulating condition, a low KL-1/KL-2 mRNA ratio favored the growth of the oocyte. The presence of the soluble form of KL (KL-1) was demonstrated to favor the proteolysis and the down regulation of the membrane receptor (c-Kit) whereas the presence of the membrane-bound form (KL-2) favored a more sustained c-Kit signaling in myeloid cell lines [104].

The development of the cryopreservation of ovarian tissue aroused the interest in primordial follicles as a source material. Efficient use of primordial follicle could help the assisted reproduction of domestic animals and endangered species. It could also help women exposed to radiotherapy or chemotherapy to recover their fertility [101]. Thus it is important to better understand what are the mechanisms underlying the exit of the follicle from the resting pool and the following folliculogenesis. KL is considered as one of few

known factors to be involved in this process [49] even though its precise role remains unclear. It has been reported that the activation of the primordial follicles is prompted by KL during ovarian organ culture [51]. Yoshida et al. (1997) [50] demonstrated that the KL/c-Kit interaction is important for primordial follicle activation.

However, little is known about the KL role in the activation of primordial follicles in *in vitro* culture system. This is partially due to the need of developing a new *in vitro* culture system where KL-2 (the membrane-bound form of KL) is tethered to physiologically compatible substrate to mediate a relevant biological answer. It is here required to create a microenvironment that allows the c-Kit signaling, as it was the case for other immobilized signaling factors such as notch [40]. Tailored PEG-hydrogel systems allowing the covalent immobilization of proteins by Michel type addition [19] or by enzymatic reaction [105] could be of good use for this purpose.

In this study, three different constructs coding for recombinant extracellular domains of the KL were prepared: 1) extracellular domain only (KLs), 2) extracellular domain plus a substrate sequence for Factor XIIIa, (KLstg) and 3) extracellular domain plus a substrate sequence for Factor XIIIa plus an RCG tag for Michel type addition immobilization (KLstag). The purified proteins were immobilized onto PEG-hydrogels. This system served for culturing four days-old ovarian tissues. The activation of the primordial follicles was monitored across all the studied conditions.

4.2. Materials and methods

4.2.1. Animals

Female F1 hybrid (C57BL/6 x CBA/caj) mice bred and housed in temperature-, lighting-, and humidity-controlled room and given food and water *ad libidum*. All animal

experimental procedures were approved by the veterinary authorities of the canton de Vaud in accordance with the Swiss Academy of Medical Science (SAMS) and the Swiss Academy of Sciences (SCNAT) guidelines.

4.2.2. Polymerase chain reaction amplification (PCR) and sequence determination

Total RNA from adult mouse ovaries were extracted by using the total RNA extraction kit (Invitrogen, Switzerland). The purity and the quality of the extracted totRNA were assessed with a Bio-nano chip (Bioanalyzer 2000, Agilent Technologies, Switzerland). The concentration of the totRNA was determined with Nano-drop (ND-1000, Witec AG, Switzerland) by reading the absorbance at 260 and 280 nm. The synthesis of cDNA was performed by using the SuperscriptTMIII First-strand reverse transcriptase. Briefly, 3 µg of total totRNA, 50 µM oligo(dT)₂₀, 10 mM dNTP mix, 0.8 µl of DEPC-treated water in 10 µL of total volume were denaturated at 65 °C for 5 min and then placed on ice. The first cDNA strand was obtained by adding 2 µL of a 10X RT buffer, 4 µl of 25 mM MgCl₂, 2 µL of 0.1 M DTT, 1 µL of RNaseOUT (40U/µL) and 1 µL of SuperscriptTMIII. The mix was incubated at 50 °C for 50 min. The reaction was stopped by rising the temperature to 85 °C for 5 min. The reaction was then chilled on ice and 1 µL of RNase H was added. The degradation of the totRNA was performed at 37 °C for 20 min. A double brand cDNA was obtained by performing a PCR with 10 µM of both sense and antisense primers (Table 4.1), 1/10 volume of 10 x PCR buffer, 20 mM of dNTPmix and 1 unit of pfx 50 DNA polymerase (Invitrogen, Switzerland). After a denaturation phase of 5 min at 95°C, the amplification proceeded for 30 cycles with an elongation time of 1 min 30 sec. The PCR products for pKL (only the full length extracellular domain) and pKLs (full length extracellular domain including the signaling peptide sequence) were cloned into a pDrive cloning vector (Qiagen, Switzerland) according to the manufacturer recommendations. *E. coli* (strain DH5α) competent cells were transfected with the obtained plasmids. Positive

clones were identified by PCR on single colonies. The PCR parameters for identifying the positive clones were identical to the parameters used for producing the cDNA. Positive colonies were cultured overnight in LB liquid medium. The plasmids containing the sequences were obtained with a miniprep extraction kit (Promega, city, Switzerland).

Table 4.1. PCR primers used for generating the various KL constructs.

Name	Primers	sense	Annealing temp. (°C)	Length (bp)
		antisense		
β-actin	5'-AAGTGTGACGTTGACATCCG-3'	5'-GATCCA CATCTGCTGGAAGG-3'	55	253
pKL: F+R From part of signal region to extracellular domain	5'-TCACTTGCATTATCTCCAAGTGC-3'		59	617
	5'-TTGTAGGCCCGAGTCTTCA-3			
pKLs:Utr+R From untranslated region to extracellular domain	5'-AAA GCA ACG GCC AAG GAC G -3'		57	794
	5'-TTGTAGGCCCGAGTCTTCAAGG-3			
Frag (SGNQEVSPLRCG)	5'-GGGCCTACA ATCTGG TAA TCA GGA GCA GGT ATC ACC ACT ACG TTGTGG A-3'		95	~58
	5'-TCCACAACGTAGTGGTGATACCTGC TCCTG ATTACCAGA TTGTAG -3'			
pEFKLs: UPKPN+DownNOTI Kpn and Not I cleavage site at the ends, from signal. Thrombin cleavage site.	5'-GGT ACC ATG AAG AAG ACA CAA ACT TGG ATT AT-3'		58	676
	5'-GCGGCCGCCAAGAACCACGCGGAA CCAGTTGTAGGCC-3'			
pEFKLstg: UPKPN+DownNOTITAG Kpn and Not I cleavage site at the ends, from signal. Thrombin cleavage site and SGNQEVSPLRCG)	5'-GGT ACC ATG AAG AAG ACA CAA ACT TGG ATT AT-3'		58	715
	5'-GCGGCCGCCAAGAACCACGCGGA ACCAGTC CACAACG -3'			
pEFKLstg: UPKPN+ DOWNNOT1TAGNORCG Kpn and Not I cleavage site at the ends, from signal. Thrombin cleavage site and SGNQEVSPL)	5'-GGT ACC ATG AAG AAG ACA CAA ACT TGG ATT AT-3'		58	706
	5'-GCGGCCGCCAAGAACCACGCGGAAC CAGTAGTGGTG A-3'			

To introduce the sequence corresponding to the tags NQEVSPL+ RCG, synthetic and complementary single strand DNA fragments coding for the needed sequence were ordered from Mircrosynth (Geneva, Switzerland). Care was taken to ensure that the ends were compatible with BbsI and HincIII digested fragments.

Both strands were mixed (200 pmol/μL each), denaturated at 95 °C for 10 min and annealed at room temperature for 2 h. One μg of plasmid containing KL was digested with BbsI (5 U/ul) and HincIII (10 U/ul) in NEB buffer 2 (New England Biolabs, Ipswich, MA,

USA). The digestion reaction was stopped by incubation 20 min at 65°C. The digestion products were separated on 1% w/v agarose gel in TEA. The band corresponding to the plasmid (~4 kb) was excised and purified with a gel extraction kit (Qiagen, Switzerland). The linearized plasmid and the synthetic DNA sequence were mixed (mole to mole) in 10 x ligase buffer. T4 ligase (1 ug /uL) was added to the mix and incubation at 4 °C proceeded overnight. The mix was used for transforming DH5 α competent *E. coli* cells. Positive clones were identified as mentioned above. This procedure is described in Fig. 4.1.

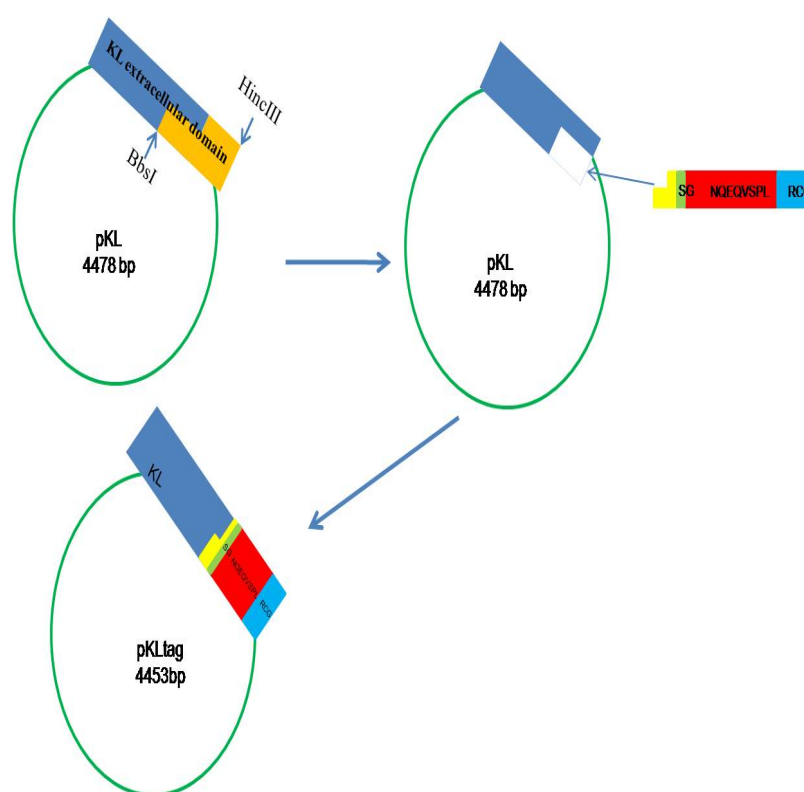


Fig.4.1. Cloning of the tag sequence into pKL vectors

To sub-clone pKL and pKLs into pEF1mycHis mammalian expression vector (Invitrogen, Switzerland) special primers embedding KpnI or NotI cleavage sites at the 5' ends and spanning over the desired sequence were designed. PCR products for pEFKLs, pEFKLstag, and pEFKLstg (Fig. 4.2) were generated and digested with KpnI and NotI to produce compatible ends with the expression vector. The linearized (KpnI and NotI) expression

plasmid and the digested PCR products were mixed (mole to mole) and ligated with T4 DNA ligase (Promega, Switzerland). The plasmids were amplified in *E. coli* and maxipreps (Qiagen, Switzerland) were made to produce large quantities of the three KL expression vectors.

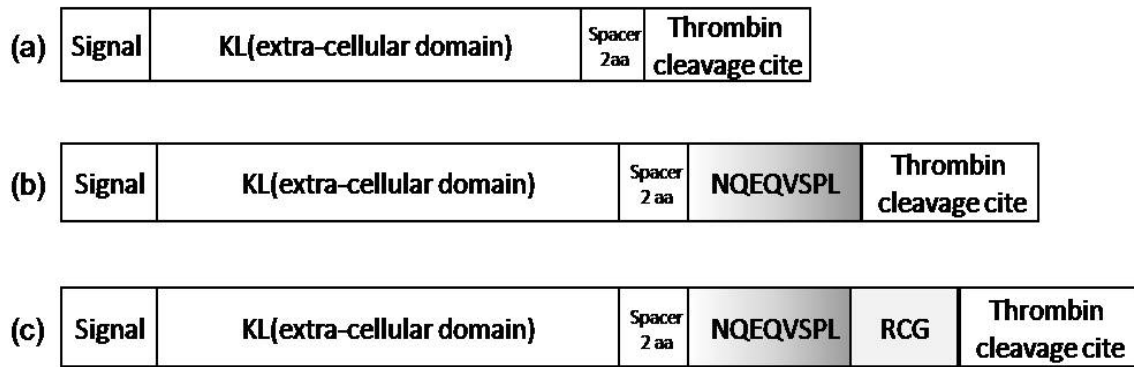


Fig.4.2. Cloning of KL variant constructs: (a) wild type extracellular domain of KL, pEFKLs, (b) pEFKLstg, and (c) pEFKLstag.

All the DNA quantifications were made with an ND-1000 spectrophotometer (Nanodrop, Witec AG, Switzerland). The sequence of all the obtained plasmids were controlled by sequencing (Microsynth, Geneva, Switzerland) and aligned versus the correct theoretical sequence using the Multalign software [106].

4.2.4. Production of recombinant KL fusion proteins

Suspension-adapted HEK-293 cells were routinely maintained in serum-free Ex-Cell 293 medium (SAFC Biosciences, St. Louis, MO) with 4 mM glutamine as described [107]. On the day before transfection, cells were inoculated into fresh medium at a density of 1×10^6 cells/ml. The next day, the cells were harvested by centrifugation at 1,200 rpm for 5 min and resuspended at a density of 20×10^6 cells/ml in 200 ml of Ex-Cell 293 medium with 4 mM glutamine in a 500-ml glass bottle [108]. Plasmid DNA and linear 25 kDa polyethylenimine (1 mg/ml in H_2O ; Polysciences, Eppenheim, Germany) were

sequentially added to concentrations of 25 µg/ml and 75 µg/ml, respectively. The culture was agitated by orbital shaking at 110 rpm in an ISF-4-W incubator (Kühner AG, Birsfelden, Switzerland) at 37°C in the presence of 5% CO₂. The bottle cap remained open about one-quarter of a turn. After 3 h the culture, the transfected cells were divided equally into two 5-liter glass bottles (Schott Glass, Mainz, Germany) each containing 1.9 l of prewarmed Pro293s medium (Lonza, Verviers, Belgium) with 4 mM glutamine. Valproic acid (500 mM in H₂O) (SAFC Biosciences) was added to a final concentration of 4 mM [109]. The two cultures were incubated with agitation as before. At 7 d post-transfection, the cell culture medium was recovered by centrifugation at 1,500 rpm for 10 min.

4.2.5. Purification and quantification of the expressed proteins

Purification of the expressed protein was carried out by his-tag affinity chromatography on a Biorad Profinia (Biorad, Hercules, CA, USA) system with the Bio-Scale Mini Profinity IMAC cartridge with 5 mL column volume. The medium was filtered prior to purification with a low-binding 0.25 µm filter. 200 mL of the medium was injected into the column and the flow-through collected. Buffers of the native IMAC purification kit (Biorad, Switzerland) were used for all subsequent steps. After injecting, a washing step with two column volumes with 25 mM imidazole was performed of a 50 mM sodium phosphate buffer with 100 mM NaCl. Fractions were buffer exchanged sodium phosphate buffer on a Bio-Scale Mini Bio-Gel P-6 desalting cartridge. The first two fractions contained the protein according to the chromatogram. The flow-through, wash and elution fractions were analyzed by SDS-PAGE and the protein identity confirmed by LC-MS/MS and MALDI-TOF

The expressed proteins were quantified with a BCA protein assay by using a BCA protein assay kit (Perbio, Switzerland). ELISA was performed with an SCF Elisa kit (R & D system, UK).

4.2.6. Immunodetection of the expressed and purified recombinant proteins

The expressed proteins were analyzed by Western blot under both reducing and non-reducing conditions. The samples were separated by SDS-PAGE and electrochemically transferred onto nitrocellulose membrane. Immublot analysis was carried out according to the protocol recommended by Bio-Rad. Rabbit polyclonal antibodies against mouse SCF (Abcam, Switzerland) were used as the primary antibody and horseradish peroxidase conjugated anti-rabbit IgG antibody as the detecting reagent.

4.2.7. Mass spectrometry analysis

Samples were reduced-alkylated (DTE/Iodoacetamide) in order to maintain the disulfide bounds in the reduced form. Trypsin digestion was then performed for at least 12 hours at 37 °C. Finally, resulting peptides were concentrated by Speed-Vac evaporation.

Samples were resuspended in LC-MS starting solvent (2%ACN, 0.1%FA) for LC-MS measurement. Around 500 femtomoles were captured on a precolumn, desalted and separated on a C18 100µm x 10mm capillary column. MS measurements were performed on a LTQ linear ion trap (Thermo). A sub-database containing edited sequences of KL was used for the search (Fig. 4.3).

4.2.8. Preparation of PEG-hydrogels functionalized with rKL proteins

PEG vinyl-sulfone (PEG-VS) was synthesized by adapting previously published protocol [18]. The PEG hydrogels were formed by Michael-type addition of thiol-containing peptides onto VS-functionalized PEG. TG-lys peptide (Ac-FKGERCG-NH₂) was synthesized by solid phase peptide synthesis using an automated peptide synthesizer

(Chemspeed, Augst, Switzerland). NovaSyn TGR resin (Merck Biosciences, Laeufelfingen, Switzerland) with a standard Fmoc chemistry was used. Purification was performed by mass-directed reverse phase-C₁₈ HPLC using a Water Autopurification System. Separation and collection of the samples were performed upon UV with broad wavelength detection (210-400 nm) (Water PDA 996 UV photodiode array) and mass directed software (Waters Masslynx software). Peptide sequences were confirmed by MALDI-TOF in the proteomics core facility of EPFL. The cross-linking peptide that contains a sequence sensitive to for MMP cleavage (Ac-GCREGPQG ↓IWGQERCG-NH₂) was obtained from Neosystem S.A. (Strasbourg, France).



Fig.4.3. Alignment of KL sequences quality assessment in LC-MS

First, the PEG-VS necessary for forming hydrogels was functionalized with a substrate peptide for Factor XIIIa, Ac-FKGERCG-NH₂ (named TG-lys, mol wt 837.4 g/mol) via Michael-type addition as well. Thirty-nine µL of 10 % PEG hydrogel (in Tris buffered saline, TBS pH 8) solution were mixed with 6 µL of TG-lys peptide solution (in H₂O). The addition proceeded at room temperature for 30 min. The solutions of PEG functionalized with TG-lys were stored in aliquots of 22.5 µL at -20 °C. In a second time, the KLstg which had the factor XIIIa substrate sequence NQEQVSPL (TG-Gln), was

reacted with TG-lys bound to the PEG in the presence of the activated factor XIIIa [105]. For example, 100 μ M of KLstg was mixed with 1 μ L of TBS containing 100 μ M of calcium chloride and 0.5 μ L of activated factor XIIIa. The mixture was added to the prepared PEG aliquot and reacted for 30 min at room temperature. Briefly, the activated factor XIIIa was prepared such that a 100 μ L of the factor XIII (273 U/mL, a generous gift of Baxter Biosurgery, Vienna Austria) was activated with 10 μ L of thrombin (200 U/mL, GEHealthcare, Switzerland) for 30 min at 37 °C. Small aliquots of activated factor XIIIa were stored at -80 °C.

4.2.9. Immobilization assay of KLstg onto PEG hydrogel

The binding assay was carried out with different concentration of KLstg recombinant protein. The functionalized with KLstg PEG-VS was cross-linked with the MMP sensitive peptide (0.3 M TEOA, pH 8.0) at 37 °C for 18 min. The obtained PEG hydrogels were placed into 150 μ L of distilled water in a 96-well plate. After, the gels were incubated at 37 °C for 3 days. The amount of KL protein that diffused out of the gels was quantified by performing an SCF ELISA kit assay (R&D system, UK) to detect the presence of the recombinant protein in the water.

