Characterization of the Mechanistic Target of Rapamycin in the Mouse Ovary

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ABSTRACT

The reproductive disorders in high producing farm animals are mainly due to nutrition-associated problems. While metabolic signals have been shown to affect reproductive processes, it is not clear how such multifactorial signals are integrated at the ovary. The mechanistic target of rapamycin (Mtor) is a highly conserved kinase that acts as a master integrator of signals from growth factors, nutrients, as well as energy and oxygen availability to regulate cell cycle and development. Although *in vitro* studies have implicated Mtor in the regulation of ovarian granulosa cell function, the *in vivo* expression pattern and function of Mtor in the ovary remain unclear. We investigated the expression pattern of Mtor-pathway genes at specific stages of gonadotropin stimulated granulosa and luteal development and examined whether ovarian Mtor pathway is sensitive to altered energy intake. In the present study we used mouse as the animal model and laser microdissection to procure pure population of ovarian somatic cells to characterize ovarian Mtor pathway. The results of the present study indicate that the expression of Mtor and its signaling pathway genes remains constant throughout the follicular and luteal development. However, the dramatic upregulation of Rps6ka2, the gene implicated in activation of Mtor signaling, in ovulating follicles is suggestive of the potential regulation of Mtor function during ovulation. Low energy intake (fasting) did not affect the mRNA abundance of Mtor pathway genes and phosphorylation of Mtor at S2448 and S2481. It also did not affect the mRNA abundance of the granulosa genes (Ccnd2, Cyp19, Fshr, etc.). Surprisingly, there was a remarkable downregulation of Ccnd2 protein in the granulosa cells of fasted mice. These data suggest that the down regulation of Ccnd2 protein may involve Mtor, as Mtor is the main regulator of cellular translation in response to nutrient availability. Indeed, our preliminary results demonstrated a dramatic reduction of phosphorylated form of Rps6kb1 – an Mtor target and a reliable indicator of the Mtor activity – in the ovaries of fasted mice. Taken together, the present study demonstrates that the expression of Mtor-pathway genes is not developmentally regulated in the mouse ovary. However, Mtor activity appears to be modulated by nutritional status of the animal and therefore Mtor may act as a metabolic integrator at the ovarian level, which is essential for normal ovarian function.

RÉSUMÉ

Les problèmes de reproduction associés aux animaux d'élevage en production intensive sont principalement dus à des problèmes nutritionnels. Alors qu'il a été montré que certains signaux métaboliques affectent le processus de reproduction, il reste à savoir de quelle manière de tels signaux multifactoriels sont intégrés au niveau de l'ovaire. Mtor (mechanistic target of rapamycin) est une protéine kinase hautement conservée, qui agit comme un intégrateur des signaux provenant des facteurs de croissance, des nutriments ainsi que de la disponibilité en énergie et en oxygène pour réguler le cycle cellulaire et le développement. Bien que des études *in vitro* aient montré une implication de Mtor dans la régulation de la fonction des cellules de la granulosa ovarienne, le mode d'expression et la fonction de Mtor *in vivo* dans l'ovaire restent à élucider. Nous avons étudié le mode d'expression des gènes de la voie de signalisation de Mtor à des stages spécifiques du développement de la granulosa stimulée par des gonadotropines et du développement lutéal. Nous avons cherché à savoir si la voie de signalisation de Mtor dans l'ovaire est sensible à une altération de l'apport énergétique. Dans cette étude, nous avons utilisé des souris comme modèle animal et nous avons effectué des microdissections par laser dans le but d'obtenir une population pure de cellules somatiques ovariennes pour caractériser la voie de signalisation de Mtor dans l'ovaire. Les résultats obtenus ont montré que l'expression de Mtor et des gènes impliqués dans sa voie de signalisation restent constants durant les développements folliculaire et lutéal. Cependant, l'importante régulation positive de Rps6ka2 dans les follicules ovariens, gène impliqué dans l'activation de Mtor, suggère une potentielle régulation de la fonction de Mtor durant l'ovulation. Un faible apport d'énergie (jeûne) n'affecte pas l'abondance d'ARNm codés par les gènes de la voie de signalisation de Mtor ni la phosphorylation de Mtor à S2448 et S2481. L'abondance d'ARNm codés par les gènes des cellules de la granulosa (Ccnd2, Cyp19, Fshr, etc.) n'a pas non plus été affectée. Étonnamment, nous avons remarqué une forte régulation négative de la protéine Ccnd2 dans les cellules de la granulosa chez des souris à jeun. Ces résultats suggèrent que la régulation négative de la protéine Ccnd2 implique Mtor puisque Mtor est le principal régulateur de la traduction en réponse à la disponibilité de nutriments. En effet, des résultats préliminaires ont montré une importante réduction de la forme phosphorylée de Rps6kb1 – une cible de Mtor et un marqueur de son activité – dans les ovaires de souris à jeun. Ainsi,

cette étude démontre que la régulation de l'expression des gènes de la voie de signalisation de Mtor n'est pas liée au stage de développement dans l'ovaire de souris. Cependant, l'activité de Mtor semble être modulée par le statut nutritionnel de l'animal et par conséquent, Mtor pourrait agir comme un intégrateur métabolique au niveau de l'ovaire, ce qui est essentiel pour la fonction ovarienne normale.