4.2.10. Mouse ovarian tissue culture in PEG-hydrogel

The mouse ovaries were obtained from four-day old mice after scarifying by cervical displacement. The ovaries were moved in L-15 medium (Gibco, Switzerland) and tore, under a stereomicroscope, in 100-200 μ m size pieces with an insulin syringe. The prepared ovarian tissues were seeded into the mixture of the PEG solution containing diverse concentrations of KLstg protein or crosslinker. Hydrogel precursors were mixed and incubated at 37 °C for 18 min to gel. The formed PEG-hydrogel with embedded ovarian tissue were placed into 150 μ L of MEM- α medium (Gibco, Switzerland) containing 100 x

ITS (Sigma, Switzerland), 1% P/S, 5 % FBS (Hyclone, Switzerland), and 100 mU/mL FSH (Organon, Switzerland). The medium was completely changed with fresh medium next day. Every two days, the half of medium was refreshed for 10 or 20 days.

After 10 days, PEG-hydrogels were harvested by digesting the gels with collagenase I (Sigma, Switzerland). The obtained ovarian tissues were embedded in paraffin, sectioned (5 μ m sections), and stained with hematoxylin and eosin. The number of viable follicles was counted with an Olympus AX 70 upright monitored microscope (20X magnification).

4.3. Results and discussion

4.3.1. Cloning of rKL variant constructs

Human KL is a protein that contains 273 amino acids divided into a signal sequence, an extracellular, a transmembrane and an intracellular domain. Murine and Human KL share 83% of sequence homology. It was demonstrated that murine KL efficiently replaces the Human one in activity tests performed on Human cells [100] (the opposite remaining not true). Thus the produced recombinant proteins could be used for future experiments with Human oocytes. The different constructs of KL contained the signal sequence (to allow the production in mammalian cells) and the extracellular domain. However they were different for the type of tags added to allow their immobilization onto PEG-hydrogels (Fig. 4.2). It is of interest to note that all of the recombinant KL included a thrombin cleavage site to allow the cleavage of the His tag used for the purification step. Two different KL constructs were designed to allow different strategies of immobilization onto PEG-hydrogel. One had only a substrate sequence for factor XIIIa, (NQE QVSPL), the other had both NQE QVSPL and RCG to allow a direct reaction with the vinylsulfone groups of the PEG via Michel type

addition. Transglutaminase (TG) is a family of enzyme that forms ϵ -(α -glutamyl) lysine isopeptide side-chain bridges by catalyzing an acyl-transfer reactions between the α -carboxamide group of protein-bound glutamyl (Gln, or Q) residues and the ϵ -amino group of lysyl (Lys, or K) residues [110]. An activated TG cross-linking enzyme; Factor XIIIa, plays a role in fibrin clot formation in damaged tissues [111]. We employed the Factor XIIIa substrate, the heptapeptide NQEQVSP named TG-Gln, for mobilizing KL onto the PEG-hydrogel. This sequence is derived from the N-terminus of the regulatory protein α_2 -plasmin inhibitor (α_2 PI). The full-length α_2 PI proteins are incorporated into fibrin networks during fibrinogen polymerization [112, 113]. Fig. 4.4 shows the scheme of PEG-hydrogel functionalized with KL by using the TG enzymatic cross-linking.

The cDNA of KL was produced from the totRNA of adult mouse ovaries. The obtained RNA qualities and quantities are shown in Fig. 4.5. The peaks in the electropherogram show 18S and 28S RNA without noise and the gel represented both 18S and 28S. The rRNA ratio of the extracted RNA was 2.67. The RNA quality is assessed by the 28S to 18S ribosomal RNA (rRNA) ratio. Ratios higher than 2 indicates that the quality is satisfying [114]. Table 4.1 shows the primers used for the cloning of the different constructions of KL. Fig. 4.6 shows the amplification bands corresponding to pKL and β -actin as a control.

4.3.2. Expressions and purification of rKL variants

The structure of the extracellular domain of KL was proven to be stabilized with disulfide bonds [115]. Thus a reduction step was not possible during the purification process in order for the recombinant protein to retain its bioactivity. This consideration made the expression of the third KL (NQEQVSP+RCG) construct difficult as it adds an extra free cysteine to the sequence. Dimerization and loss of activity were then anticipated for that construct.

The two other KL constructs were transfected on the HEK-293. Three and 0.6 mg of purified rKLs and rKLstg were respectively obtained.

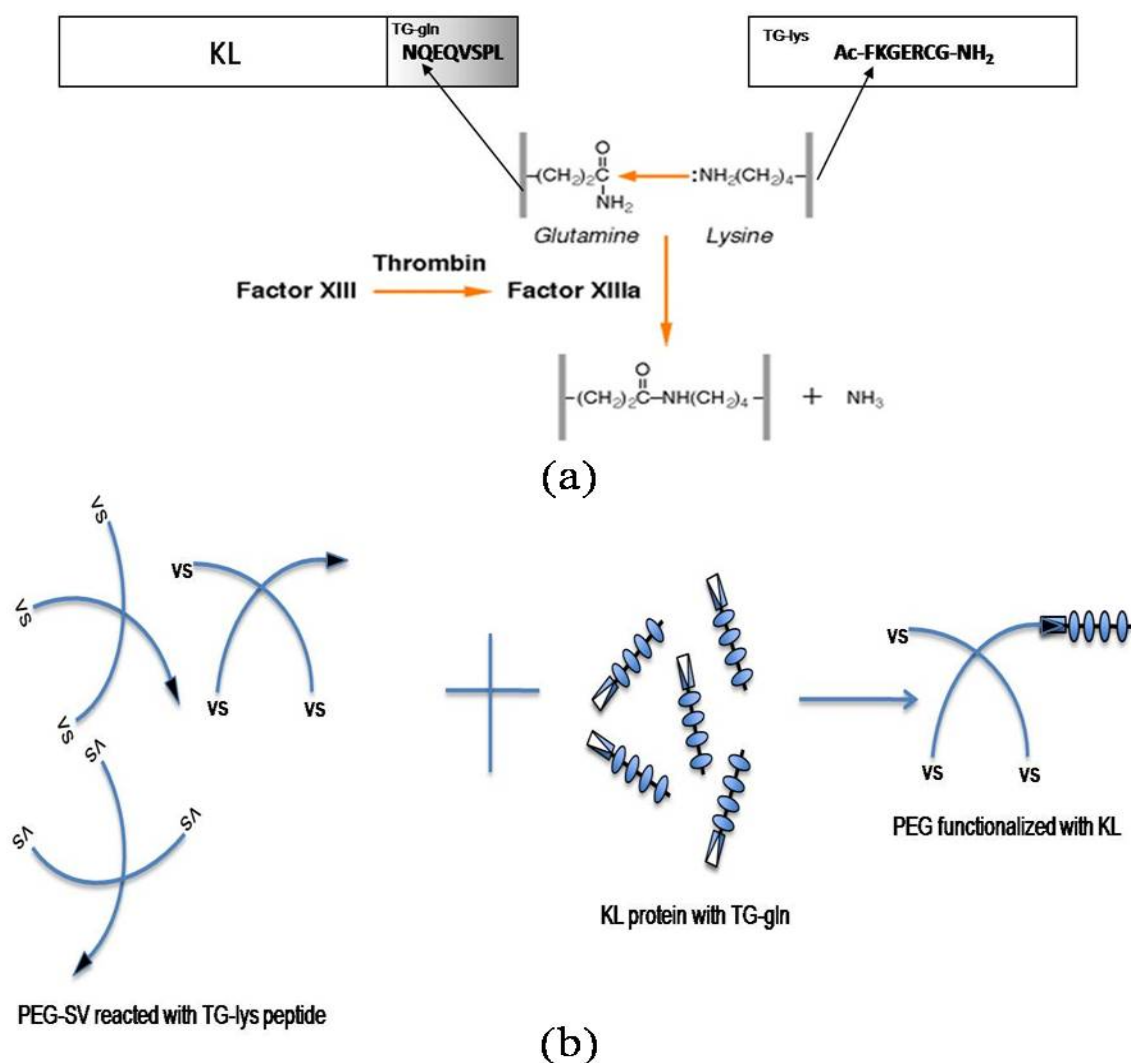


Fig.4.4. Scheme of PEG-hydrogel functionalization with KLtg. (a) Transglutaminase cross-linking reaction. (b) PEG-hydrogel functionalized with TG-lys reacted with KLstg by TG reaction.

4.3.3. Characterization of KL proteins

The produced proteins, KL wild type extracellular domain (KLs) and KL with the TG-Gln sequence (KLstg) were found at the concentrations of 315.30 µg/mL and 60.08 µg/mL respectively in the culture media. SDS-PAGE and western blotting analysis showed that the

molecular weight of KLS and KLSstg were of ~33 kDa under reducing and non-reducing conditions (Fig. 4.7). This indicates that both recombinant proteins were produced as monomers. KLS and KLSstg were expected at 20856.7 and 21896.8 Da respectively. The observed higher molecular weight is certainly due to glycosylation. This post-translational modification was reported as critical for the biological activity of KL. Huang et al. 1992 [116] reported the occurrence of four N-linked glycosylation site in KL. Natural and recombinant KL from chinese hamster was reported to be 30 % overweighed with carbohydrates [117, 118]. The glycosylated status did not block the binding of KL to c-Kit, even when the protein is heavily glycosylated [49]. The LC-MS analyzed peptides covered 71 % of the sequence of both produced proteins (Fig. 4.8) confirming the efficiency of the production/purification method.

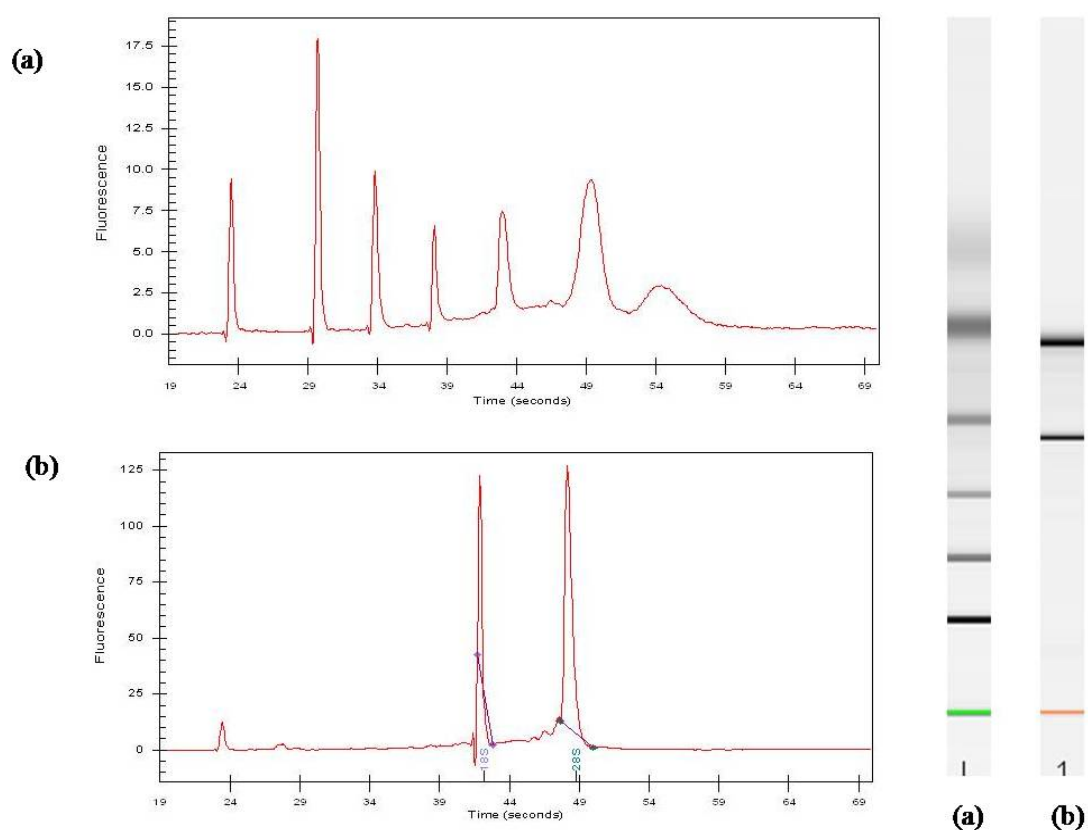


Fig. 4.5. The RNA extraction from mice ovaries. The quality and quantities were measured with Bio-nano chip. (a) ladder and (b) the extracted total RNA. The peaks in the electropherogram show 18S and 28S RNA without noise and the gel represented both 18S and 28S. The ribosomal RNA ratio is the extracted RNA was 2.67.

4.3.4. Immobilization of rKL onto the PEG-hydrogel

Different concentrations of KLstg were bound onto the PEG-hydrogel as showed in the schemes of PEG-hydrogel formation (Fig. 4.4). The released KLstg from the hydrogels after 3 days of incubation were measured (Table 4.2). In all the conditions about 15 % of the immobilized KLstg was released. The results indicate that the amount of TG-lysin peptide bound on PEG was enough even for the high concentrations of KLstg. The non-bound KLstg could be due to the short reaction time with the activated factor XIIIa or to the calcium concentration

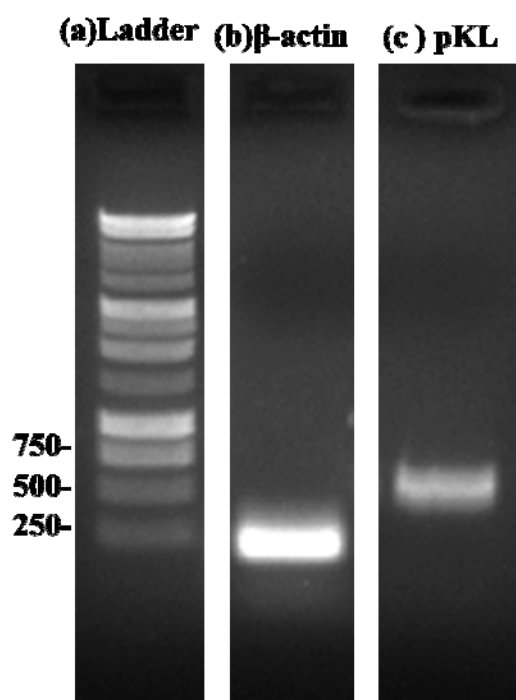


Fig. 4.6. Gel electrophoresis of PCR products. (a) Ladder, (b) β -actin, (C) pKL. The PCR products were loaded into a 1 % agarose gel in TEA.

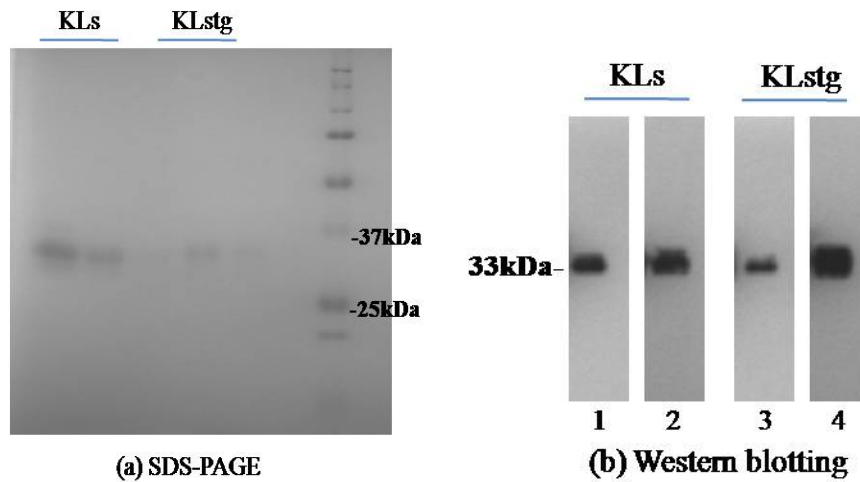


Fig. 4.7. The expressed rKL proteins were analyzed by (a) SDS-PAGE and (b) western blotting. The 1 and 3 in western blotting are the non-reducing condition and 2 and 4 are the reducing condition. SDS-PAGE and western blotting analysis showed that the molecular weight of KLs and KLstag were of ~ 33 kDa under reducing and non-reducing conditions.

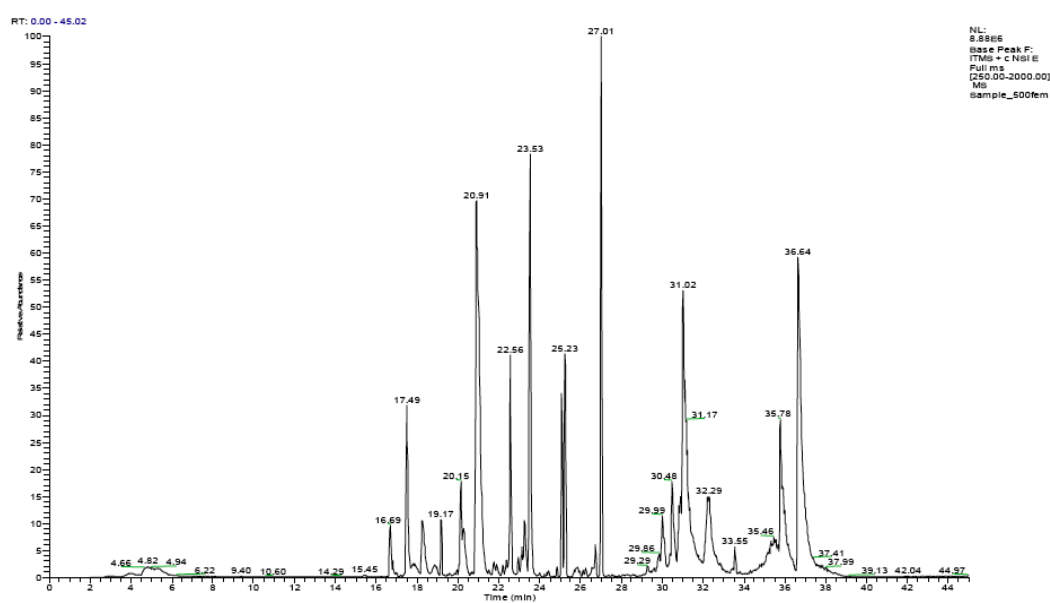
Table 4.2. Immobilization assay of the KLstg. The releasing KLstg from the hydrogels after 3 days of incubation were measured by SCF Elisa kit. In all of the conditions about 15 % of KLstg were released from the hydrogel.

Used concentration of KLtg (60 ng/ μ L)				KL (ng)
KLtg(μ M)	ul/aliquot	ng/aliquot	ng/gel	from Water
50.00	1.125	67.50	11.25	1.69
100.00	2.350	141.00	23.50	3.53
200.00	4.633	277.98	46.33	6.95
300.00	6.933	415.98	69.33	10.40

4.3.5. Ovarian tissue culture in the PEG-hydrogel system

Ovarian tissues from four days old mice were embedded in PEG-hydrogels functionalized with different concentration of KLstg and cultured for 10 days. The cultured ovarian tissues were compared to the condition supplied with 150 ng/mL of free KL in the medium. Fig.

4.9 shows that visible primordial and primary follicles were seen in the fresh ovary collected from four days old mice. More primordial follicles were present on the cortical side of the ovary and only some of the primary follicles were in the center of the ovary. Thus, the best effort was made to tear the ovaries in homogeneous parts; all including the middle part of the ovary. After 10 days in culture, the follicle growth was assessed from the cultured ovarian tissues (Fig. 4.10). Secondary follicles were observed only in the conditions: “without KLstg” and “100 μ M KLstg” and the number of secondary follicle was slightly higher in the “100 μ M KLstg” condition (Table 4.3).



Sequence Coverage: **71%**

Matched peptides shown in **Bold Red**

```

1  MKKTQTWIIIT CIYLQLLLFN PLVKTKEICG NPVTDNVKDI TKLVANLPND
51 YMITLNYVAG MDVLPSHCWL RDMVIQLSLS LTTLLDKFSN ISEGLSNYSI
101 IDKLGKIVDD LVLCMEENAP KNIKESPKRP ETRSFTPEEF FSIFNRSIDA
151 FKDFMVASDT SDCVLSSTLG PEKDSRVSVT KPFMLPPVAA SSLRNDSSSS
201 NRKAAKAPED SGLQLVPRGS EQKLISEEDL HHHHHH

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Fig. 4.8. Mass spectrum of the generated KL peptides in LC-MS. The sequence coverage was 71% in the both produced proteins. It confirmed the efficiency of the production/purification method.

When the ovarian tissue was cultured with higher concentration of KLstg (300 μ M), primary follicles were more present when compared with the other conditions. The

primordial follicles were present in the condition where soluble KL (KLs) was added to the medium. This result suggests that transmembrane form of KL can induce primordial development in 4 day-old mouse ovarian tissue. The observations suggest also that an optimal amount of the transmembrane form of KL is needed to activate the primordial follicles and allow the transition to the primary follicle state. However, due to the unknown initial number of primordial or primary follicle, it is difficult to comment on the rate of the primordial follicle activation induced by KL. Moreover, the sum of the visible number of oocytes in the dissected ovary was much smaller when compared to the number of oocytes found in a histological section of a four-day old mouse ovary. The activation of primordial follicle induces granulosa cell proliferation, morphogenesis and oocyte growth [49]. The granulosa cells in murine primordial follicle express only minimal amounts of KL mRNA and protein while the oocytes of primordial follicles express high amounts of c-Kit mRNA and proteins [20, 47]. This consideration rises the question of whether all of the oocytes in primordial follicle can be activated and resume growth by a KL stimulation? *In vivo*, primordial follicles are activated and exit the resting pool in a gradual manner [119]. However, it was observed *in vitro* that primordial follicles of species including cattle [120], primate [121], rat [51] and mouse [49] were spontaneously activated. Wandji et al. (1996) [120] proposed that this spontaneous activation was due to the absence of an inhibitor. Even though enough data are produced to assume that KL may have a role in primordial follicles activation and/or in promoting oocyte growth, it is still interesting to 1- elucidate how primordial follicles respond when over exposed to KL and to 2- define the respective roles of the soluble and membrane-bound in order to better understand KL signaling in the context of reproduction.

Table 4.3. Number of visible in ovarian tissue cultured in PEG-hydrogel functionalized with KLstg. High concentration of KL in the hydrogel and soluble KL did not have any visible secondary follicles.

Concentration of KL (μ M)	Follicle stages			
		primordial	primary	Secondary
0	ave. Number	1.4	2	1.2
	S.D.	1.95	1.22	1.3
100	ave. Number	0.8	2	2.6
	S.D.	1.032	1.49	0.96
300	ave. Number	0.56	6	0
	S.D.	0.88	3.04	0
150 ng/mL in the medium	ave. Number	2.33	3.5	0
	S.D.	3.5	2.17	0

4.4. Conclusion

The three different constructions of KL (KLs, KLstg, and KLstag) were successfully cloned. KLs and KLstg were expressed in HEK-293 cells, purified and tested. Ovarian tissues from four-day old mice were cultured in PEG-hydrogels functionalized with KLstg and compared with the condition where soluble KLs were added to the medium. The primordial follicles were activated and grew when they were cultured in the presence of immobilized KLstg but not when KL was supplied under a soluble form. Further investigations using this platform will certainly be of help to better understand the respective roles of the membrane-bound and the soluble form of KL during folliculogenesis. Furthermore, the established systems would be easily transferable for exploring the *in vitro* development of human ovarian follicles once the ethical clearance is obtained.

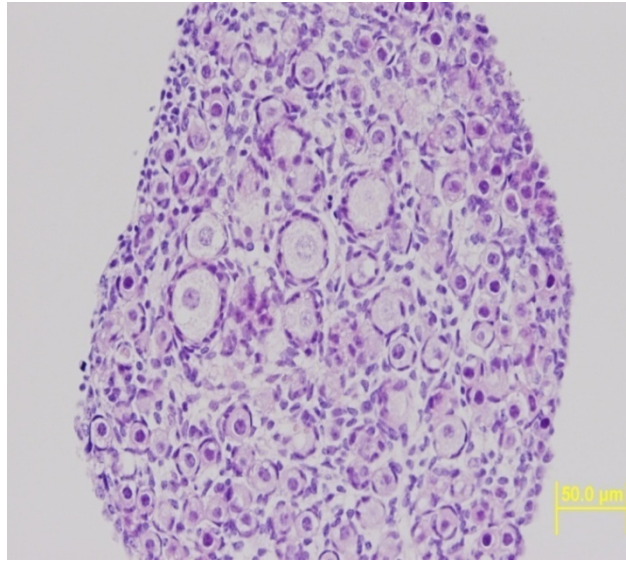


Fig. 4.9. Histology section of the whole ovary from four-day old mice.

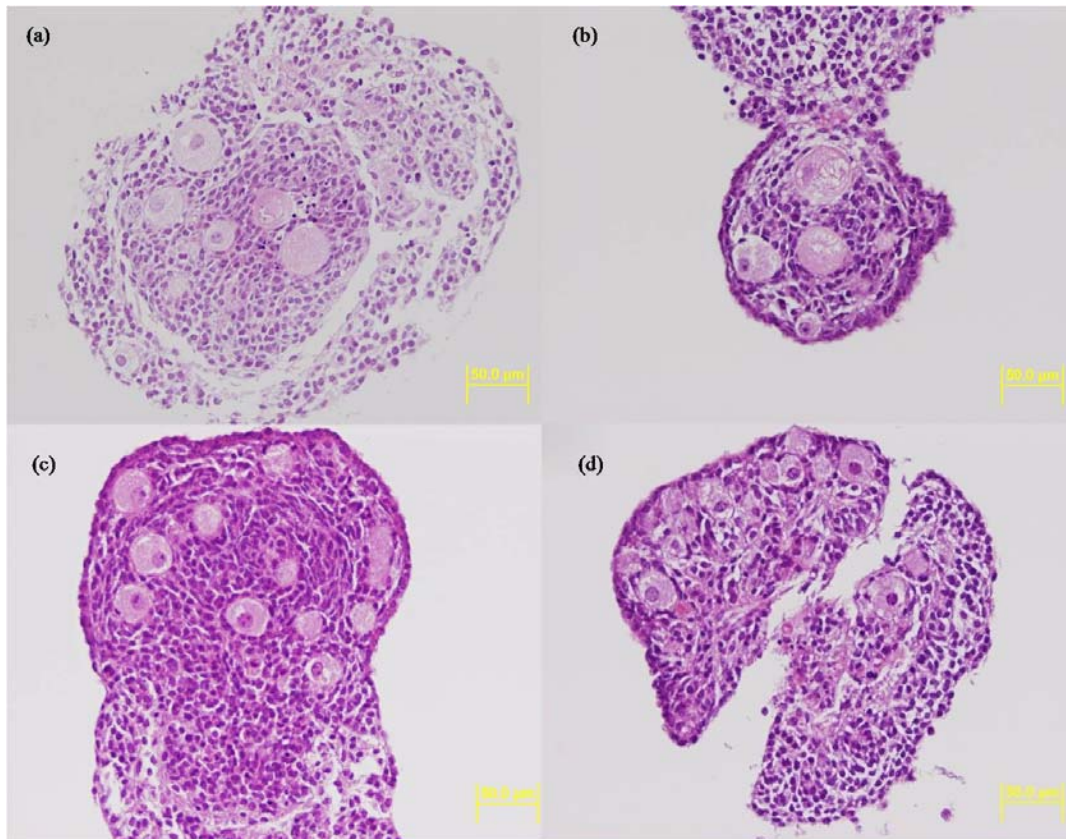


Fig. 10. Histology section of cultured ovarian tissues for 10 days in PEG-hydrogel functionalized with the different concentrations of KLstg. (a) no KLstg, (b) 100 μm of KLstg, (c) 300 μm of KLstg, and (d) 150 ng/mL of KLs was added in the medium.

Chapter V (original manuscript)

Ovarian lymphangiogenesis is necessary for hormonal maintenance and fetal development during murine pregnancy

Jong Eun Ihm^{1*}, Joseph M. Rutkowski^{1*}, Seung Tae Lee¹, Alexandra Liagre-Quazzola^{2,3},
Veronique I. Greenwood¹, Miriella C. Pasquier¹, Didier Trono^{2,3}, Jeffrey A. Hubbell¹, and
Melody A. Swartz¹

1: Institute of Bioengineering, School of Life Sciences, École Polytechnique Fédérale de Lausanne (EPFL), Switzerland

2: Global Health Institute, School of Life Sciences, École Polytechnique Fédérale de Lausanne (EPFL), Switzerland

3: "Frontiers in Genetics" National Center for Competence in Research, École Polytechnique Fédérale de Lausanne (EPFL), Switzerland

* These authors contributed equal work

Abstract

Lymphatic vessels surround ovarian follicles, but the roles of lymphatics and lymphangiogenesis in folliculogenesis and pregnancy are undefined. Here we demonstrate a critical role for ovarian lymphatics in murine reproduction by blocking lymphangiogenesis with mF4-31C1, a specific antagonist antibody to vascular endothelial growth factor receptor (VEGFR)-3. VEGFR-3 neutralization for two weeks prior to mating blocked ovarian lymphangiogenesis throughout folliculogenesis without limiting blood angiogenesis. While the number of oocytes ovulated and fertilized and uterine implantations were normal, all pregnancies were unsuccessful due to fetal defects and miscarriage. Preantral follicles isolated from treated ovaries grew and matured normally *in vitro*. When embryos from mF4-31C1 treated mice were transferred to untreated surrogate mothers, pregnancies were normal and came to term. Conversely, normal embryos transferred to treated surrogate mothers led to the same fetal deficiencies as previously observed *in situ*, suggesting that lymphatic capillaries maintain an ovarian hormonal environment necessary for fetal development and pregnancy maintenance. Indeed, pregnant mice with blocked follicular lymphangiogenesis also exhibited significantly reduced progesterone and estradiol, hormones sourced from the ovarian corpora lutea during pregnancy. In total, these results demonstrate that lymphangiogenesis is a necessary process for ovarian lymphatic capillaries that transport hormones and thereby critical for successful reproduction.

Keywords: corpus luteum, folliculogenesis, lymphatic, VEGF-C, VEGFR-3, blastocyst

Acknowledgments:

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5.1. Introduction

Lymphatic vessels are present within the ovary and surround follicles during maturation [122, 123], but the importance of the lymphatic vasculature and lymphangiogenesis in the ovary is unclear. Consequently, the potential roles of lymphatic vessels in follicle maturation and pregnancy, and the extent or even necessity of lymphangiogenesis in reproduction, are undefined. This contrasts with ovarian blood angiogenesis, whose critical roles in follicular nourishment and maturation as well as the formation and maintenance of the corpus luteum is well appreciated; indeed, oocyte fertilization, embryonic implantation, and pregnancy all require blood angiogenesis [124-126]. Lymphangiogenesis, which is often concurrent with blood angiogenesis [127], may play an equally important role in these processes.

Adult blood angiogenesis requires signaling via vascular endothelial growth factor (VEGF) receptor -2 (VEGFR-2), most potently by VEGF ligation [128, 129]. In murine ovaries, VEGF expression increases during angiogenic growth phases [130], and blockade of VEGFR-2 signaling effectively prevents angiogenesis, resulting in a marked decrease in ovarian weight, blood vessel density, number of corpora lutea, and infertility [131-133]. Since gonadatropin treatment apparently does not correct these deficiencies [134], it is likely that follicle maturation and successful pregnancy are highly dependent on VEGFR-2-mediated neovascularization [124, 135].

VEGFR-3 is expressed primarily on lymphatic endothelial cells (LECs) in adult tissue [54, 136], and its signaling, via ligation by VEGF-C or VEGF-D, is necessary for lymphangiogenesis by inducing LEC proliferation and migration [56, 57, 136, 137]. Blockade of VEGFR-3 signaling, using a function blocking antibody such as mF4-31C1 (ImClone Systems), completely blocks the initiation of new lymphatic vessels in adult mice

without affecting pre-existing lymphatic morphology or function and without apparently affecting blood angiogenesis [56, 138]. Here we investigate the roles of lymphatic vessels and lymphangiogenesis in reproductive functions of the ovary. Specifically, we hypothesize that lymphangiogenesis within the ovary parallels blood angiogenesis during reproductive cycles [139-141] and that these new lymphatic capillaries may serve to balance hormones produced within the ovary, transport hormones from the ovary and corpus luteum, and aid in hormonal communication between the uterus and ovaries during pregnancy [142-144]. Using combined *in vivo*, *ex vivo*, and *in vitro* methods, we examined which aspects of fertility are influenced by inhibited lymphangiogenesis, including oocyte and follicular development and maturation, embryonic implantation in the uterus, and embryonic development. We show that blocking ovarian lymphangiogenesis prevents viable, full-term pregnancies due to decreased systemic hormone levels, thereby demonstrating a critical role for the ovarian lymphatic vasculature in reproduction.

5.2. Materials and Methods

5.2.1. Animal procedures

All protocols were approved by the Veterinary Authorities of the Canton Vaud according to Swiss law (protocols 1687, 1988, and 1988.1). The function-blocking antibody against murine VEGFR-3, mF4-31C1, was kindly provided by ImClone Systems [57]. For two weeks prior to mating, 0.25 mL of 2.5 mg/mL mF4-31C1 was injected intraperitoneally every two days. 0.25 mL saline was similarly injected for some control groups with no adverse effects on reproductive potential.

For studies without fertilization, 3 week old female F1 hybrid mice (C57Bl/6JxCBA/caj, Charles River Laboratories, France) were treated for two weeks and then sacrificed.

Follicles and ovaries were collected for subsequent *in vitro* culture and histological examination, respectively.

In studies requiring fertilization, 4-6 week old female F1 hybrid (C57Bl/6JxCBA/caj) mice were treated for two weeks before mating (to ensure spanning two full menstrual cycles). At approximately 6-8 weeks of age, mice in estrus were mated and coitus was evaluated by the presence of a vaginal plug 16 hours post-mating. For embryo retrieval, *ex vivo* culture, and transplantation, mice were sacrificed 42 hours post-mating and two-cell embryos collected by oviduct flushing with M2 medium (Sigma-Aldrich, St. Louis, MO).

Embryos were implanted into pseudo-pregnant recipient NMRI mice (Charles River) following standard implantation protocols. Embryos from mF4-31C1 treated and untreated F1 hybrid donors were implanted into treated and untreated recipients. Recipient mice were anesthetized using an intraperitoneal injection of ketamine (100mg/kg) and xylazine (10mg/kg). A small midsagittal incision, over each oviduct, was made and the donor embryos were deposited into each oviduct by mouth pipetting under a stereomicroscope. The incision was then sutured and pregnancies were permitted to continue through day 17. Implantation success was consistently >90%.

For examination of fetal development and uterine implantation, mice were sacrificed at pregnancy day 17 or after birth. Implantation spots were counted in the uterus and fetuses were graded as either (a) normal, (b) grade i – normal sized but abnormal coloration, (c) grade ii –under-developed fetus (in size or limb development), and (d) grade iii – implanted cell mass or necrotic fetus (refer to Figure 5.2B for examples).

5.2.2. *In vitro* follicle culture and maturation

To determine the direct effects of *in vivo* mF4-31C1 treatment on normal follicle maturation potential, ovaries were isolated from 5-week-old mice following two weeks of

antibody treatment. *In vitro* maturation of preantral follicles was performed as previously described [3, 145]. Briefly, whole ovaries were placed in 3 mL of L-15 Leibovitz-glutamax medium (Gibco, Carlsbad, CA) with 10% FBS (HyClone Laboratories, Logan, UT) and 1% penicillin-streptomycin (Gibco) solution. Preantral follicles with diameters of 100-130 μ m were mechanically separated from the ovaries, washed, transferred to individual 10 μ L droplets of MEM-alpha-glutamax medium containing 5% FBS, 1% ITS (5 μ g/mL, insulin, 5 μ g/mL, transferrin, and 5 μ g/mL selenium mixture solution; all Gibco), 1% penicillin-streptomycin, and 100 MIU/mL recombinant human follicle stimulating hormone (hFSH) (Organon, Switzerland).

On day 12 of *in vitro* culture, follicle maturation was induced by exposing to medium lacking hFSH, but supplemented with 2.5 IU/mL hCG-Pregnyl (Organon) and 5 ng/mL murine epidermal growth factor (Sigma). After 16 hours, oocytes were retrieved by removing the follicular cumulus cells using 200 IU/mL of hyaluronidase (Sigma). Oocytes were classified by the following maturation states: germinal vesicle (GV), germinal vesicle breakdown (GVBD) and metaphase II (MII).

In groups where VEGFR-3 was neutralized directly on normal follicles, follicles were cultured using the above reagents in a method modified from West, et al. [73]. 10 μ g/mL mF4-31C1 was added to the culture medium of the test group.