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CONTRIBUTION OF AUTHORS

Dr. Raj Duggavathi contributed in a supervisory role in this research and he reviewed the thesis. All experiments and data analysis were carried out by Anitha Kalaiselvanraja. Tamara Cohen and Dayananda Siddappa contributed to the laboratory work by assisting in western blot analyses.

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LIST OF ABBREVIATIONS

Ar	Androgen receptor
ARC	Arcuate nucleus
Areg	Amphiregulin
bHLH	basic helix- loop- helix transcription factor
Bmp	bone morphogenic protein
Btc	Betacellulin
Ca^{2+}	Calcium
cAMP	Cyclic Adenosine mono phosphate
Cebpb	CCAAT/enhancer-binding protein β
CHO	Chinese hamster ovary
Ccnd2	Cyclin D2
CI	Calving interval
CL	Corpus Luteum
COC	Cumulus-oocyte complex
Cyp11a1	Cytochrome P450 cholesterol side chain cleavage
Cyp17a1	17α-hydroxylase/$C_{17–20}$lyase cytochrome P450
Cyp19a1	Aromatase
Egf	Epidermal growth factor
4EBP1	4E binding protein 1
Ereg	Epiregulin
Erk	Extracellular signal-regulated kinase
Fgf2	Fibroblast growth factor
Figla	Factors in the germline α
FKBP	FK506-binding protein
FRAP	FKBP12-rapamycin-associated protein
FSH	Follicle Stimulating hormone
FTC-CR	Failure to conceive culling rate
GAP	GTPase-activating protein
GC	Granulosa cell
Gdf9	Growth differentiation factor 9
Gja1	Connexin 43
Gja4	Connexin 37
GnRH	Gonadotropin releasing hormone
GPCR	G-protein coupled receptor
Gsk3b	Glycogen synthase kinase 3β
HDC	Heat manifestation and detection
Hif1	Hypoxia-inducible factor 1
Hsd3b	3β hydroxysteroid dehydrogenase
Hsd17b	17 β hydroxysteroid dehydrogenase
Insr	Insulin receptor
Irs1	Insulin receptor substrate -1
Kitl	Kit ligand
KO	Knockout
Lepr	Leptin receptor
Igf-1	Insulin like growth factor-1
LH	Luteinizing hormone

Lhcgr	LH/Choriogonadotropin receptor
LMD	Laser Microdissection
M7G	7-Methylguanosine
Mapk	Mitogen-Activated Protein Kinases
Amh	Müllerian - inhibiting substance
Mtor	Mechanistic target of Rapamycin
MTORC1	MTOR complex 1
MTORC2	MTOR complex 2
NEB	Negative energy balance
Nobox	Newborn ovary homeobox
PA	Phosphatidic acid
Pdgf	Platelet-derived growth factor
PGC	Primordial germ cell
PGE2	Prostaglandin E
Pgr	Progesterone receptor
PKC	Protein kinase C
PI3-K	Phosphatidylinositol 3-kinase
POF	Premature ovarian failure
Pparg	Peroxisome proliferator activated receptor gamma
PR	Pregnancy rate
Prr5	Proline-rich repeat protein 5
PVN	Paraventricular Nucleus
Rapalogs	Rapamycin analogs
RAFT	Rapamycin and FKBP12 target
RAPT	*Rap*amycin *t*arget
Rheb	Ras-homology enriched in brain
Rictor	Rapamycin-insensitive companion of Mtor
Rptor	Raptor
SCF	Stem cell factor
SEP	Sirolimus effector protein
Star	Steroidogenic acute regulatory protein
Tgfb	Transforming growth factor beta
TOR	Target of rapamycin
Tsc1/Tsc2	Tuberous sclerosis complex 1/2
WT1	Wilms tumor suppressor 1
ZPs	Zona pellucida

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I. INTRODUCTION

Reproductive performance in modern farms has steadily declined despite improvement in production traits, because the contemporary intensive farm management practices have significant effect on metabolic and nutritional status of food animals. Nutrition is an important determinant of reproductive success, which in turn is of great economic importance to animal industry. All the processes in female reproduction including ovarian growth, oocytes maturation, ovulation, conception, pregnancy and lactation are energetically expensive (Wade and Schneider 1992), and are therefore dependent on the body energy reserves and metabolic responses to nutrition. The link between reproduction and nutrition is mediated through numerous physiological and molecular mechanisms. The regulatory system that connects metabolic input to reproductive process involves complex integration of multiple metabolic and hormonal signals emanating from the central regulators of the reproductive axis (hypothalamus and pituitary) as well as various somatic tissues of the body including adipose tissue, liver and pancreas (Chagas, Bass et al. 2007).