5.2.3 *Ex vivo* development of 2-cell embryos

To determine the direct effects of VEGFR-3 inhibition on preimplantation embryonic development, two-cell embryos were retrieved from the oviducts and cultured in 4-well dishes containing 400 μ L of M16 medium (Sigma). The number of embryos that developed into the 8-cell, morula, and blastocyst stages were quantified and imaged under a Nikon

SMZ1000 stereomicroscope with a Nikon DS-5M monochrome camera at 66, 90 and 114 hours after mating, respectively.

5.2.4 Immunofluorescence, immunohistochemistry, and histology

To visualize lymphatic vessels, 6- μ m-thick ovary and uterus cryosections were labeled with a primary antibody to the lymphatic-specific marker LYVE-1 (1:500; 07-538; Upstate, Charlottesville, VA). For blood vessels, ovaries were labeled with a FITC-conjugated primary antibody to CD31/PECAM-1 (1:200; 550274; BD Pharmingen, San Jose, CA). Vessels were also co-labeled for VEGFR-3 (1:100; AF743; R&D Systems, Minneapolis, MN). Sections were also labeled for the macrophage-specific surface marker F4/80 (1:50; MCA497; AbD Serotec, Oxford, UK) and collagen IV (1:1000, 10760; MP Biomedicals, Irvine, CA). These antibodies were detected with Alexafluor 488 or 594-conjugated donkey, rabbit, or goat IgG secondary antibodies (1:200, Molecular Probes) and nuclei were labeled with DAPI mounting medium (Vector Labs, Burlingame, CA). Fluorescent labeling was observed and imaged using a Zeiss Axiovert 200M microscope with a Zeiss MRm camera. Slides were then rinsed and counterstained with hematoxylin and eosin, dehydrated, mounted with Eukitt (Fluka Chemie, AG, Buchs, Switzerland), and imaged again using a color Zeiss MRc camera. The corresponding fluorescence and chromogenic images were then compared for identification, and subsequent quantification, of follicular development.

To label apoptotic cells, a fluorescence TUNEL kit was used according to manufacturer's instructions (Roche Diagnostics, Rotkreuz, Switzerland). Oil red O (Sigma-Aldrich, Buchs, Switzerland) was used to stain lipids in ovarian frozen sections. Sections were counterstained with hematoxylin and immediately mounted with M  wiol-based mounting medium for imaging.

5.2.5 Serum Analysis

Serum was collected from all mice when sacrificed and analyzed using ELISA kits for estradiol (Calbiotech, Spring Valley, CA) and progesterone (BioSource, Carlsbad, CA) levels according to manufacturer's instructions. Absorbance was measured using a Tecan Safire2 plate reader (Tecan, Männedorf, Switzerland).

5.2.6 Image analysis and quantification

To quantify macrophages, Oil red O, and TUNEL labeled slides, images of entire ovaries were assembled, the ovary body was outlined, and the percentage of positive area measured using Metamorph 6.3 (Molecular Devices Corp., Sunnyvale, CA). For lymphatic and blood vessel quantification, we considered each follicular maturation state. Each follicle was outlined using a Wacom CintiQ freehand graphic monitor (Wacom Co., Ltd., Saitama, Japan) for accuracy. Vessel labeling was defined by fluorescence threshold and the number of positive pixels for each follicle was measured. The average vessel area for each maturation state is reported. Follicle maturation states were scored as follows: (i) preantral follicle (secondary follicle), (ii) antral follicle (small follicle with formed atrium), (iii) Graffian follicle (large follicle with significant atrium), and (iv) corpora lutea (Supplemental Figure 1). These divisions were consistently identified across multiple examiners.

5.2.7 Statistical Methods

For determining statistical significance between treatments in follicle vascularization over the stages of maturation, follicle survival and maturation, and embryo development, ANOVA followed by DUNCAN was used. Students't-tests were used to compare other factors in treated vs. untreated plasma or in ovaries as a whole.

5.3. Results

5.3.1 VEGFR-3 neutralization prevents successful murine pregnancy

Female mice were mated at 6-8 weeks of age following 2 weeks of treatment with either (i) anti-VEGFR-3 neutralizing antibody (mF4-31C1), (ii) saline, or (iii) no injection (normal). All treatments were ceased before mating. Mice receiving saline or no injection were equally successful in giving birth to normal and healthy pups. Mice treated with mF4-31C1, however, failed to produce a single live birth in the animals tested. This unexpected response to VEGFR-3 blockade prior to mating led to the hypothesis that lymphangiogenesis within the ovary helps mediate reproductive ability.

5.3.2 Ovarian lymphangiogenesis but not blood angiogenesis is inhibited by VEGFR-3 blockade

First, we examined blood and lymphatic vessels in the ovaries of saline-treated mice using CD31 and LYVE-1 co-labeling, and examined their relative expression of VEGFR-3. While all lymphatic (LYVE-1⁺) vessels expressed VEGFR-3 (Fig. 5.1A), limited VEGFR-3 expression was found on follicular blood vessels (Fig. 5.1B). Lymphatic vessels were observed surrounding nearly every follicle at all maturation states and did not penetrate into the follicular body or thecal layers (Fig. 5.1A, C). The extent of lymphatic vascularization was dependent on the maturation stage of the follicles, with preantral follicles displaying only a few sparse lymphatics and corpora lutea displaying a significantly higher degree of peripheral lymphatic vessels. In ovaries from mice treated with mF4-31C1, the extent of lymphatic vascularization was greatly reduced at all stages of follicular maturation, as measured by vessel density (Fig. 5.1D). There was not, however, a complete lack of lymphatic vessels. This was consistent with our previous studies where VEGFR-3

neutralization prevented lymphangiogenesis but had no morphological effects on pre-existing lymphatic vessels [56, 57].

While mature blood vessels do not express VEGFR-3, angiogenic blood vessels have been observed to express VEGFR-3 during normal development as well as in tumors and healing wounds [146-148], and there is evidence that VEGFR-3 inhibition may limit tumor angiogenesis [60, 149]. Since blood angiogenesis in the ovary is necessary for pregnancy [124-126, 134, 150], we examined the blood vessels to assess whether mF4-31C1 had any effects on ovarian blood angiogenesis. We found VEGFR-3 expression primarily limited to lymphatic vessels, with the exception of the blood vasculature within the corpora lutea (Fig. 5.1B). More importantly, the extent of blood vascularization, as quantified by vessel density, was not significantly affected by VEGFR-3 blockade (Fig. 5.1E). Therefore, ovarian blood angiogenesis appeared to be unaffected by VEGFR-3 neutralization, consistent with our findings in dermal wound healing and regeneration [56, 57].

5.3.3 VEGFR-3 neutralization pre-fertilization leads to retarded embryonic development

Since pregnancies were not successful in VEGFR-3 neutralized mice, we sought to determine at what stage post-fertilization observable differences could be seen in embryonic and fetal development. Mice were treated for two weeks and then mated, and the uteri examined at pregnancy day 17. We observed no differences in the number of implantation sites in the uteri of treated vs. control mice, but fewer fetuses remained in mF4-31C1-treated mice at pregnancy day 17 (Fig. 5.2A). More strikingly, those fetuses remaining were dramatically smaller and underdeveloped (Figs. 5.2A,B), threatening future miscarriage. Combined, these results indicate that multiple abortions had already occurred by day 17, and that the remaining fetuses were likely not viable.

To determine whether the numbers of ovulated oocytes were normal and to examine blastocyst development from VEGFR-3-blocked mice, mice were sacrificed 42 hr after mating and 2-cell embryos were flushed from the oviduct. The numbers of harvested 2-cell embryos per mouse were the same in normal vs treated mice, with an average of 7.1 ± 2.5 and 8.3 ± 1.5 taken from normal and treated mice, respectively. Additionally, all 2-cell embryos were cultured *in vitro* to blastocysts with 100% success, regardless of treatment (Fig. 5.2C). Thus, although VEGFR-3 neutralization pre-mating dramatically affected embryonic development, it did not appear to reduce ovulation quantity or fertilization potential of ovulated oocytes.

5.3.4 VEGFR-3 neutralization decreases number of healthy follicles but has no direct effect on their quality

To explore the possible effects of VEGFR-3 signaling and lack of lymphatic vasculature on follicular maturation potential in virgin mice, preantral follicles (100-130 μm in size) were retrieved after 2 weeks of treatment. The numbers of healthy preantral follicles successfully retrieved was lower ($P < 0.01$) from mF4-31C1-treated mice than from saline-treated mice (Fig. 5.3A). This was due to a noted fragile contact between the oocyte and granulosa cells in mF4-31C1 treated mice (noting that preantral follicles require interactions between surrounding granulosa and theca cell layers and the oocyte [145]). These contacts were apparently not, however, due to any loss of integrity of the basal lamina as examined by collagen IV staining (Fig. 5.3B), since no differences were observed in the granulosa-thecal boundary. While these discrepancies may impact the local hormonal environment *in vivo*, once separated and cultured *in vitro*, both groups of preantral follicles exhibited similar survival rates ($P = 0.763$, Fig. 5.3C). Surviving follicles were able to mature normally, as defined by the method [3], through the GVBD and MII phase with equal success (Fig. 5.3C).

Finally, to determine whether VEGFR-3 blocking had any direct effects on folliculogenesis, we isolated preantral follicles from untreated mice and cultured them in the presence of 10 $\mu\text{g/mL}$ mF4-31C1 *in vitro*. All follicles survived (Fig. 5.3D) and grew to similar sizes ($P=0.299$, Fig. 5.3E). Thus, VEGFR-3 blocking had no direct effect on *in vitro* growth and maturation of secondary follicles.

5.3.5 Loss of lymphatic capillaries does not alter macrophage recruitment, lipid accumulation, or apoptosis within the ovary

Our combined *in vivo* and *in vitro* results suggest that failed pregnancies derive from alterations in the follicular environment due to the lack of lymphatic vessels. Since immune function may be important in mediating the balance between hormone accumulation [151] and follicle maturation and ovulation within the ovary [152, 153], and since lymphatic capillaries may be important in ovarian immune cell trafficking [139, 140], we examined macrophage populations in the ovaries (Fig. 5.4A). We found no significant differences in macrophage numbers within the ovaries ($P=0.362$; Fig. 5.4B). Therefore, the lack of lymphatic capillaries did not visibly alter overall macrophage recruitment in the ovary.

Furthermore, lymphatic insufficiencies have been linked to excessive tissue lipid accumulation in skin [154, 155]. Since lipids are necessary for hormone synthesis by granulosa and luteal cells in the ovary [156, 157], we examined gross lipid content in the ovaries via oil red O staining (Fig. 5.4C), but no differences were observed between mF4-31C1-treated and control animals ($P=0.532$; Fig. 5.4D).

Finally, we sought to determine whether blocking VEGFR-3 would affect cellular apoptosis, which normally occurs within certain bodies of the ovary throughout the menstrual cycle [158]. Analysis of apoptotic cells revealed a similar distribution and number of TUNEL-positive cells within the ovary (Fig. 5.4F) in both groups. Consistent

with earlier findings [158, 159], apoptotic cells were confined primarily to the interior layer granulosa of regressing antral follicles, regressing corpora lutea, and post-ovulatory cells at the ovarian wall in both groups (Fig. 5.4E). Thus, neither the direct blockade of VEGFR-3 nor the resultant lack of a significant lymphatic vasculature led to abnormal cell apoptosis in the ovary.

5.3.6 Loss of ovarian lymphatics results in significantly reduced hormone levels during pregnancy

With few other differences between ovaries from treated and untreated mice noted, we sought to determine whether the lack of ovarian lymphatics altered hormone levels during pregnancy. Serum collected from systemic circulation 42 hours after mating revealed no change in progesterone levels (Fig. 5.5A), but a significant decrease in estradiol levels (Fig. 5.5B). As estradiol is sourced from the granulosa/luteal cells in the corpus luteum during pregnancy, and since mF4-31C1 treatment had the greatest effect on decreasing follicle-associated lymphatic capillaries around the corpora lutea (Fig. 5.1D), these data suggest that ovarian lymphangiogenesis is critical for hormone transport and that decreased follicular lymphatics lead to decreased progesterone and estrogen transport, critical for maintaining pregnancy, from the ovary. These findings also support the hypothesis that intraovarian lymphatic capillaries are the entry point of ovarian sourced hormones to the systemic circulation [144].

As hormone secretion by the murine corpora lutea has also been linked to proper blood angiogenesis, we verified the blood vasculature of pregnant mouse ovaries at day 17. In both untreated and treated mice, the blood vasculature of the corpora lutea appeared normal (Fig. 5.5C) while the lymphatic vasculature surrounding these bodies in mF4-31C1 treated ovaries was notably deficient.

5.3.7 Reduced ovarian hormone production by recipient mothers results in poor fetal development and miscarriage of transplanted normal embryos

Finally, to demonstrate an ovarian, and not uterine, cause to pregnancy failures, we isolated two-cell embryos from normal and treated mothers and implanted them into normal and treated pseudo-pregnant recipient mothers. Regardless of the treatment of the donor mother, transplantation of embryos into normal mothers resulted in normal implantation and fetal development with only normal, viable fetuses found in the uterus at day 17 (Fig. 5.6A). Conversely, the deficient ovarian hormone signaling demonstrated in treated recipient mothers led to retarded fetal development of implanted embryos (Fig. 6A). In fact, the developmental deficiencies observed (Fig. 5.6B) were nearly identical to those found during *in situ* pregnancies (Fig. 5.2A,B). This supports the hypothesis of early ovarian lymphangiogenesis being necessary for subsequent pregnancy success.

Further verification of an ovarian source to failed pregnancies was found upon examination of the uterine blood and lymphatic vasculature from normal and treated mothers. No changes in the blood or lymphatic vessel network of the ovarian wall were noted in early pregnancy (Fig. 5.6D). This lack of differences in the uterine vasculature supported the findings of normal implantation rates, and reinforced that ovarian lymphangiogenesis is the likely process affecting hormone maintenance.

5.4. Discussion

Taken in total, these results demonstrate that ovarian lymphatics, particularly those that develop during folliculogenesis, are necessary for maintaining pregnancy by providing a conduit for hormone transport. We saw that blockade of VEGFR-3 effectively halted

lymphangiogenesis of maturing follicles within the ovary while not visibly affecting blood angiogenesis, macrophage recruitment, lipid accumulation, or overall cell apoptosis. In the absence of lymphangiogenesis, there were fewer patent secondary follicles, but those that were patent could mature normally, were ovulated, and could be fertilized. Embryonic masses naturally implanted in the uterus and partially developed, but all eventually miscarried; there were no successful births despite a normal number of uterine implantation spots. The absence of new lymphatics in the ovary appears mainly to disturb progesterone and estradiol levels during pregnancy. As these hormones are sourced from the corpora lutea, it is likely the follicular lymphatic capillaries are necessary in regulating a hormonal environment conducive to normal pregnancy maintenance.

The ovarian microvasculature is critical in regulating hormonal transport during pregnancy. Normally, as follicles mature, the theca layers become vascularized by blood vessels [160] and support follicles by synthesizing estrogen [161]; abnormalities in the theca cell layers can result in infertility [162]. Post-implantation, proper blood vascularization is necessary for successful pregnancy [124] and blocking blood vessel formation in the corpus luteum leads to pregnancy failures [126]. The developing blood vasculature of the corpus luteum permits this pseudo-organ to function properly, supplying increased progesterone and estrogen to the uterus to maintain pregnancy [126, 150, 163]. New lymphatic capillaries must supply a route by which hormones produced within the ovary enter systemic circulation [144]. Additionally, it has also been suggested that retrograde transfer of prostaglandin E₂ – involved in many crucial processes of pregnancy, including maintenance of the corpora lutea - from the uterus to the ovary may occur via lymphatic transport [143, 150]. Indeed, our data demonstrates that ovarian lymphatic vessels and lymphangiogenesis are essential for reproduction. The poorly connected granulosa of isolated follicles and lower levels of progesterone and estradiol during pregnancy are likely

related [164]. The blocked growth of lymphatic capillaries during folliculogenesis disturbs the hormonal balance, as evidenced by reduced estradiol with VEGFR-3 neutralization. The lymphatic vasculature of these follicles is then insufficient to modulate the corpus lutea and their hormone secretions during pregnancy. As VEGFR-3 signaling, and therefore, lymphangiogenesis, was only blocked prior to mating, oocytes are ovulated and fertilized and embryos implant normally in the uterus, we have isolated a developmental period in which lymphangiogenesis appears to most critically occur.

Another important role of lymphatic vasculature is to maintain fluid balance and interstitial fluid pressure (IFP). Throughout the body, these roles are inherently tied to lymphatic function. In the ovary, follicles become increasingly vascularized as they grow and a fluid-filled antrum is formed. The IFP in antra of developing follicles is approximately 15 mmHg regardless of size and drops rapidly to 5mm Hg immediately preceding ovulation [165]. Post-ovulation, the IFP in the highly vascularized corpus luteum has been reported at a very high 50 mmHg [165]. Ovarian lymphatics clearly must play a role in modulating fluid pressures. Furthermore, concurrent lymphangiogenesis is likely necessary to drain extravasated fluid from the newly formed blood capillaries [140] and may help to regulate morphogenetic processes and signaling on the luteal cells by controlling interstitial flow, an important morphoregulator for many cell types [166].

Lack of ovarian lymphatics have also been reported in ADAMTS-1 knockout mice [122] and Frizzled4 knockout mice exhibit low levels of ovarian VEGF-C, the primary ligand to VEGFR-3 [167]. Both of these strains are infertile, despite normal mating behavior; infertility in these mice was concluded to be the result of failed hormone transport, intrafollicular pressure modulation, or maintenance of the corpus luteum. Mice possessing mutations in VEGFR-3 such that their lymphatic capillaries are present but poorly functional can reproduce, albeit at a lower success rate than wildtype mice [168, 169].

In conclusion, our data demonstrate that VEGFR-3-mediated lymphangiogenesis in the ovary is necessary for pregnancy by modulating levels of progesterone and estrogen from the corpora lutea. With anti-lymphangiogenic therapies aimed at preventing tumor metastases proposed as a cancer therapy [60, 149, 170] and pro-lymphangiogenic therapies proposed for treating lymphedema [168, 171, 172], it is critical to understand the role of lymphangiogenesis in the ovary and the role of lymphatics in fertility. Moreover, an increased knowledge of the physiologic role of lymphangiogenesis in the ovaries may provide insight into causes of infertility (and potential therapeutic strategies) and permit a more careful examination of vasculogenesis and lymphangiogenesis inherent with ovarian cancers.

Figures

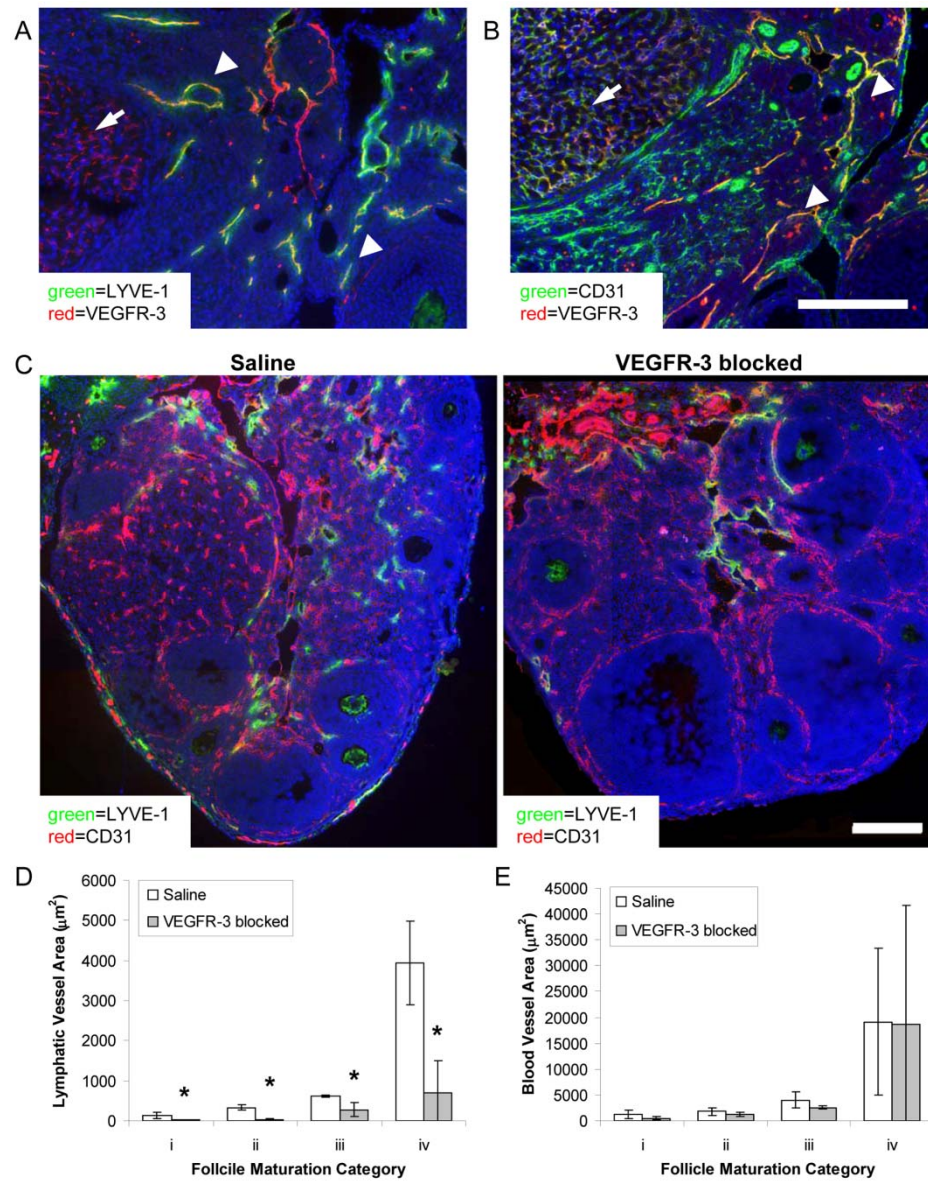


Fig. 1. Ovarian and follicular lymphangiogenesis, but not blood angiogenesis, was inhibited by VEGFR-3 blockade in the ovary, preventing successful pregnancy. A) Ovarian lymphatic vessels (green, LYVE-1) are VEGFR-3 (red) positive (arrowheads). Some non-lymphatic associated VEGFR-3 is found on the blood vessels within the corpora lutea (arrows). B) Blood vessels (green, CD31) are mostly negative for VEGFR-3 (red), with the exception of those within the corpora lutea (arrow). As lymphatic vessels also express CD31, the strong VEGFR-3 colocalization marks lymphatics (arrowheads). Bar=200 μm . C) The extent of lymphatic vasculature (green, LYVE-1) normally developing within the ovary, left, was reduced in VEGFR-3 blocked ovaries, right; the blood vasculature (red, CD31) appeared to be unaffected by the treatment. Bar=200 μm . D) Quantification of lymphatic vessel coverage in (i) preantral follicles, (ii) small follicles with formed atrium, (iii) large follicles with significant atrium and (iv) corpora lutea demonstrated the significant reduction in lymphatic vessels at each maturation state with VEGFR-3 blockade. E) Blood vessel coverage was not affected by VEGFR-3 blockade, despite some blood vessels expressing VEGFR-3. Notice the normal increase in vascularization of the follicles with maturation for both lymphatic and blood vessels. * $P < 0.05$ between treatments.

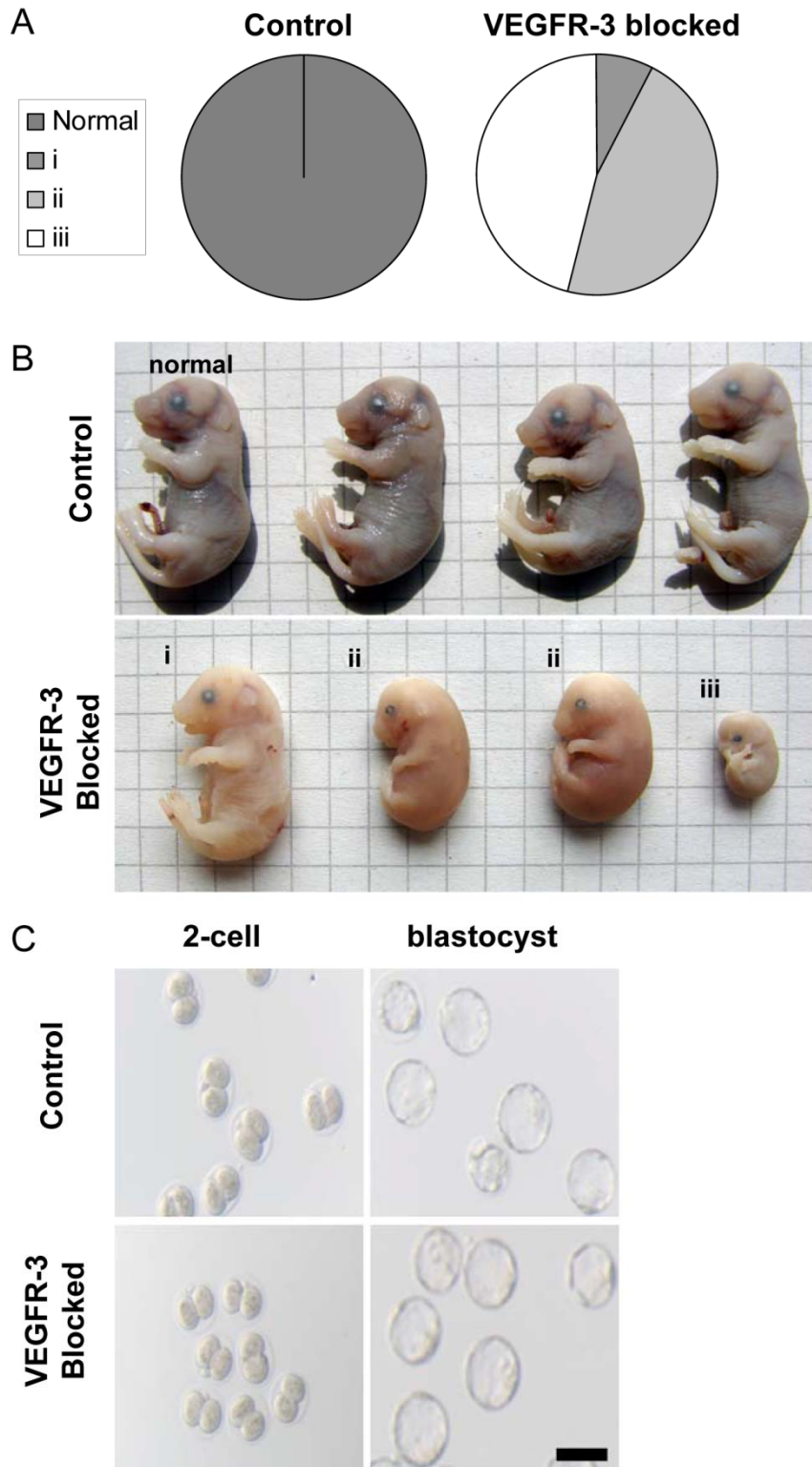


Fig. 2. Pregnancies occurred after lymphangiogenesis was blocked, but embryonic development was severely impaired in the womb. A) In mice treated with mF4-31C1 prior to fertilization, few bodies remained within the uterus at pregnancy day 17, and they were scored as normal, (i) normal sized but discolored, (ii) deficient size and underdeveloped, and (iii) identifiable cell masses. B) Fetuses extracted at pregnancy day 17 from treated animals displayed a marked deficiency from control animals. Grid=4mm x 4mm. C) 2-cell embryos were extracted from fertilized VEGFR-3 treated mice and culture in vitro. The appearance of both 2-cell embryos (left) and blastocysts (right) appeared identical to those taken from control animals. Scale bar=100 μ m.

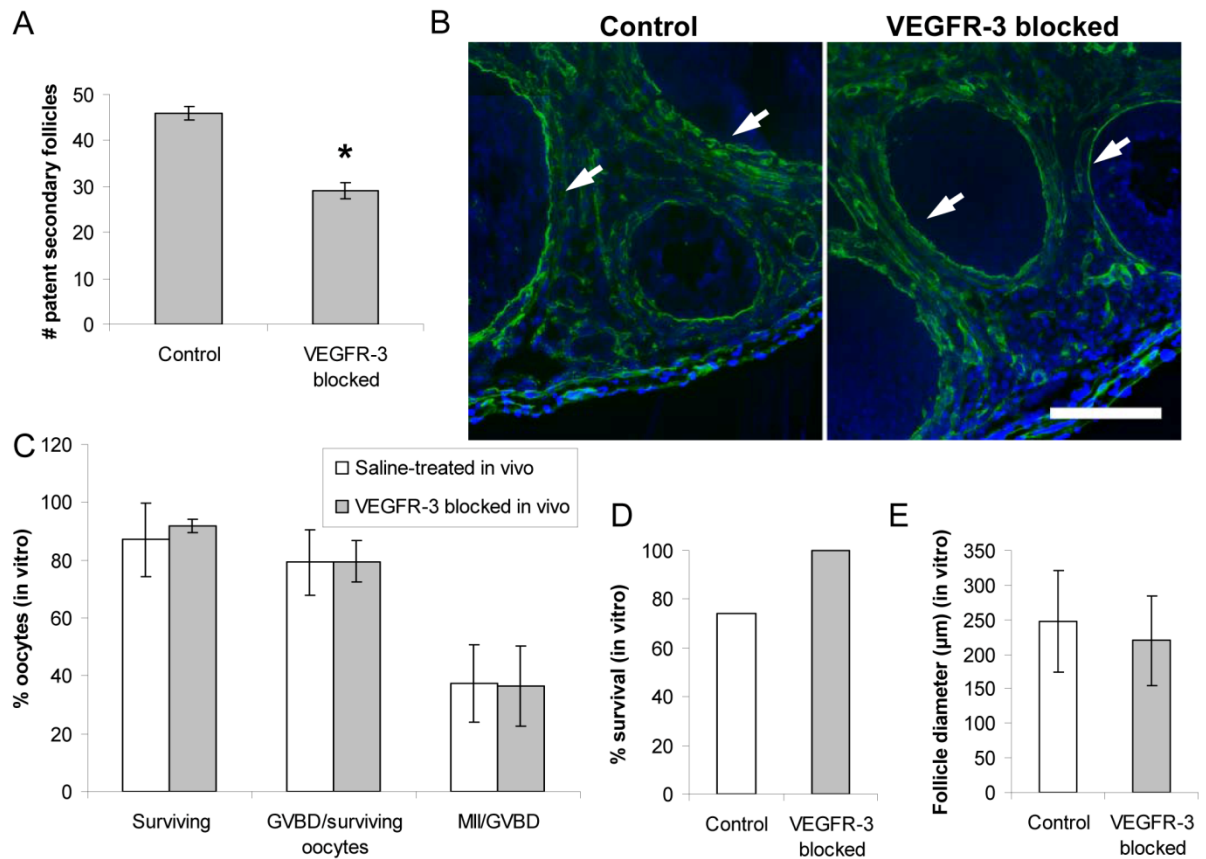


Fig. 3. Secondary follicles, after being retrieved from the ovaries of treated mice, matured normally in vitro. A) Fewer patent follicles could be successfully separated from collected ovaries in treated mice. B) Patency was not determined by basal lamina quality, as collagen IV staining (green) displayed intact basement membranes (arrows) in both control and treated follicles. Bar=100μm. C) Follicles and their oocytes separated from ovaries of VEGFR-3 treated mice survived normally in vitro. Surviving germinal vesicle (GV) oocytes from treated and control mice mature to germinal vesicle breakdown (GVBD) and metaphase II (MII) stages using the in vitro drop culture technique at equal rates. D) Survival of secondary follicles directly treated with the VEGFR-3 blocking antibody in vitro was uninhibited. E) Maturation potential, as determined by follicle size, was also unaffected by direct VEGFR-3 treatment on the follicles.

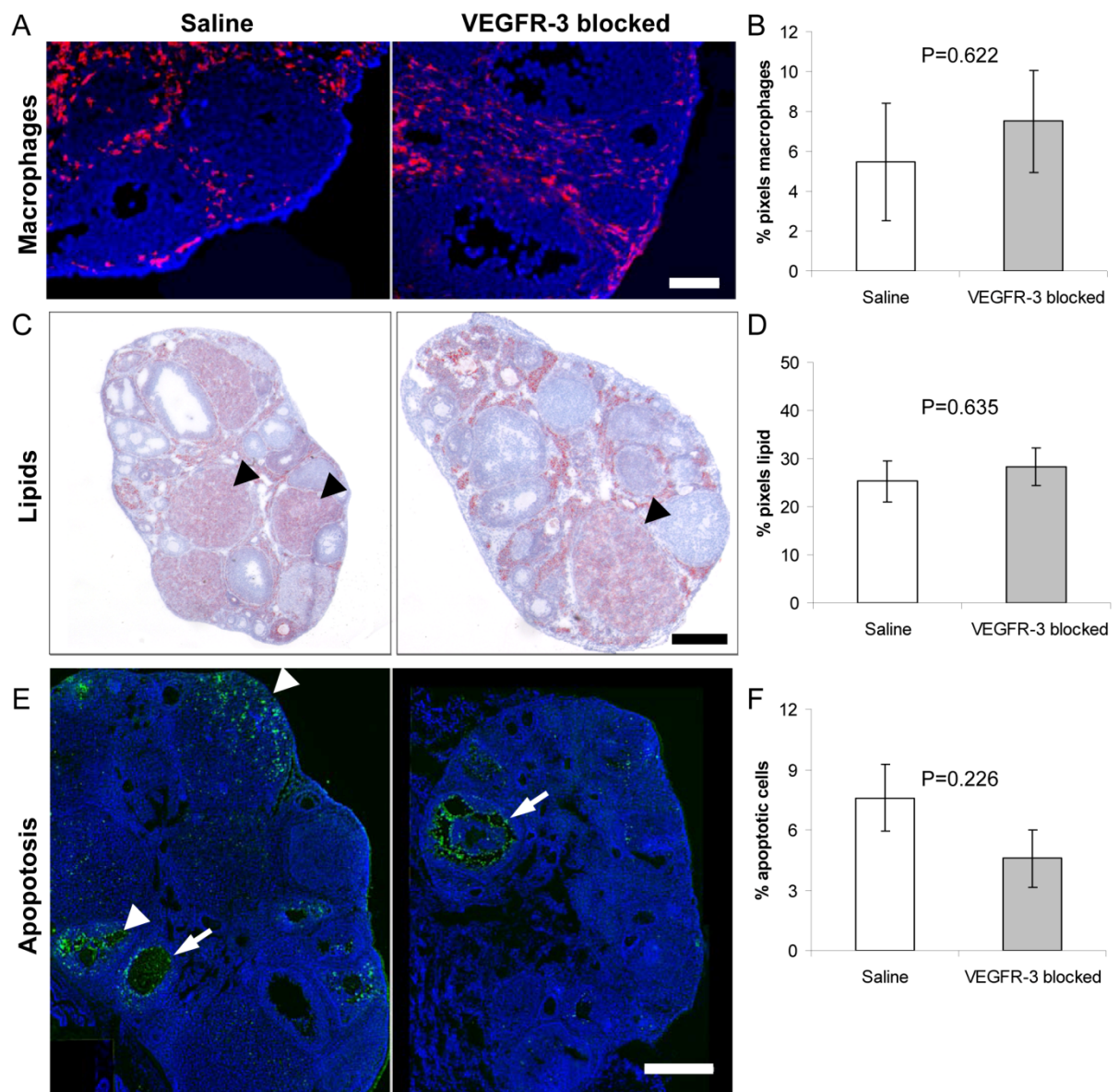


Fig. 4. Macrophage recruitment, lipid accumulation, and cell apoptosis in the ovary appeared to be unaffected by VEGFR-3 blockade. A) Macrophages (red, F4/80) present in the ovary was limited to follicular peripheries in both control and treated ovaries. Bar=100 μ m. B) The total number quantified within the ovary was unchanged. C) The ovaries contain large amount of lipids (red, Oil Red O), particularly in the corpora lutea (arrowheads). Bar=300 μ m. D) Lipid accumulation in the ovary was not significantly different without lymphatics. E) Apoptotic cells (green, TUNEL) were limited to interior granulosa cells (arrows) of regressing follicles and regular apoptosis of regressing corpora lutea and corpus hemorrhagicum (arrowheads) in both treatments. Bar=100 μ m. F) No difference was measured in the number of apoptotic cells within the ovary with mF4-31C1 treatment.

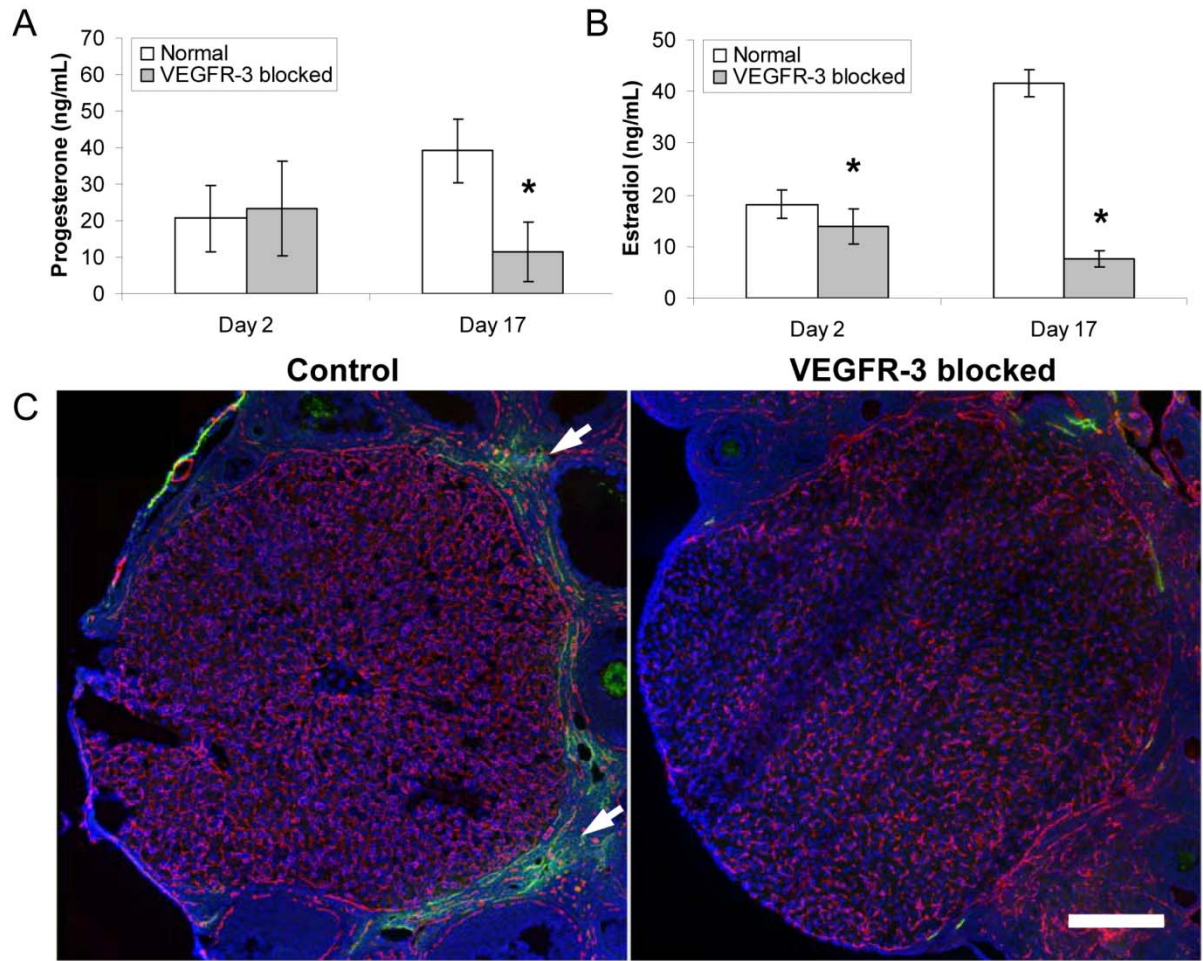


Fig. 5. Blockade of lymphangiogenesis led to reduced systemic estradiol levels in mice following fertilization and decreases in both estradiol and progesterone during pregnancy. A) Serum progesterone levels in control and mF4-31C1 treated mice were normal at pregnancy day 2, but significantly reduced at pregnancy day 17. Since treatment was halted before mating, and since no difference was seen in serum levels at day 2, the mF4-31C1 treatment did not directly affect these hormones. B) Estradiol levels were significantly reduced during pregnancy when ovarian lymphangiogenesis was blocked. * $P < 0.05$ between treatments. C) The blood vasculature (red, CD31) corpora lutea in the ovaries of pregnant mothers at day 17 appeared normal in mF4-31C1 treated mice. In control mothers, lymphatic vessels (arrows) (green, LYVE-1) surround the corpus luteum and other follicles. Bar=200μm.

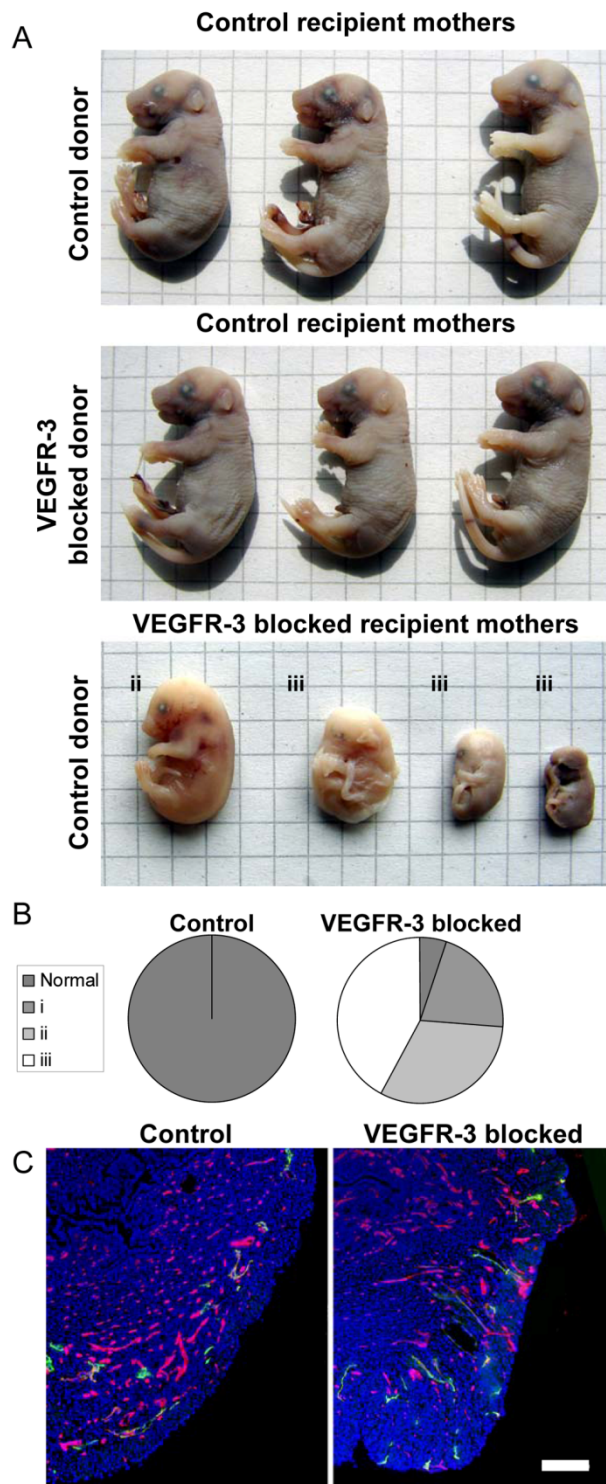
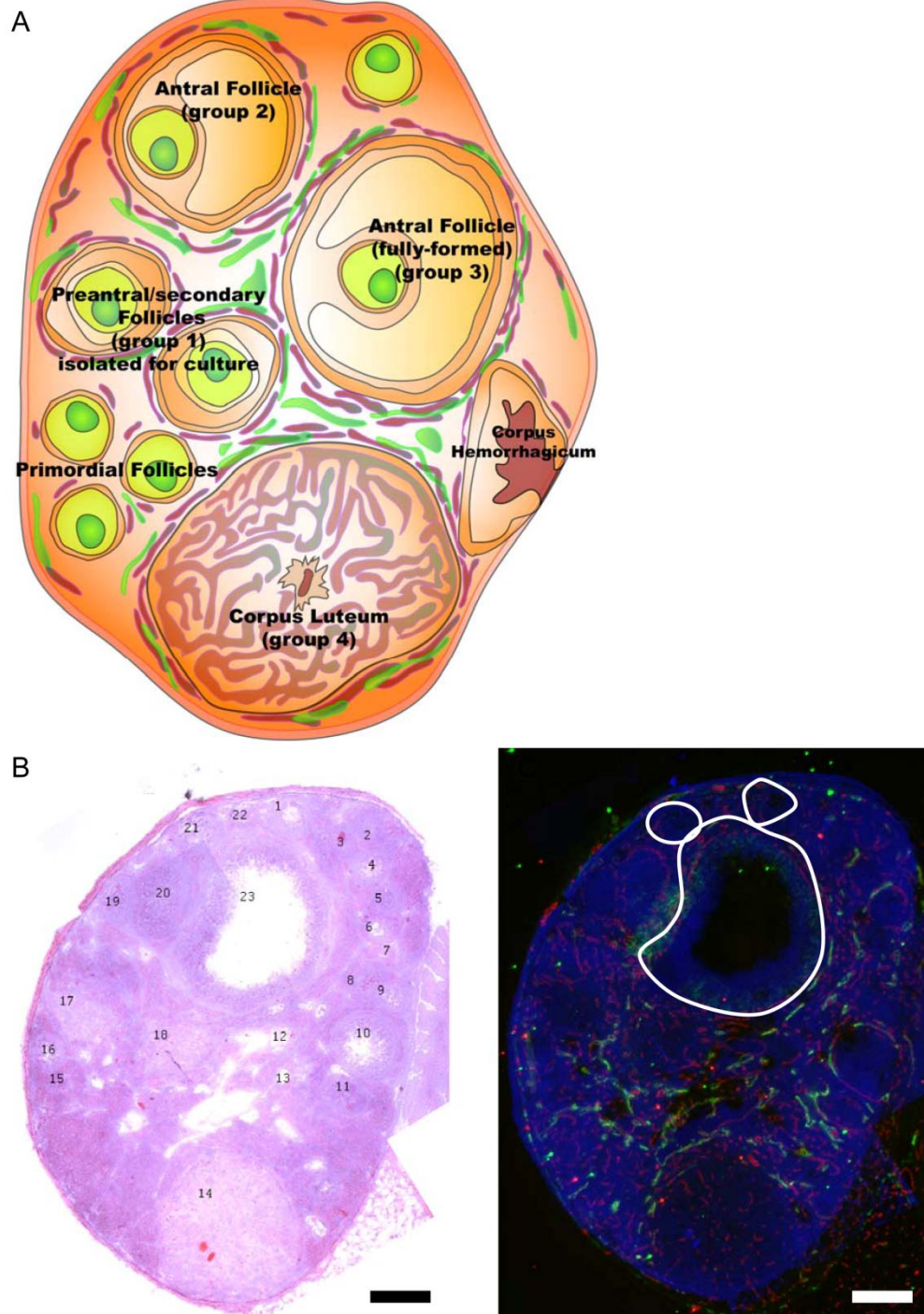


Fig. 6. All embryos – from both control and mF4-31C1 treated mothers – implanted into treated recipient mothers were insufficiently developed despite normal uterine vasculature. A) 2-cell embryos from saline and mF4-31C1 treated mothers implanted into normal, pseudo-pregnant recipient mothers developed into normal, viable fetuses by day 17. In pseudo-pregnant mothers pre-treated with mF4-31C1, embryos from control mothers failed to develop into normal fetuses, despite normal implantations. Grid=4mm. B) The distribution of fetal quality at day 17 following normal embryo transfer to mF4-31C1 treated recipients closely replicated that of in situ fetal development, as reported in Figure 2. C) Blood (red, CD31) and lymphatic (green, LYVE-1) capillaries in the uterine wall appeared normal in both mF4-31C1 treated and untreated mothers, further supporting an ovarian cause. Bar=200 μ m.

Supplemental Figure 1



Supplemental Figure 1. Follicular vasculature was calculated for four stages of follicle maturation. A) Follicles exist in various maturation states within the murine follicle with increasing vasculature with maturation. Follicles that had reached preantral status were identified for quantifying their surrounding vasculature. B) Each follicle was first identified and numbered on hematoxylin labeled sections, where follicular structure was easier to recognize. C) On the corresponding fluorescence image, each follicle was then outlined in Metamorph software (white lines indicate the concept) and the percent area of lymphatic and blood vessel coverage within each region of interest was quantified. Bars=200 μ m.

Chapter 6: Retrospective and outlooks

The need for efficient follicles culture systems becomes obvious when the actual lack of a reliable source of mature oocytes is considered. The culture systems constitute also a key tool for the understanding of mechanisms such as oogenesis, folliculogenesis, and embryogenesis. Last two decades, a substantial effort was made to establish an efficient culture system and to acquire fundamental knowledge about the oocyte and follicle growth. The *in vitro* production of mature oocyte from primordial follicles was reported once in mouse [30]. The primordial follicles were fully matured through a two-step culture, first neonatal ovarian organ culture and after preantral follicle culture. The produced oocytes could be fertilized and gave offspring. However, this technique was limited by a low yield and offspring plagued with multiple disorders such as premature aging. Although, the experiment could not be repeated, it brought the hope of an achievable growth and maturation of the oocyte *in vitro*.