Nutrition associated ovarian dysfunction is encountered in both animals (infertility in dairy cows) and humans (PCOS). Both overfeeding and under nutrition (both acute and chronic dietary restriction) have been shown to affect the reproductive performance. Several studies have investigated nutrition mediated fertility problems in many mammals including dairy cow (Lucy 2001; Chagas, Bass et al. 2007), sows (Etienne, Camous et al. 1983), ewe (Lozano, Lonergan et al. 2003), laboratory rodents (Krackow 1989) and humans(Linne 2004). For example in dairy cow, infertility during early lactation has been attributed to dramatic alterations in the metabolic status such as Negative Energy Balance(NEB), decreased leptin, insulin-resistance, etc., (Lucy 2001). Though several metabolic signals have been shown to underlie such ovarian pathologies, how such multifactorial signals are interpreted at the ovarian level has been a difficult question to resolve.

A great body of recent evidence indicates that the Mechanistic target of Rapamycin (Mtor) could be the candidate molecule to integrate multiple external signals at the ovary. Mtor is a highly conserved serine/threonine protein kinase, known to regulate cell growth and metabolism (Yang, Yang et al. 2008) in response to nutrients, growth factors, cellular energy, mitogens, phosphatidic acid (PA), oxygen availability and stresses. Mtor was first

identified in yeast *Sacchromyces Cerevisiae* (as TOR) in 1991 and later identified in other eukaryotic cells including those of humans and mice (Bai and Jiang 2010). Mtor is an important mediator of cellular signals and acts as a master integrator of metabolic/nutrient signals to regulate cellular processes such as gene expression, protein synthesis, ribosome biogenesis, cell cycle, apoptosis and hypoxic adaptation in many tissues including the liver, muscle and brain. It exists in two distinct multi-protein complexes referred as MTORC1 and MTORC2. Mtor has been reported as a cellular fuel sensor in several organs and its activity is linked to the regulation of energy intake through hormone related signaling such as leptin, ghrelin and insulin (Cota, Proulx et al. 2006; Xu, Li et al. 2009).

Since follicular and corpus luteum (CL) growth involves a complex but tightly regulated integration of numerous autocrine, endocrine and paracrine signals within follicular and luteal microenvironment, Mtor may act as the molecular hub of such integration. Recent *in vitro* studies have shown that Mtor expression is predominantly cytoplasmic/perinuclear and may mediate granulosa cell proliferation and survival (Kayampilly and Menon 2007; Yaba, Bianchi et al. 2008). However, the *in vivo* pattern of Mtor expression and function in different cell-types of ovary that are critical for proliferation, steroidogenesis, oocyte maturation, ovulation and fertility remain to be deciphered.

Given these observations, our **overall hypothesis** was that the expression of Mtor and its signaling pathway genes, and their activity would vary according to the developmental stage of the ovary. In addition, we also hypothesized that Mtor signaling pathway within ovarian granulosa cells can detect changes in the availability of nutrients during follicular growth. Therefore, the main objective of the present study was to systematically characterize the expression pattern of Mtor-pathway genes in the granulosa and luteal cells of the mouse ovary as a first step toward elucidation of the role of Mtor in the regulation of ovarian functions. The second aim of the study was to examine if Mtor within the follicular granulosa cells senses reduced energy intake (fasting) during follicular growth. We used immature super stimulated C57BL/6NCrl mice, because of short generation time and easy to breed, mouse has been used as a primary model for both human and animals since many years in various biomedical research applications. Moreover, its wealth of genetic information and most advanced genetic engineering methods among mammals motivated us to choose this model for the present study.

II. REVIEW OF LITERATURE

1. Ovary - Female reproductive physiology

The Ovary is a highly complex and organized female reproductive organ. Its main function is to produce female gametes (oocyte) and steroid (estrogens and progesterone) production (Gougeon 1996). In most species, the ovarian cortex contains specialized structures called follicles and corpus luteum (CL) at different stages of development. Ovarian follicle is composed of oocytes surrounded by closely associated somatic cells (granulosa and theca cells) (Richards and Pangas 2010). These two somatic cells are structural components of the follicles, of which granulosa cells perform as ovarian "nurse cells" whereas theca cells provide estrogenic precursor "androstenedione" to the granulosa cells. The follicular microenvironment is essential for the growth and development of the oocyte (Edson, Nagaraja et al. 2009). Successful reproduction requires that the oocyte acquires, within the growing ovarian follicle, the ability to be fertilized. In fact, the major cause of female infertility is the defect at the ovarian follicular level, which calls for a better understanding of the physiology of ovarian follicular growth.

In the modern dairy cows, success in milk yield is associated with reproductive problems (Lucy 2001). Although, reproductive failure is a multifaceted problem, one of the main causes of infertility is dramatic alterations in the metabolic status of the cow during early postpartum (Chagas, Bass et al. 2007). Metabolic changes in early lactation, for instance Negative energy balance (NEB) inhibits follicular growth, postpartum LH pulsality, resumption of ovarian activity, oocytes maturation, competence and ovulation (Lucy 2000; Leroy, Vanholder et al. 2004), which ultimately results in infertility.

The fertility indices as well as economic efficiency of the dairy farms are normally measured by maintaining the herd performances including calving interval (CI) and Failure to conceive culling rate (FTC-CR) at the standard level. The standards of these performances are determined by the fertility factors called heat manifestation and detection (HDC) and pregnancy rate (PR). Long or extended CI and high CR are the results of infertility that produce severe financial losses to the dairy farmers and animal production industry (R J Esslemont1 2001). Hence, it is essential to delineate the metabolic and endocrine signals that

are responsible for lower conception rates in dairy cows and the mechanism by which these metabolic signals are translated into nutritional related ovarian dysfunction.