Most *in vitro* follicle culture systems are conventional 2-D culture system as extensively used in biology. The follicles in 2-D culture system remodel their structure in order to readapt to the hindrances caused by such an environment. This remodeling leads to disconnections between the oocyte and the granulosa cells or between the granulosa cells themselves. Despite a relative success in terms of secondary oocyte growth and maturation, the studies investigating the role of hormones or growth could hardly cope with such a bias and thus often reported contradictory results. The first introduced 3-D culture system used alginate bead and demonstrated that the structure of the follicles was maintained and that the oocyte maturation was possible. However, in this system it was difficult to observe the

morphology of the follicle during the culture because of the bead shape. This system was also limited by the possibility to load a unique follicle per bead. Moreover, functionalizing alginate with interesting growth factors was not straightforward. For overcoming these problems, a culture system based on tailored PEG-hydrogels is herein reported.

In a first step, the mechanical properties of PEG-hydrogels could be tuned by varying the ratio of SV/TH thus modulating the crosslinks density of the hydrogel. The goal was to approach the mechanical properties of the ovarian tissue. The PEG-hydrogel systems was also functionalized with an integrin binding peptide (RGD) to mediate cell attachment as it could happen in environment where the ECM is present. Optimal conditions for oocyte growth, maturation and quality were established and compared with results obtained from 2-D culture systems. Once these “basal” optimal conditions defined, the PEG-hydrogels culture system was used to study the effects of the most relevant gonadotropins for oocyte maturation (FSH and LH). This experiment intended to address the contradictory results published until now. The measured effects of LH and FSH in the PEG-hydrogels system were found to be relatively close to what is observed *in vivo* and drastically different from what is observed in the 2-D systems.

This study highlights the importance of putting the studied material in a situation close to the *in vivo* microenvironment for investigating the effects of growth factor, hormones, drugs, etc.

Better characterizing the function of the membrane-bound form of KL can potentially bring new insights in folliculogenesis and/or hematopoiesis understandings. Here, the ease of signal immobilization onto the PEG-hydrogel can provide a valuable tool for studying such processes. In this context, various recombinant forms of KL (soluble and attached) were expressed in the mammalian cells. The tetherable form (KLstg) of KL was successfully

purified, tested and immobilized onto PEG-hydrogel. This system was used to culture ovarian tissues. After 10 days of culture, the results demonstrated that secondary follicles were only visible when a low amounts (100 μ M) of KL was bond to the PEG matrix. Most primordial follicles did not develop when KL was present in a soluble form. The engineered forms of KL in combination with the PEG-hydrogel systems will certainly be of use to gain further knowledge in folliculogenesis and might serve to better understand hemetopoiesis as well. In addition, because of the cross-bioactivity of KL between mice and Humans, the reported platform could be of use for future clinical applications.

In parallel, a new role for the lymphatic circulation in the modulation of hormone levels during pregnancy was identified. First, we have demonstrated that lymphangiogenesis occurs within the ovary during folliculogenesis and is essential for pregnancy. This is likely to modulate the systemic estrogen during pregnancy. Herein we proposed that the lymphatic circulation could influence the complex processes of folliculogenesis, ovulation, and pregnancy. Lymphatic vessels are critical in inflammation and fluid balance. The ovulation is considered to be a massive inflammatory event with the modulation of the pressure within the antrum of developed follicles, the massive secretion of hormones, and the eventual rupture of the ovarian capsule should present an almost unsustainable inflammatory environment. Lymphatic vessels should have the potential to modulate these factors as would occur in other tissues. However, further investigations are needed to assess the role of ovarian lymphatic capillaries in the views of examination of intrafollicular pressures, granulosa cell molecular expression, hormone secretion and hormonal rescue therapy.

In this thesis, we demonstrate the potential of a tailored PEG-hydrogel system to study complex processes such as folliculogenesis. Breaking down the complexity of the microenvironment and reconstructing it in a combinatorial assay will certainly allow better

understanding of the key events governing events such as, activation, maturation and ovulation of the oocyte. In this context the PEG-hydrogels constitute an attractive platform because it combines advantages such dimensionality and relevant mechanical properties, cell responsiveness, ease of functionalization, compatibility with microscopic observation and single follicle analysis. Thus, it is safe to assume that that the efficiency of the PEG-hydrogel system will be fully exploited when used in high throughput assays. This will certainly be of great use to the scientific community in its effort to bring innovative solutions to fertility-related questions.

In a closer future it will be interesting to continue optimizing the system till embryonic development is obtained. In this context, continuing the investigations on the role of KL has the potential to yield valuable information. Other strategies such as genomic studies on cultured follicle can also be considered for identifying the remaining “bottlenecks”.

Bibliographic references

1. McGee EA, Hsueh AJW. Initial and cyclic recruitment of ovarian follicles. *Endocr Rev* 2000 Apr;21(2):200-214.
 2. Senbon S, Hirao Y, Miyano T. Interactions between the oocyte and surrounding somatic cells in follicular development: Lessons from in vitro culture. *J Reprod Develop* 2003 Aug;49(4):259-269.
 3. Liu J, Van Der Elst J, Van Den Broecke R, Dumortier F, Dhont M. Maturation of mouse primordial follicles by combination of grafting and in vitro culture. *Biol Reprod* 2000 May;62(5):1218-1223.
 4. Liu J, Van der Elst J, Van den Broecke R, Dhont M. Live offspring by in vitro fertilization of oocytes from cryopreserved primordial mouse follicles after sequential in vivo transplantation and in vitro maturation. *Biol Reprod* 2001 Jan;64(1):171-178.
 5. O'Brien MJ, Pendola JK, Eppig JJ. A revised protocol for in vitro development of mouse oocytes from primordial follicles dramatically improves their developmental competence. *Biology of Reproduction* 2003 May;68(5):1682-1686.
 6. Telfer EE, Binnie JP, Jordan LB. Effect of follicle size on the onset of apoptotic cell death in cultured bovine ovarian follicles. *Theriogenology* 1998 Jan 1;49(1):357-357.
 7. Roy SK, Greenwald GS. Methods of separation and in-vitro culture of pre-antral follicles from mammalian ovaries. *Hum Reprod Update* 1996 May-Jun;2(3):236-245.
 8. Smitz JEJ, Cortvrindt RG. The earliest stages of folliculogenesis in vitro. *Reproduction* 2002 Feb;123(2):185-202.
 9. Lenie S, Cortvrindt R, Adriaenssens T, Smitz J. A reproducible two-step culture system for isolated primary mouse ovarian follicles as single functional units. *Biol Reprod* 2004 Nov;71(5):1730-1738.
 10. Donnez J, Dolmans MM. Livebirth after cryopreserved ovarian tissue autotransplantation - Reply. *Lancet* 2004 Dec 11;364(9451):2092-2093.
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11. Picton HM, Danfour MA, Harris SE, Chambers EL, Huntriss J. Growth and maturation of oocytes in vitro. *Reprod Suppl* 2003;61:445-462.
 12. Murray A, Spears N. Follicular development in vitro. *Semin Reprod Med* 2000;18(2):109-122.
 13. Otsuka F, Yamamoto S, Erickson GF, Shimasaki S. Bone morphogenetic protein-15 inhibits follicle-stimulating hormone (FSH) action by suppressing FSH receptor expression. *J Biol Chem* 2001 Apr 6;276(14):11387-11392.
 14. Otsuka F, Shimasaki S. A negative feedback system between oocyte bone morphogenetic protein 15 and granulosa cell kit ligand: Its role in regulating granulosa cell mitosis. *P Natl Acad Sci USA* 2002 Jun 11;99(12):8060-8065.
 15. Nilsson EE, Kezele P, Skinner MK. Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries. *Mol Cell Endocrinol* 2002 Feb 25;188(1-2):65-73.
 16. Driancourt MA, Reynaud K, Cortvrindt R, Smitz J. Roles of KIT and KIT LIGAND in ovarian function. *Rev Reprod* 2000 Sep;5(3):143-152.
 17. Nilsson E, Parrott JA, Skinner MK. Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. *Mol Cell Endocrinol* 2001 Apr 25;175(1-2):123-130.
 18. Lutolf MP, Hubbell JA. Synthesis and physicochemical characterization of end-linked poly(ethylene glycol)-co-peptide hydrogels formed by Michael-type addition. *Biomacromolecules* 2003 May-Jun;4(3):713-722.
 19. Lutolf MP, Lauer-Fields JL, Schmoekel HG, Metters AT, Weber FE, Fields GB, et al. Synthetic matrix metalloproteinase-sensitive hydrogels for the conduction of tissue regeneration: Engineering cell-invasion characteristics. *Proceedings of the National Academy of Sciences of the United States of America* 2003 Apr 29;100(9):5413-5418.
 20. Doneda L, Klinger FG, Larizza L, De Felici M. KL/KIT co-expression in mouse fetal oocytes. *Int J Dev Biol* 2002 Dec;46(8):1015-1021.
 21. Cortvrindt R, Smitz J. In vitro follicle growth: Achievements in mammalian species. *Reproduction in Domestic Animals* 2001 Feb;36(1):3-9.
-

22. Braw-Tal R. The initiation of follicle growth: the oocyte or the somatic cells? *Mol Cell Endocrinol* 2002 Feb 22;187(1-2):11-18.
 23. Kaipia A, Hsueh AJW. Regulation of ovarian follicle atresia. *Annual Review of Physiology* 1997;59:349-363.
 24. Smith GD. In vitro maturation of oocyte. *Current women's health reports* 2001;1:143-151.
 25. Gosden RG, Mullan J, Picton HM, Yin H, Tan SL. Current perspective on primordial follicle cryopreservation and culture for reproductive medicine. *Hum Reprod Update* 2002 Mar-Apr;8(2):105-110.
 26. Miyano T. In vitro growth of mammalian oocytes. *Journal of Reproduction and Development* 2005 Apr;51(2):169-176.
 27. Carroll J, Whittingham DG, Wood MJ. Effect of Dibutyryl Cyclic Adenosine-Monophosphate on Granulosa-Cell Proliferation, Oocyte Growth and Meiotic Maturation in Isolated Mouse Primary Ovarian Follicles Cultured in Collagen Gels. *J Reprod Fertil* 1991 May;92(1):197-207.
 28. Lee ST, Choi MH, Gong SP, Han JY, Lim JM. Establishment of a basic method for manipulating preantral follicles: effects of retrieval method on in vitro growth of preantral follicles and intrafollicular oocytes. *Zygote* 2007 May;15(2):109-116.
 29. Cortvrindt R, Smitz J, VanSteirteghem AC. In-vitro maturation, fertilization and embryo development of immature oocytes from early preantral follicles from prepuberal mice in a simplified culture system. *Hum Reprod* 1996 Dec;11(12):2656-2666.
 30. Eppig JJ, Schroeder AC. Capacity of Mouse Oocytes from Preantral Follicles to Undergo Embryogenesis and Development to Live Young after Growth, Maturation, and Fertilization In vitro. *Biol Reprod* 1989 Aug;41(2):268-276.
 31. Spears N, Boland NI, Murray AA, Gosden RG. Mouse Oocytes Derived from in-Vitro Grown Primary Ovarian Follicles Are Fertile. *Hum Reprod* 1994 Mar;9(3):527-532.
 32. Cortvrindt RG, Hu YX, Liu J, Smitz JEJ. Timed analysis of the nuclear maturation of oocytes in early preantral mouse follicle culture supplemented with recombinant gonadotropin. *Fertil Steril* 1998 Dec;70(6):1114-1125.
-

33. Nayudu PL, Osborn SM. Factors Influencing the Rate of Preantral and Antral Growth of Mouse Ovarian Follicles Invitro. *Journal of Reproduction and Fertility* 1992 Jul;95(2):349-362.
 34. Boland NI, Humpherson PG, Leese HJ, Gosden RG. Pattern of Lactate Production and Steroidogenesis during Growth and Maturation of Mouse Ovarian Follicles Invitro. *Biology of Reproduction* 1993 Apr;48(4):798-806.
 35. Hartshorne GM, Sargent IL, Barlow DH. Growth-Rates and Antrum Formation of Mouse Ovarian Follicles in-Vitro in Response to Follicle-Stimulating-Hormone, Relaxin, Cyclic-Amp and Hypoxanthine. *Hum Reprod* 1994 Jun;9(6):1003-1012.
 36. Pangas SA, Saudye H, Shea LD, Woodruff TK. Novel approach for the three-dimensional culture of granulosa cell-oocyte complexes. *Tissue Engineering* 2003 Oct;9(5):1013-1021.
 37. Xu M, Kreeger PK, Shea LD, Woodruff TK. Tissue-engineered follicles produce live, fertile offspring. *Tissue Eng* 2006 Oct;12(10):2739-2746.
 38. Elbert DL, Pratt AB, Lutolf MP, Halstenberg S, Hubbell JA. Protein delivery from materials formed by self-selective conjugate addition reactions. *J Control Release* 2001 Sep 11;76(1-2):11-25.
 39. Lutolf MP, Tirelli N, Cerritelli S, Cavalli L, Hubbell JA. Systematic modulation of Michael-type reactivity of thiols through the use of charged amino acids. *Bioconjugate Chem* 2001 Nov-Dec;12(6):1051-1056.
 40. Seliktar D, Zisch AH, Lutolf MP, Wrana JL, Hubbell JA. MMP-2 sensitive, VEGF-bearing bioactive hydrogels for promotion of vascular healing. *Journal of Biomedical Materials Research Part A* 2004 Mar 15;68A(4):704-716.
 41. Zsebo KM, Williams DA, Geissler EN, Broudy VC, Martin FH, Atkins HL, et al. Stem-Cell Factor Is Encoded at the Si-Locus of the Mouse and Is the Ligand for the C-Kit Tyrosine Kinase Receptor. *Cell* 1990 Oct 5;63(1):213-224.
 42. Sattler M, Salgia R. Targeting c-Kit mutations: basic science to novel therapies. *Leukemia Res* 2004 May;28:S11-S20.
 43. Gougeon A, Busso D. Morphologic and functional determinants of primordial and primary follicles in the monkey ovary. *Mol Cell Endocrinol* 2000 May 25;163(1-2):33-41.
-

44. Hoyer PE, Byskov AG, Mollgard K. Stem cell factor and c-Kit in human primordial germ cells and fetal ovaries. *Mol Cell Endocrinol* 2005 Apr 29;234(1-2):1-10.
 45. Stoop H, Honecker F, Cools M, de Krijger R, Bokemeyer C, Looijenga LHJ. Differentiation and development of human female germ cells during prenatal gonadogenesis: an immunohistochemical study. *Hum Reprod* 2005 Jun;20(6):1466-1476.
 46. Clark DE, Tisdall DJ, Fidler AE, McNatty KP. Localization of mRNA encoding c-kit during the initiation of folliculogenesis in ovine fetal ovaries. *J Reprod Fertil* 1996 Mar;106(2):329-335.
 47. Horie K, Takakura K, Taii S, Narimoto K, Noda Y, Nishikawa S, et al. The Expression of C-Kit Protein during Oogenesis and Early Embryonic-Development. *Biol Reprod* 1991 Oct;45(4):547-552.
 48. Manova K, Huang EJ, Angeles M, Deleon V, Sanchez S, Pronovost SM, et al. 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* 1993 May;157(1):85-99.
 49. Hutt KJ, McLaughlin EA, Holland MK. Kit ligand and c-Kit have diverse roles during mammalian oogenesis and folliculogenesis. *Mol Hum Reprod* 2006 Feb;12(2):61-69.
 50. Yoshida H, Takakura N, Kataoka H, Kunisada T, Okamura H, Nishikawa S. Stepwise requirement of c-kit tyrosine kinase in mouse ovarian follicle development. *Dev Biol* 1997 Apr 1;184(1):122-137.
 51. Parrott JA, Skinner MK. Kit-ligand/stem cell factor induces primordial follicle development and initiates folliculogenesis. *Endocrinology* 1999 Sep;140(9):4262-4271.
 52. Gilchrist RB, Ritter LJ, Cranfield M, Jeffery LA, Amato F, Scott SJ, et al. Immunoneutralization of growth differentiation factor 9 reveals it partially accounts for mouse oocyte mitogenic activity. *Biol Reprod* 2004 Sep;71(3):732-739.
 53. Jin X, Han CS, Yu FQ, Wei P, Hu ZY, Liu YX. Anti-apoptotic action of stem cell factor on oocytes in primordial follicles and its signal transduction. *Mol Reprod Dev* 2005 Jan;70(1):82-90.
-

54. Adams RH, Alitalo K. Molecular regulation of angiogenesis and lymphangiogenesis. *Nat Rev Mol Cell Bio* 2007 Jun;8(6):464-478.
55. Oliver G, Alitalo K. The lymphatic vasculature: Recent progress and paradigms. *Annu Rev Cell Dev Bi* 2005;21:457-483.
56. Goldman J, Rutkowski JM, Shields JD, Pasquier MC, Cui Y, Schmokel HG, et al. Cooperative and redundant roles of VEGFR-2 and VEGFR-3 signaling in adult lymphangiogenesis. *Faseb J* 2007 Apr;21(4):1003-1012.
57. Pytowski B, Goldman J, Persaud K, Wu Y, Witte L, Hicklin DJ, et al. Complete and specific inhibition of adult lymphatic regeneration by a novel VEGFR-3 neutralizing antibody. *J Natl Cancer I* 2005 Jan 5;97(1):14-21.
58. Baluk P, Tammela T, Ator E, Lyubynska N, Achen MG, Hicklin DJ, et al. Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. *J Clin Invest* 2005 Feb;115(2):247-257.
59. Bock F, Onderka J, Dietrich T, Bachmann B, Pytowski B, Cursiefen C. Blockade of VEGFR3-signalling specifically inhibits lymphangiogenesis in inflammatory corneal neovascularisation. *Graef Arch Clin Exp* 2008 Jan;246(1):115-119.
60. Roberts N, Kloos B, Cassella M, Podgrabinska S, Persaud K, Wu Y, et al. Inhibition of VEGFR-3 activation with the antagonistic antibody more potently suppresses lymph node and distant metastases than inactivation of VEGFR-2. *Cancer Res* 2006 Mar 1;66(5):2650-2657.
61. Smith ML, Gourdon D, Little WC, Kubow KE, Eguiluz RA, Luna-Morris S, et al. Force-induced unfolding of fibronectin in the extracellular matrix of living cells. *Plos Biology* 2007 Oct;5(10):2243-2254.
62. Rodgers RJ, Irving-Rodgers HF, van Wezel IL. Extracellular matrix in ovarian follicles. *Molecular and Cellular Endocrinology* 2000 May 25;163(1-2):73-79.
63. Irving-Rodgers HF, Rodgers RJ. Extracellular matrix in ovarian follicular development and disease. *Cell and Tissue Research* 2005 Oct;322(1):89-98.
64. Smith MF, Ricke WA, Bakke LJ, Dow MPD, Smith GW. Ovarian tissue remodeling: role of matrix metalloproteases and their inhibitors. *Molecular and Cellular Endocrinology* 2002 May 31;191(1):45-56.
-

65. Liu K, Wahlberg P, Leonardsson G, Hagglund AC, Ny A, Boden I, et al. Successful ovulation in plasminogen-deficient mice treated with the broad-spectrum matrix metalloproteinase inhibitor galardin. *Developmental Biology* 2006 Jul 15;295(2):615-622.
66. Hagglund AC, Ny A, Leonardsson G, Ny T. Regulation and localization of matrix metalloproteinases and tissue inhibitors of metalloproteinases in the mouse ovary during gonadotropin-induced ovulation. *Endocrinology* 1999 Sep;140(9):4351-4358.
67. Goxe B, Flechon JE, Delasalle S, Salesse R. Rgd-Mediated Adhesion of Porcine Granulosa-Cells Modulates Their Differentiation Response to Fsh in-Vitro. *Biology of the Cell* 1995;83(2-3):169-177.
68. Eppig JJ, OBrien M, Wigglesworth K. Mammalian oocyte growth, and development in vitro. *Molecular Reproduction and Development* 1996 Jun;44(2):260-273.
69. Kraehenbuehl TP, Zammaretti P, Van der Vlies AJ, Schoenmakers RG, Lutolf MP, Jaconi ME, et al. Three-dimensional extracellular matrix-directed cardioprogenitor differentiation: systematic modulation of a synthetic cell-responsive PEG-hydrogel. *Biomaterials* 2008 Jun;29(18):2757-2766.
70. Eichenlaubritter U, Betzendahl I. Chloral Hydrate Induced Spindle Aberrations, Metaphase-I Arrest and Aneuploidy in Mouse Oocytes. *Mutagenesis* 1995 Nov;10(6):477-486.
71. Assoian RK, Klein EA. Growth control by intracellular tension and extracellular stiffness. *Trends in Cell Biology* 2008 Jul;18(7):347-352.
72. Brandl F, Sommer F, Goepferich A. Rational design of hydrogels for tissue engineering: Impact of physical factors on cell behavior. *Biomaterials* 2007 Jan;28(2):134-146.
73. West ER, Xu M, Woodruff TK, Shea LD. Physical properties of alginate hydrogels and their effects on in vitro follicle development. *Biomaterials* 2007 Oct;28(30):4439-4448.
74. Drury JL, Mooney DJ. Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials* 2003 Nov;24(24):4337-4351.
-

75. Richardson MC, Davies DW, Watson RH, Dunsford ML, Inman CB, Masson GM. Cultured Human Granulosa-Cells as a Model for Corpus-Luteum Function - Relative Roles of Gonadotropin and Low-Density-Lipoprotein Studied under Defined Culture Conditions. *Human Reproduction* 1992 Jan;7(1):12-18.
76. Luck MR. The gonadal extracellular matrix. *Oxf Rev Reprod Biol* 1994;16:33-85.
77. Huet C, Pisselet C, Mandon-Pepin B, Monget P, Monniaux D. Extracellular matrix regulates ovine granulosa cell survival, proliferation and steroidogenesis: relationships between cell shape and function. *Journal of Endocrinology* 2001 May;169(2):347-360.
78. Goxe B, Salesse R, Remy JJ, Genty N, Garnier J. Lh Receptor Rna and Protein-Levels after Hormonal Treatment of Porcine Granulosa-Cells in Primary Culture. *Journal of Molecular Endocrinology* 1992 Apr;8(2):119-129.
79. Kreeger PK, Woodruff TK, Shea LD. Murine granulosa cell morphology and function are regulated by a synthetic Arg-Gly-Asp matrix. *Molecular and Cellular Endocrinology* 2003 Jul 31;205(1-2):1-10.
80. Eppig JJ. Analysis of Mouse Oogenesis Invitro Oocyte Isolation and Utilization of Exogenous Energy-Sources by Growing Oocytes. *Journal of Experimental Zoology* 1976;198(3):375-381.
81. Heller DT, Cahill DM, Schultz RM. Biochemical-Studies of Mammalian Oogenesis - Metabolic Cooperativity between Granulosa-Cells and Growing-Mouse Oocytes. *Developmental Biology* 1981;84(2):455-464.
82. Anderson E, Albertini DF. Gap Junctions between Oocyte and Companion Follicle Cells in Mammalian Ovary. *Journal of Cell Biology* 1976;71(2):680-686.
83. Hsueh AJW, Billig H, Tsafiriri A. Ovarian Follicle Atresia - a Hormonally Controlled Apoptotic Process. *Endocrine Reviews* 1994 Dec;15(6):707-724.
84. Abel MH, Wootton AN, Wilkins V, Huhtaniemi I, Knight PG, Charlton HM. The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. *Endocrinology* 2000 May;141(5):1795-1803.
85. Adriaens I, Cortvrindt R, Smits J. Differential FSH exposure in preantral follicle culture has marked effects on folliculogenesis and oocyte developmental competence. *Human Reproduction* 2004 Feb;19(2):398-408.
-

86. Urban RJ, Garmey JC, Shupnik MA, Veldhuis JD. Follicle-Stimulating-Hormone Increases Concentrations of Messenger-Ribonucleic-Acid Encoding Cytochrome-P450 Cholesterol Side-Chain Cleavage Enzyme in Primary Cultures of Porcine Granulosa-Cells. *Endocrinology* 1991 Apr;128(4):2000-2007.
87. Silva JM, Hamel M, Sahmil M, Price CA. Control of oestradiol secretion and of cytochrome P450 aromatase messenger ribonucleic acid accumulation by FSH involves different intracellular pathways in oestrogenic bovine granulosa cells in vitro. *Reproduction* 2006 Dec;132(6):909-917.
88. Demeestere I, Centner J, Gervy C, Englert Y, Delbaere A. Impact of various endocrine and paracrine factors on in vitro culture of preantral follicles in rodents. *Reproduction* 2005 Aug;130(2):147-156.
89. Mao JD, Wu GM, Smith MF, McCauley TC, Cantley TC, Prather RS, et al. Effects of culture medium, serum type, and various concentrations of follicle-stimulating hormone on porcine preantral follicular development and antrum formation in vitro. *Biology of Reproduction* 2002 Oct;67(4):1197-1203.
90. Cortvrindt R, Smits J, VanSteirteghem AC. Assessment of the need for follicle stimulating hormone in early preantral mouse follicle culture in vitro. *Human Reproduction* 1997 Apr;12(4):759-768.
91. Mitchell LM, Kennedy CR, Hartshorne GM. Effects of varying gonadotrophin dose and timing on antrum formation and ovulation efficiency of mouse follicles in vitro. *Human Reproduction* 2002 May;17(5):1181-1188.
92. Galway AB, Lapolt PS, Tsafiriri A, Dargan CM, Boime I, Hsueh AJW. Recombinant Follicle-Stimulating-Hormone Induces Ovulation and Tissue Plasminogen-Activator Expression in Hypophysectomized Rats. *Endocrinology* 1990 Dec;127(6):3023-3028.
93. Cortvrindt R, Hu Y, Smits J. Recombinant luteinizing hormone as a survival and differentiation factor increases oocyte maturation in recombinant follicle stimulating hormone-supplemented mouse preantral follicle culture. *Human Reproduction* 1998 May;13(5):1292-1302.
94. Lee ST, Choi MH, Lee EJ, Gong SP, Jang M, Park SH, et al. Establishment of autologous embryonic stem cells derived from preantral follicle culture and oocyte parthenogenesis. *Fertil Steril* 2007;19(4S):1193-1203.
95. Liu J, Rybouchkin A, Van der Elst J, Dhont M. Fertilization of mouse oocytes from in vitro-matured preantral follicles using classical in vitro fertilization or intracytoplasmic sperm injection. *Biology of Reproduction* 2002 Aug;67(2):575-579.
-

96. Tisdall DJ, Watanabe K, Hudson NL, Smith P, McNatty KP. Fsh Receptor Gene-Expression during Ovarian Follicle Development in Sheep. *Journal of Molecular Endocrinology* 1995 Dec;15(3):273-281.
97. Oktay K, Briggs D, Gosden RG. Ontogeny of follicle-stimulating hormone receptor gene expression in isolated human ovarian follicles. *Journal of Clinical Endocrinology and Metabolism* 1997 Nov;82(11):3748-3751.
98. Hsueh AJW, Adashi EY, Jones PBC, Welsh TH. Hormonal-Regulation of the Differentiation of Cultured Ovarian Granulosa-Cells. *Endocrine Reviews* 1984;5(1):76-127.
99. Sirard MA, Desrosier S, Assidi A. In vivo and in vitro effects of FSH on oocyte maturation and developmental competence. *Theriogenology* 2007 Sep 1;68:S71-S76.
100. Sette C, Dolci S, Geremia R, Rossi P. The role of stem cell factor and of alternative c-kit gene products in the establishment, maintenance and function of germ cells. *Int J Dev Biol* 2000;44(6):599-608.
101. Fortune JE. The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. *Anim Reprod Sci* 2003 Oct 15;78(3-4):135-163.
102. Motro B, Bernstein A. Dynamic Changes in Ovarian C-Kit and Steel Expression during the Estrous Reproductive-Cycle. *Dev Dynam* 1993 May;197(1):69-79.
103. Thomas FH, Ethier JF, Shimasaki S, Vanderhyden BC. Follicle-stimulating hormone regulates oocyte growth by modulation of expression of oocyte and granulosa cell factors. *Endocrinology* 2005 Feb;146(2):941-949.
104. Miyazawa K, Williams DA, Gotoh A, Nishimaki J, Broxmeyer HE, Toyama K. Membrane-Bound Steel Factor Induces More Persistent Tyrosine Kinase Activation and Longer Life-Span of C-Kit Gene-Encoded Protein Than Its Soluble Form. *Blood* 1995 Feb 1;85(3):641-649.
105. Ehrbar M, Rizzi SC, Schoenmakers RG, San Miguel B, Hubbell JA, Weber FE, et al. Biomolecular hydrogels formed and degraded via site-specific enzymatic reactions. *Biomacromolecules* 2007 Oct;8(10):3000-3007.
106. Corpet F. Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* 1988 Nov 25;16(22):10881-10890.
-

107. Muller N, Girard P, Hacker DL, Jordan M, Wurm FM. Orbital shaker technology for the cultivation of mammalian cells in suspension. *Biotechnol Bioeng* 2005 Feb 20;89(4):400-406.
108. Backliwal G, Hildinger M, Hasija V, Wurm FM. High-density transfection with HEK-293 cells allows doubling of transient titers and removes need for a priori DNA complex formation with PEI. *Biotechnol Bioeng* 2008 Feb 15;99(3):721-727.
109. Backliwal G, Hildinger M, Kuettel i, Delegrange F, Hacker DL, Wurm FM. Valproic acid – an alternative to sodium butyrate for enhancing recombinant gene expression in mammalian cultures. *Biotechnol Bioeng* 2008;submitted.
110. Lorand L, Graham RM. Transglutaminases: Crosslinking enzymes with pleiotropic functions. *Nat Rev Mol Cell Bio* 2003 Feb;4(2):140-156.
111. Sperinde JJ, Griffith LG. Synthesis and characterization of enzymatically-cross-linked poly(ethylene glycol) hydrogels. *Macromolecules* 1997 Sep 8;30(18):5255-5264.