Estrous cycle: is a recurrent set of physiological and behavioral changes in most mammalian females that occurs between successive periods of estrus. It is a cyclic pattern of ovarian activity; each cycle has a follicular and a luteal phase, dominated respectively by the hormone estradiol from ovarian follicles and progesterone from the corpus luteum (Forde, Beltman et al. 2010).

Oogenesis: is the process of meiosis in female organisms from an oogonium to a primary oocyte, to a secondary oocyte, and then to an ovum. The process begins as primordial germ cells (PGCs) soon after fertilization during embryogenesis (Bukovsky, Caudle et al. 2005).

1.1. Primordial Germ cells (PGCs) migration

During embryogenesis, the PGCs migrate from the yolk sac and colonize into indifferent gonads. After colonization in the ovary, the female PGCs undergo proliferation by mitotic division, and subsequently differentiation into oocytes, which enter meiosis (Richards and Pangas 2010). Then the oocytes are blocked in the first meiotic prophase throughout the folliculogenesis (Richard 2007). Afterwards, the meiotically arrested oocytes that present in the form of germ cell cluster develop into primordial follicles as soon as they are individually surrounded with somatic cells (Edson, Nagaraja et al. 2009).

1.2. Folliculogenesis

In most of the species, a large reserve of primordial follicles is established during fetal life (e.g. large mammals) or at the time of birth (e.g. rodents). Primordial follicles serve as a non-growing or resting pool of oocytes, Within these primordial follicles, immature oocyte is surrounded by a single layer of flattened or Squamous pre-granulosa cells (Aerts and Bols 2010). This formation of primordial follicles requires the presence of the oocytes and their interaction with surrounding somatic cells. Follicles begin to grow from the pool of primordial follicle, continuously throughout the life span, and most of them undergo atresia, which is otherwise known as degeneration. For example, the mouse ovary contains a pool of 11,000 oocytes at birth and almost half of them degenerated at 30 days old. The remaining,

those are enclosed within small follicles, beginning to grow from the pool. With age, the number of oocytes and so potential follicles are considerably reduced in the ovary. Hence, only few follicles of about 800 from 30 to 60 days and 30 between 570 and 600 days of age have been identified to emerge from the pool (Edwards, Fowler et al. 1977). Basal follicular growth and terminal follicular growth are the two phases of follicular development, in which the former is dependent of various growth factors of paracrine origin and the later is under the control of gonadotropins (Monniaux, Huet et al. 1997).

Activation of primordial follicles leads to the formation of primary follicles that are characterized by a single layer of cuboidal instead of flattened granulosa cells (Aerts and Bols 2010). During subsequent growth stages, the oocyte within the follicle increases in diameter and Zona pellucida (ZPs) forms between the oocyte and granulosa cells (van Wezel and Rodgers 1996). The Zona pellucida, composed of 3 glycoproteins (ZP1, 2 and 3), is an extra cellular matrix essential for the organization of the granulosa cells around the oocyte (Richards 2001). After the formation of Zona pellucida, communication between granulosa cells and the oocyte occurs via "Gap junctions". As the oocyte gets its nutrition via the granulosa cells and granulosa cells rely on oocyte for their proliferation and functions, the communication between both cell types is bidirectional (Oktem and Oktay 2008; Edson, Nagaraja et al. 2009). Granulosa cells are the key to follicle growth and they proliferate in a stereotyped manner and become steroidogenic in later stages (from antral stage). Subsequent to the granulosa proliferation, basal lamina is formed, upon which theca cell layer is organized (Hirshfield 1991; Elvin and Matzuk 1998).

The next stage of follicular growth is referred to as preantral to early antral transition, which is most susceptible to follicle atresia and is crucial for theca cell layer formation. Several recent findings suggest that the theca cell layer is derived exclusively from cortical stromal cell (Orisaka, Tajima et al. 2009). Theca forms into two layers, of that, theca interna functions as a source of androgens for adjacent granulosa cells and theca externa is essential for ovulation (Edson, Nagaraja et al. 2009).

Tertiary or Antral follicles develop as a result of formation of fluid filled cavity called "antrum" and are further sub divided into small and large antral follicles. It consists three distinct layers called granulosa cell layer, theca interna and theca externa. According to a

previous report (Lussier, Matton et al. 1987), an average of 42 days is required for the development of antral follicles in the cow. During antrum formation, initially follicular fluid also known as liquor folliculi accumulates within the granulosa cells and develops into multiple fluid filled foci and then these multiple foci coalesce to form a single antral cavity (Rodgers and Irving-Rodgers 2010). The antrum separates the granulosa cell population into two functionally distinct subtypes namely mural and cumulus granulosa cells. Mural granulosa cells are required for steroidogenesis and ovulation, while cumulus cells are crucial for oocyte growth and competence(Edson, Nagaraja et al. 2009).