112. Schense JC, Hubbell JA. Cross-linking exogenous bifunctional peptides into fibrin gels with factor XIIIa. *Bioconjugate Chem* 1999 Jan-Feb;10(1):75-81.
113. Schense JC, Bloch J, Aebischer P, Hubbell JA. Enzymatic incorporation of bioactive peptides into fibrin matrices enhances neurite extension. *Nat Biotechnol* 2000 Apr;18(4):415-419.
114. Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *Bmc Mol Biol* 2006 Jan 31;7:-.
115. Jiang XL, Gurel O, Mendiaz EA, Stearns GW, Clogston CL, Lu HS, et al. Structure of the active core of human stem cell factor and analysis of binding to its receptor Kit. *Embo J* 2000 Jul 3;19(13):3192-3203.
116. Huang EJ, Nocka KH, Buck J, Besmer P. Differential Expression and Processing of 2 Cell Associated Forms of the Kit-Ligand - Kl-1 and Kl-2. *Mol Biol Cell* 1992 Mar;3(3):349-362.
117. Arakawa T, Yphantis DA, Lary JW, Narhi LO, Lu HS, Prestrelski SJ, et al. Glycosylated and Unglycosylated Recombinant-Derived Human Stem-Cell Factors Are Dimeric and Have Extensive Regular Secondary Structure. *J Biol Chem* 1991 Oct 5;266(28):18942-18948.
-

118. Lu HS, Clogston CL, Wypych J, Parker VP, Lee TD, Swiderek K, et al. Posttranslational Processing of Membrane-Associated Recombinant Human Stem-Cell Factor Expressed in Chinese-Hamster Ovary Cells. *Archives of Biochemistry and Biophysics* 1992 Oct;298(1):150-158.
119. Gougeon A. Regulation of ovarian follicular development in primates: Facts and hypotheses. *Endocr Rev* 1996 Apr;17(2):121-155.
120. Wandji SA, Srsen V, Voss AK, Eppig JJ, Fortune JE. Initiation in vitro of growth of bovine primordial follicles. *Biol Reprod* 1996 Nov;55(5):942-948.
121. Wandji SA, Srsen V, Voss AK, Eppig JJ, Fortune JE. Initiation in vitro of growth of bovine primordial follicles in serum-free medium. *Biol Reprod* 1996;54:161-161.
122. Brown HM, Dunning KR, Robker RL, Pritchard M, Russell DL. Requirement for ADAMTS-1 in extracellular matrix remodeling during ovarian folliculogenesis and lymphangiogenesis. *Dev Biol* 2006 Oct 14.
123. Gaytan F, Tarradas E, Bellido C, Morales C, Sanchez-Criado JE. Prostaglandin E(1) inhibits abnormal follicle rupture and restores ovulation in indomethacin-treated rats. *Biol Reprod* 2002 Oct;67(4):1140-1147.
124. Fraser HM. Regulation of the ovarian follicular vasculature. *Reprod Biol Endocrinol* 2006;4:18.
125. Kaczmarek MM, Schams D, Ziecik AJ. Role of vascular endothelial growth factor in ovarian physiology - an overview. *Reprod Biol* 2005 Jul;5(2):111-136.
126. Pauli SA, Tang H, Wang J, Bohlen P, Posser R, Hartman T, et al. The vascular endothelial growth factor (VEGF)/VEGF receptor 2 pathway is critical for blood vessel survival in corpora lutea of pregnancy in the rodent. *Endocrinology* 2005 Mar;146(3):1301-1311.
127. Skobe M, Hamberg LM, Hawighorst T, Schirner M, Wolf GL, Alitalo K, et al. Concurrent induction of lymphangiogenesis, angiogenesis, and macrophage recruitment by vascular endothelial growth factor-C in melanoma. *Am J Pathol* 2001 Sep;159(3):893-903.
128. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004 Aug;25(4):581-611.
-

129. Shibuya M. Differential roles of vascular endothelial growth factor receptor-1 and receptor-2 in angiogenesis. *J Biochem Mol Biol* 2006 Sep 30;39(5):469-478.
130. Iijima K, Jiang JY, Shimizu T, Sasada H, Sato E. Acceleration of follicular development by administration of vascular endothelial growth factor in cycling female rats. *J Reprod Dev* 2005 Feb;51(1):161-168.
131. Gomez R, Simon C, Remohi J, Pellicer A. Vascular endothelial growth factor receptor-2 activation induces vascular permeability in hyperstimulated rats, and this effect is prevented by receptor blockade. *Endocrinology* 2002 Nov;143(11):4339-4348.
132. Hazzard TM, Rohan RM, Molskness TA, Fanton JW, D'Amato RJ, Stouffer RL. Injection of antiangiogenic agents into the macaque preovulatory follicle: disruption of corpus luteum development and function. *Endocrine* 2002 Apr;17(3):199-206.
133. Zimmermann RC, Hartman T, Bohlen P, Sauer MV, Kitajewski J. Preovulatory treatment of mice with anti-VEGF receptor 2 antibody inhibits angiogenesis in corpora lutea. *Microvasc Res* 2001 Jul;62(1):15-25.
134. Zimmermann RC, Hartman T, Kavic S, Pauli SA, Bohlen P, Sauer MV, et al. Vascular endothelial growth factor receptor 2-mediated angiogenesis is essential for gonadotropin-dependent follicle development. *J Clin Invest* 2003 Sep;112(5):659-669.
135. Stouffer RL, Xu F, Duffy DM. Molecular control of ovulation and luteinization in the primate follicle. *Front Biosci* 2007;12:297-307.
136. Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. *Nature* 2005 Dec 15;438(7070):946-953.
137. Tammela T, Enholm B, Alitalo K, Paavonen K. The biology of vascular endothelial growth factors. *Cardiovasc Res* 2005 Feb 15;65(3):550-563.
138. Pytowski B, Goldman J, Persaud K, Wu Y, Witte L, Hicklin DJ, et al. Complete and specific inhibition of adult lymphatic regeneration by a novel VEGFR-3 neutralizing antibody. *J Natl Cancer Inst* 2005 Jan 5;97(1):14-21.
139. Otsuki Y, Magari S, Sugimoto O. Lymphatic capillaries in rabbit ovaries during ovulation: an ultrastructural study. *Lymphology* 1986 Jun;19(2):55-64.
-

140. Otsuki Y, Magari S, Sugimoto O. Fine structure and morphometric analysis of lymphatic capillaries in the developing corpus luteum of the rabbit. *Lymphology* 1987 Jun;20(2):64-72.
141. Ichikawa S, Uchino S, Hirata Y. Lymphatic and blood vasculature of the forming corpus luteum. *Lymphology* 1987 Jun;20(2):73-83.
142. Heap RB, Fleet IR, Davis AJ, Goode JA, Hamon MH, Walters DE, et al. Neurotransmitters and lymphatic-vascular transfer of prostaglandin F₂ alpha stimulate ovarian oxytocin output in sheep. *J Endocrinol* 1989 Jul;122(1):147-159.
143. Stefanczyk-Krzymowska S, Chlopek J, Grzegorzewski W, Radomski M. Local transfer of prostaglandin E₂ into the ovary and its retrograde transfer into the uterus in early pregnant sows. *Exp Physiol* 2005 Nov;90(6):807-814.
144. Stefanczyk-Krzymowska S, Krzymowski T. Local adjustment of blood and lymph circulation in the hormonal regulation of reproduction in female pigs--facts, conclusions and suggestions for future research. *Reprod Biol* 2002 Jul;2(2):115-132.
145. Demeestere I, Delbaere A, Gervy C, Van den Bergh M, Devreker F, Englert Y. Effect of preantral follicle isolation technique on in-vitro follicular growth, oocyte maturation and embryo development in mice. *Human Reproduction* 2002 Aug;17(8):2152-2159.
146. Inai T, Mancuso M, Hashizume H, Baffert F, Haskell A, Baluk P, et al. Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. *Am J Pathol* 2004 Jul;165(1):35-52.
147. Partanen TA, Paavonen K. Lymphatic versus blood vascular endothelial growth factors and receptors in humans. *Microsc Res Tech* 2001 Oct 15;55(2):108-121.
148. Witmer AN, van Blijswijk BC, Dai J, Hofman P, Partanen TA, Vrensen GF, et al. VEGFR-3 in adult angiogenesis. *J Pathol* 2001 Nov;195(4):490-497.
149. Laakkonen P, Waltari M, Holopainen T, Takahashi T, Pytowski B, Steiner P, et al. Vascular endothelial growth factor receptor 3 is involved in tumor angiogenesis and growth. *Cancer Res* 2007 Jan 15;67(2):593-599.
150. Reynolds LP, Grazul-Bilska AT, Redmer DA. Angiogenesis in the corpus luteum. *Endocrine* 2000 Feb;12(1):1-9.
-

151. Pate JL, Landis Keyes P. Immune cells in the corpus luteum: friends or foes? *Reproduction* 2001 Nov;122(5):665-676.
152. Hedger MP, Qin JX, Robertson DM, de Kretser DM. Intraovarian regulation of immune system functions. *Reprod Fertil Dev* 1990;2(3):263-280.
153. Shakil T, Whitehead SA. Inhibitory action of peritoneal macrophages on progesterone secretion from co-cultured rat granulosa cells. *Biol Reprod* 1994 May;50(5):1183-1189.
154. Rockson SG. Lymphedema. *Am J Med* 2001 Mar;110(4):288-295.
155. Rutkowski JM, Moya M, Johannes J, Goldman J, Swartz MA. Secondary lymphedema in the mouse tail: Lymphatic hyperplasia, VEGF-C upregulation, and the protective role of MMP-9. *Microvasc Res* 2006 Jul 27.
156. Skarzynski D, Mlynarczuk J, Kotwica J. Involvement of high-density lipoprotein in stimulatory effect of hormones supporting function of the bovine corpus luteum. *Acta Vet Hung* 2003;51(1):111-120.
157. Veldhuis JD. Follicle-stimulating hormone regulates low density lipoprotein metabolism by swine granulosa cells. *Endocrinology* 1988 Sep;123(3):1660-1667.
158. Rolaki A, Drakakis P, Millingos S, Loutradis D, Makrigiannakis A. Novel trends in follicular development, atresia and corpus luteum regression: a role for apoptosis. *Reprod Biomed Online* 2005 Jul;11(1):93-103.
159. Nourani MR, Owada Y, Kitanaka N, Sakagami H, Hoshi H, Iwasa H, et al. Occurrence of immunoreactivity for adipocyte-type fatty acid binding protein in degenerating granulosa cells in atretic antral follicles of mouse ovary. *J Mol Histol* 2005 Oct;36(8-9):491-497.
160. Vollmar B, Laschke MW, Rohan R, Koenig J, Menger MD. In vivo imaging of physiological angiogenesis from immature to preovulatory ovarian follicles. *Am J Pathol* 2001 Nov;159(5):1661-1670.
161. Acosta TJ, Miyamoto A. Vascular control of ovarian function: ovulation, corpus luteum formation and regression. *Anim Reprod Sci* 2004 Jul;82-83:127-140.
162. Magoffin DA. Ovarian theca cell. *Int J Biochem Cell Biol* 2005 Jul;37(7):1344-1349.
-

163. Jabbour HN, Kelly RW, Fraser HM, Critchley HO. Endocrine regulation of menstruation. *Endocr Rev* 2006 Feb;27(1):17-46.
164. Drummond AE, Findlay JK. The role of estrogen in folliculogenesis. *Mol Cell Endocrinol* 1999 May 25;151(1-2):57-64.
165. Espey LL, Lipner H. Measurements Of Intrafollicular Pressures In The Rabbit Ovary. *Am J Physiol* 1963 Dec;205:1067-1072.
166. Rutkowski JM, Swartz MA. A driving force for change: interstitial flow as a morphoregulator. *Trends Cell Biol* 2007 Jan;17(1):44-50.
167. Hsieh M, Boerboom D, Shimada M, Lo Y, Parlow AF, Luhmann UF, et al. Mice null for *Frizzled4* (*Fzd4*^{-/-}) are infertile and exhibit impaired corpora lutea formation and function. *Biol Reprod* 2005 Dec;73(6):1135-1146.
168. Karkkainen MJ, Saaristo A, Jussila L, Karila KA, Lawrence EC, Pajusola K, et al. A model for gene therapy of human hereditary lymphedema. *Proc Natl Acad Sci U S A* 2001 Oct 23;98(22):12677-12682.
169. Makinen T, Jussila L, Veikkola T, Karpanen T, Kettunen MI, Pulkkanen KJ, et al. Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat Med* 2001 Feb;7(2):199-205.
170. Achen MG, Mann GB, Stacker SA. Targeting lymphangiogenesis to prevent tumour metastasis. *Br J Cancer* 2006 May 22;94(10):1355-1360.
171. Szuba A, Skobe M, Karkkainen MJ, Shin WS, Beynet DP, Rockson NB, et al. Therapeutic lymphangiogenesis with human recombinant VEGF-C. *Faseb J* 2002 Dec;16(14):1985-1987.
172. Yoon YS, Murayama T, Gravereaux E, Tkebuchava T, Silver M, Curry C, et al. VEGF-C gene therapy augments postnatal lymphangiogenesis and ameliorates secondary lymphedema. *J Clin Invest* 2003 Mar;111(5):717-725.
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11월 3일 나의 생애 마지막 시험이 끝났다. 휴... 참 오랫동안 학교라는 울타리에서 학생이라는 이름으로 살아왔다. 그리고 그 마침표를 드디어 찍은 것이다. 그 많았던 스트레스도, 이리 쉽게 날아 갈 수 있단 말인가? 2004년 난 유난히 등에 날개가 나는 듯한 느낌이 들었다. 꼭 어디든 훨훨 날아 가야 갈 듯 했다. 이리저리 뛰어 다니며 비행 준비를 했다. 그렇게 처음 떠나온 곳이 취리히다. 2004년 7월 29일, 그 날을 잊을 수 있을까? 공항에서 취리히의 나의 기숙사로 오는 동안 택시 밖 풍경은 작은 예쁜 마을이었다. 그리고 처음으로 집을 떠나서 살게 된 나의 기숙사 방은 너무 좋았다. 나와 취리히의 첫 만남은 너무도 화창했다. 매주 토요일마다 난 취리히 시내 여기 저기를 다니고 킨더스피탈(기숙사이름) 친구들과 가끔 산에도 가고 매일 저녁 같이 해 먹으면 즐거운 여름을 보냈다. 그리고 다시 로잔이라는 곳으로 옮겨오면서 본격적인 학위에 들어 갔다. 첫 관문인 박사과정 시험은 나를 많이 긴장시켰다. 열심히 준비한 결과, 시험은 무사히 아주 잘 통과했다. 그 희망차고 야심만만한 과제를 수행하기 위해 동분서주하면 열심히 했다. 물론 난 그 과제를 내 희망대로 완벽하게 해 내진 못 했다. 안 되는 것도 있다는 것을 배웠다. 아직 여러면에서 부족하다는 것도 배웠다. 그러나, 끝낼 때는 끝내야 한다는 것, 방향을 돌릴 때는 돌려야 한다는 것, 그리고 시작전에 많이 준비하고 해야한다는 것을 배웠다. 한국과 다른 인간 관계에 때문에 힘들었다. 그러나, 이젠 알겠다. 어떻게 해야 하는 것이 좋을지. 이젠 모두와 어떻게 예쁘게 지낼 수 있는지 알 것 같다.

지난 5년은 나에게 많은 변화를 준 아주 소중한 시간들이다. 빈 손으로 이곳엔 온 내가 지금은 너무 많은 것을 가졌다. 결혼을 해서 사랑하는 남편, 사미가 있고 새로운 가족인 시집 식구들도 있고 늘 날 지지해주고 자랑스러워 해 주고 우리 식구들 있다. 그리고 이제 3주후면 나의 아들도 태어난다. 이렇게 행복한 사람도 있을까? 이 모든 것에 정말 모두에게 감사드린다.

앞으로 어떤 세상이 나에게 펼쳐질지 나두 참 궁금하다. 이제 나에 인생의 새로운 장으로 들어서는 거다. 초심을 잃고 앓고 늘 모든일에 최선을 다 하는 사람이 되었으면 한다.

Curriculum vitae

JongEun Ihm

Date of birth: July 9, 1974

Place of birth: Seoul, Republic of Korea

2003-2008: PhD candidate at EPFL Lausanne, School of Life Science,

Institute of Bioengineering, Laboratory of Regenerative Medicine and
Pharmacobiology.

1999-2001: Master of Science (Biotechnology) at Korea University, School of
Biotechnology.

Master thesis on “Modified chitosan as DNA for gene carrier”.

1999: Bachelor of Science (Chemistry) at Seoul Women’s University.

Peer-Reviewed Papers

1. *Acta Biomaterialia* **1**, 165 (2005)

[Poly \(4-vinylimidazole\) as nonviral gene carrier: *in vitro* and *in vivo* transfection.](#)

Jong Eun Ihm, Ki-Ok Han, Chang Sun Hwang, Jong Hee Kang, Kwang-Duk Ahn, In-Kwon Han, Dong Keun Han, Jeffrey A. Hubbell, Chong-Su Cho.

2. *Langmuir*, **21**, 9346 (2005)

[Thermosensitive Nanospheres with a Gold Layer Revealed as Low-Cytotoxic Drug Vehicles.](#)

Jian Qin, Yun Suk Jo, Jong Eun Ihm, Do Kyung Kim, and Mamoun Muhammed.

3. *Ultrasound in Medicine and Biology*, **31**, 857 (2005)
[Manipulation of Cells using an Ultrasonic Pressure Field.](#) Albrecht Haake, Adrian Neild, Deok-Ho Kim, Jong-Eun Ihm, Yu Sun, Jürg Dual, Bradley J. Nelson and Byeong-Kwon Ju.
4. *Bioconjugate Chem.* **14**, 707 (2003)
High transfection efficiency of poly(4-vinyl imidazole) as a new gene carrier, Jong-Eun Ihm, Ki-Ok Han, In-Kwon Han, Kwang-Duk Ahn, Dong-Kun Han, Chong-Su Cho.
5. *J. Contr. Rel.* **86**, 349 (2003)
Galactosylated chitosan(GC)-graft-poly(vinyl pyrrolidone)(PVP) as hepatocyte-targeting DNA carrier preparation and physicochemical characterization of GC-graft-PVP/DNA complex. I.K. Park, J. E. Ihm, Y. H. Park, Y. J. Choi, S. I. Kim, W. J. Kim, T. Akaike, C. S. Cho.
6. *J. Contr. Rel.* **93**, 389 (2003)
Efficient gene delivery by uronic acid-modified chitosan. Tae Hee Kim, Jong Eun Ihm, Yoo Jae Choi, Jae Woon Nah, Chong Su Cho.