1.2.1 Follicular development during the estrous cycle

The average time requirement for the development of primordial to ovulatory follicular stage is estimated at 20, 184, 180 and 205 days in the mice, (Pedersen 1970) sheep (Lundy, Smith et al. 1999), cow (Lussier, Matton et al. 1987) and human (Gougeon 1996) respectively. During an estrous cycle, a group of antral follicles develops in a wave like pattern (Evans, Adams et al. 1994) into one or more ovulatory follicle in most mammals including humans. The antral follicular dynamics involve four processes that comprise recruitment, selection, dominance and atresia (Fortune 1994; Aerts and Bols 2010). In every growth wave, because of the increased circulating Follicle stimulating hormone (FSH) level, a cohort of antral follicles is recruited for the development. Then the recruited follicles are subjected to selection process, whereby some of them develop further into dominant and the remaining regress. As the follicles enter the selection phase, FSH secretion is inhibited due to the production of estradiol and inhibin by the growing follicles (Ginther, Bergfelt et al. 2000)

Under the influence of FSH, the antral follicle grows bigger and bigger with oocyte maturing within to become the highly organized structure called pre ovulatory or Graffian follicle. Mature antral follicles are termed as Graffian follicle, in which oocyte surrounded by cumulus granulosa cells that are separated from the mural granulosa cells by a fluid-filled cavity (Richards 1980). In order to support the mature oocyte, there are tens of thousands of granulosa cells proliferated and differentiated within ovulatory follicles (Yaba, Bianchi et al. 2008).

The stage of dominance allows producing more estrogen from the largest follicle that induces preovulatory centre to release Luteinizing hormone (LH) surge. Shortly after LH surge, oocytes resume meiosis (Aerts and Bols 2010), and the preovulatory follicle undergoes the process of cumulus expansion due to hyaluronan rich matrix and ovulation. In response to LH, the preovulatory follicles produce plasminogen activator (mucoploysaccharide) (Richards 1980) and prostaglandin E (PGE2) (Filion, Bouchard et al. 2001). The former induces proteolytic enzymatic reaction necessary for ovulation, whereas later is required for the follicle rupture and release of the oocyte.

1.3. Corpus luteum development

After ovulation, the surge of LH causes transformation of granulosa and theca cells into luteal cells, a process called luteinization. Consequently, the remnant of the ovulated follicle differentiates into another endocrine gland called corpus luteum (CL). CL is also referred to as the yellow body, which secretes the hormone progesterone to maintain the pregnancy (Smith, McIntush et al. 1994).

2. Ovarian Steroidogenesis

Ovarian steroidogenesis and subsequent local steroid mediated signaling are essential for normal ovarian processes. Steroid production is tightly regulated and complex process, which appears to occur using two cell/two gonadotropin model. In this model, under the influence of gonadotropin, initially androgen substrate required for ovarian estrogen biosynthesis is produced in theca cells, where LH/choriogonadotropin receptor (*Lhcgr)* is located(Jamnongjit and Hammes 2006) .

In response to basal level of LH, key components of the steroidogenic machinery including Steroidogenic acute regulatory protein (*Star*), cytochrome P450 cholesterol side chain cleavage (*Cyp11a1*), 17α-hydroxylase/$C_{17–20}$ lyase cytochrome P450 (*Cyp17a1*) and 3β hydroxysteroid dehydrogenase (*Hsd3b*) are expressed in theca cells. During the initial stages of steroid synthesis, *Star*, a rate-limiting enzyme, transports cholesterol from outer to inner

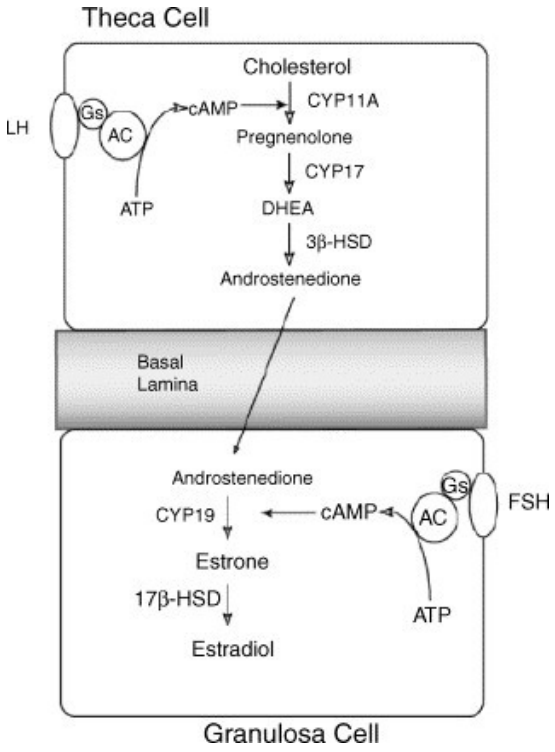

Figure 1: Steroidogenic cascades in the somatic cells of the ovary (Magoffin 2005)

mitochondrial membrane. As shown in Fig. 1, within mitochondria, the cholesterol is converted into pregnenolone followed by dehydroepiandrosterone and then androstenedione using *Cyp11a1, Cyp17a1* and *Hsd3b* respectively. Subsequently, androstenedione diffuses across basement membrane of theca cells and reaches granulosa cells, where the expression of *Fshr*, aromatase (*Cyp19a1*) and 17 β hydroxysteroid dehydrogenase (*Hsd17b*) stimulate conversion of androgen into estradiol under the influence of FSH (Gougeon 1996; Magoffin 2005; Edson, Nagaraja et al. 2009).

The mechanisms that regulate ovarian steroidogenesis include activation of Star, gonadotropin and growth factor mediated signaling as well as both genomic and non-genomic steroid mediated signaling in somatic and germ cells of the ovary respectively (Jamnongjit and Hammes 2006).

3. Regulators of ovarian growth

3.1. Granulosa cell function

Granulosa cells are considered as a most important regulator of ovarian growth because they perform several functions as follows:

Granulosa cells are required for the follicular development and production of estrogen, inhibin, follicular fluid and several growth factors. In addition, the most important role of these cells is to supply nutrients and signaling molecules to the oocyte (Oktem and Oktay 2008) and hence the rate of oocyte growth is directly associated to the number of granulosa cells coupled to the oocyte. Furthermore, they are crucial for the acquisition of oocyte nuclear and cytoplasmic meiotic competence (Fair 2003). Because of the wide range of granulosa functions through follicular development, any abnormality in granulosa cell formation and functions leads to fertility problem in both animals as well as humans. Therefore, we are most interested to understand the role of Mtor in the physiology of granulosa cell.

3.2. Role of Gonadotropins

Although the follicles grow from primordial to secondary stages independent of the pituitary gonadotropins, many events in the adult ovary are gonadotropin dependent (Roche 1996; Orisaka, Tajima et al. 2009). Late follicular development is mediated by two pituitary gonadotropins namely Follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Under the control of pulses of gonadotropin-releasing hormone (GnRH) from the hypothalamus, FSH and LH are secreted from the anterior pituitary and coordinately regulate ovarian function (Richards 1994; Richards and Pangas 2010). In turn, the ovarian steroids regulate hypothalamus and pituitary functions in both negative and positive feedback. FSH controls follicular granulosa cell growth, estradiol production and Lhcgr expression (Richards 1994), whereas LH is required for ovulation, cumulus cell oocyte complex (COC) expansion, oocyte maturation and follicular luteinization.

In rat, in the presence of basal concentration of FSH and LH, small antral follicles develop into preovulatory follicles during metestrus to proestrual stage of estrous cycle (Uilenbroek and Richards 1979). In response to tonic LH, the thecal specific Lhcgr is

increased 2 to 3 fold, and thereby increased testosterone synthesis (Fortune and Armstrong 1977), which is then aromatized into estrogen by FSH stimulated aromatase system in the granulosa cells. Subsequently, the higher concentration of estrogen stimulates further granulosa cell proliferation and modifies Fshr-adenylate cyclase (AC)-CAMP response system in the granulosa cells. This leads to enhancement of granulosa cell response to FSH and mediates FSH induction of Lhcgr in granulosa cells of developing antral follicles, which eventually results in increased responsiveness to LH as well as FSH (Richards 1980).

3.3. Local Modulators/Paracrine factors

3.3.1. Determinants of Primordial germ cell (PGC) formation and proliferation

Several factors regulate PGC formation and proliferation in embryogenesis. Bmp4 and Bmp8b from the extra embryonic ectoderm, and gap junction protein Gja1 are required for the formation of PGC (Richards 2001). The regulation of PGC proliferation is species dependent. In mice, Bmp 2, 4 and 7 increase the proliferation, while activin and Tgfb inhibit it, however in humans, activin is the positive regulator of PGC proliferation (Richards and Pangas 2010).

3.3.2. Factors for Early folliculogenesis & maintain the follicles in non-growing pool

Locally produced factors from the oocyte, granulosa and theca cells regulate follicular growth. *Figla* (Factors in the germline α) is a basic helix- loop- helix transcription factor (bHLH), expressed by oocytes within primordial follicles, facilitates follicle growth initiation (Richards 2001). Studies from *Figla* knockout mice revealed absence of primordial follicles at birth. Because, Figla is required for the expression of all the three Zona pellucida proteins essential for granulosa cell attachment (Soyal, Amleh et al. 2000). In addition, recent mutant mouse models identified that transition from primordial to primary follicles occurs in the presence of other oocyte expressed transcription factors, namely newborn ovary homeobox (*Nobox*), Spermatogenesis and oogenesis bHLH transcription factors 1 and 2 (*Sohlh1* and *Sohlh2*) (Richards and Pangas 2010). Apart from this, Connexin 37 (Gja4), Dazla and c-kit receptor (Kit) are some of the other oocyte specific local factors involve in the follicular growth initiation (Richards 2001).

Kit ligand (Kitl)/stem cell factor, secreted by the granulosa cells, is important for primordial follicle activation. Studies from mutant mice and both *in vitro* and *in vivo* studies explained the importance of Kitl/Kit interaction in the initial stages of follicular growth (Edson, Nagaraja et al. 2009; Aerts and Bols 2010). Moreover, folliculogenesis is regulated by other factors from granulosa cells such as anti-Müllerian hormone (Amh), liver receptor homologous protein -1/Nr5a2, members of forkhead box and GATA family and Wilms tumor suppressor1(WT1) (Richards 2001).

Growth factors including Epidermal growth factor (Egf), basic fibroblast growth factor (Fgf2) , platelet-derived growth factor (Pdgf)(Edson, Nagaraja et al. 2009), and members of the Transforming Growth Factors β (Tgfb) family namely Bone Morphogenic Protein 15 (Bmp 15), Growth differentiation factor 9 (Gdf 9) (Elvin, Yan et al. 2000), activin and inhibin have important role at different stages of folliculogenesis in cell and species specific manner. More specifically, these factors are essential during early stage and stimulate growth of primordial follicle (Webb, Garnsworthy et al. 2004; Orisaka, Tajima et al. 2009). Among these, Bmp15, Gdf9, Pdgf and Fgf2 are oocyte derived factors.

Connexin 43 (Gja1) and Connexin 37 (Gja4) are two gap junction core proteins critical for folliculogenesis and ovarian cell function. The ovaries of Gja1 deficient mice have defects in germ line (Juneja, Barr et al. 1999) with developmental block at the primary follicle stage (Ackert, Gittens et al. 2001), characterized by retarded granulosa cell proliferation and oocyte growth. Mice null for Gja4 do not form gap junction, absence of large antral follicles, defects in oocyte growth, meiotic competence and immature luteinization (Simon, Goodenough et al. 1997; Richards 2001; Edson, Nagaraja et al. 2009).

Preantral to early antral transition is dependent on granulosa secreted factors insulin like growth factor-1 (Igf-I) and Kitl, oocytes specific Gdf9, and is augmented androgen receptor (Ar) expression, Ar mediated androgen production and *Cyp17a1* mRNA expression. Failure of theca layer formation is observed in Gdf9 null mice (Orisaka, Tajima et al. 2009).

3.3.3. Late folliculogenesis and CL development:

In addition to gonadotropins, involvement of steroid hormones and several local factors and their interactions are crucial for folliculogenesis beyond the early antral stage, ovulation and CL development. FSH and other coregulators, for example β-catenin and Tgfb family members activin and inhibin regulate antral and preovulatory stage through multiple signaling cascades (Knight and Glister 2001; Richards and Pangas 2010).

Activin, inhibin, follistatin (Hillier 2001) and Bmp are some of the local factors play important role with regard to modulation of gonadotropins and steroidogenesis. Among them, activin promotes Fshr, Lhcgr expression and estrogen production, while inhibin suppresses FSH by negative feedback with the support of estradiol and enhances LH mediated androgen production (Knight and Glister 2001). Bmp2 has been shown to stimulate FSH secretion in mice (Lee, Khivansara et al. 2007), while BMP4 inhibits FSH release in sheep (Faure, Nicol et al. 2005). In mice, conversion of estradiol from theca cell derived androgen is regulated by means of several factors such as activin, Smad 2/3 and Smad 4 transcription factors, cell cycle regulator cyclin D2 (*Ccnd2*) and the steroidogenic enzyme aromatase (Richards and Pangas 2010).

Preovulatory follicular development and cumulus matrix formation are dependent on oocytes factors namely Bmp15 and Gdf9 and proteases called Adamts1 and cathapsin L. The Epidermal growth factor (Egf)-like family members from mural granulosa cells of preovulatory follicles including amphiregulin (Areg), epiregulin (Ereg), and betacellulin (Btc) also stimulate cumulus expansion and oocytes maturation (Edson, Nagaraja et al. 2009). Apart from this, Igf-1, Ccnd2, Cdk inhibitor (*cdkn1a* and *cdkn1b*) and several nuclear receptors including Estrogen receptors (Esr 1 and Esr 2 subtypes), Progesterone receptor (Pgr) and Peroxisome proliferator activated receptor gamma (Pparg) are additional growth supporting molecules involved in later stages of folliculogenesis and other ovarian functions like CL formation etc (Richards 2001). In consequence of LH stimulated cAMP signaling in luteal cells as well as activation of EGFR signaling cascades includes Ras and Mapk1 and 3 pathways, CL secretes the hormone progesterone to maintain the pregnancy (Richards and Pangas 2010).

3.4. Metabolic indicators/hormones

Normal ovarian function is also dependent on a group of metabolic hormones namely insulin, Igf-1, leptin, adiponectin and ghrelin (Chagas, Bass et al. 2007). Follicular function is modified with changes in peripheral concentrations of metabolic hormones (Webb, Nicholas et al. 2003; Webb, Garnsworthy et al. 2004). Being a central metabolic mediator, insulin potentiates estradiol production (Armstrong, Gong et al. 2002), granulosa cell proliferation, follicular development and ovulation (Poretsky, Cataldo et al. 1999).

There is evidence that growth hormones, for example, Insulin like growth factors (Igfs), composed of two ligands Igf-I and II, play a role in follicular development and atresia (Zhao, van Tol et al. 2000; Monget, Fabre et al. 2002). Insulin and Igf-I have been shown to act synergistically with gonadotropins and thus promote ovarian development and steroidogenesis (Lucy 2000). Within the follicles, the granulosa and theca cells respond to gonadotropin with the influence of locally produced Igf-II and circulating Igf-I (Webb, Garnsworthy et al. 2004). Igfs and Igf binding proteins are nutritionally regulated (Watson, Westhusin et al. 1999)

Leptin has been shown to inhibit ovarian steroidogenesis such as estradiol (Kendall, Gutierrez et al. 2004) theca cell derived androgen and insulin induced progesterone (Spicer and Francisco 1998). However, leptin is found to increase estradiol production when immunized passively (Kendall, Gutierrez et al. 2004) and accelerate gonadotropin stimulated ovarian steroidogenesis by in vitro cultured follicles (Swain, Dunn et al. 2004). In addition, in the later study, leptin has been found to decrease follicle growth rate significantly in dose dependent manner. The roles of leptin signaling in granulosa function seem to be controversial with contradicting data from different labs.

3.5. Signaling Cascades

A complex network of signals emanating from the hypothalamus-pituitary axis, peripheral tissues (adipose tissue, pancreas, liver, etc), and also the ovary itself (paracrine signals) regulates ovarian growth and function during different stages of follicular development, ovulation and corpus luteum development (Gougeon 1996). Among the various cell types of the ovary, granulosa cells are more important in which signaling takes

place by means of nuclear receptors, cyclic adenosine monophosphate and members of the epidermal growth factor family (Duggavathi and Murphy 2009).

3.5.1. Protein Kinase A (Akap7) pathway

Gonadotropins and other extracellular signals exert their effect through divergent signaling pathways initiated by cyclic adenosine 3´, 5´-monophosphate (cAMP) dependent or independent mechanism. In cAMP dependent mechanism, gonadotropins activate adenyl cyclase via G-protein coupled receptor followed by cAMP production and subsequent activation of protein kinase A (Akap7) (Richards 1994; Tamura, Nakagawa et al. 2004). Gonadotropin-induced cAMP-PKA pathway in granulosa and theca cells has been reported as regulators of Star, Cyp17 and Cyp19 expression and thereby promoting estrogen and androgen production (Jamnongjit and Hammes 2006). Moreover, this pathway involves in FSH stimulated granulosa cell proliferation and LH stimulated progesterone secretion in luteal cells (Richards and Pangas 2010).

However, in cAMP independent mechanism, FSH/gonadotropin mediated activation of various signaling cascade includes Phosphatidylinositol 3 kinase(Pik3r1)(via Src tyrosine kinases), Mitogen-Activated Protein Kinase(Mapk) and glycogen synthase kinase 3β (Gsk3b) regulate granulosa cell differentiation and function in the ovary (Wayne, Fan et al. 2007; Richards and Pangas 2010).

3.5.2. Mitogen-Activated Protein Kinases (Mapk-pathway)

Mitogen-activated protein kinase (Mapk) is one of the families of myelin basic protein kinases, activated by tyrosine or threonine phosphorylation. p42mapk (Mapk1/Erk2) and p44mapk (Mapk3/Erk1) are two well-known isoforms, co-expressed all mammalian tissues (Davis 1993; Das, Maizels et al. 1996). It has been observed that Mapk pathway, which is activated by FSH/LH via G protein-coupled receptor, regulates steroidogenesis in different manner (increase or decrease) depending upon the cell culture system. The pathway involves activation of several downstream targets in a sequential order Gβγ, Src , Ras , Raf , Mek , Mapk and Erk (Jamnongjit and Hammes 2006).

Mapk/Erk pathway is necessary to regulate granulosa cell survival and apoptosis (Tamura, Nakagawa et al. 2004), oocyte maturation (Villa-Diaz and Miyano 2004), FSH

mediated steroidogenesis (Moore, Otsuka et al. 2001; Tajima, Dantes et al. 2003; Yu, Han et al. 2005). In addition, in vivo Erk1/2 Knock out (KO) experiment in the mouse granulosa cells demonstrates the importance of these kinases for LH mediated ovarian functions including meiotic resumption of oocytes, ovulation and luteinization (Fan, Liu et al. 2009). Furthermore, the Mapk is also required for LH mediated progesterone secretion (Richards and Pangas 2010) and PGF2α induced luteal cell regression (Arvisais, Romanelli et al. 2006).

3.5.3. Janus Kinase - Signal transducer and activator of transcription (Jak-Stat pathway)

Jak-Stat pathway mediates its effects in response to cytokines and growth factors. Upon cytokine signaling, Jak, a cytoplasmic protein has tyrosine kinase activity, is autophosphorylated and activates Stat, a transcription factor. Activation of Stat leads to its dimerization and translocation into the nucleus and thus mediates gene transcription. There are 4 Jak family members and 7 Stat factors in mammalian cells (Imada and Leonard 2000; Hou, Zheng et al. 2002). Both i*n vitro* and *in vivo* evidence demonstrate that Jak-Stat pathway is required for haematopoiesis (Hou, Zheng et al. 2002), developmental process in many cells types including cancer cells and apoptosis (Bromberg 2001), patterning of follicular epithelium and border cell migration during oogenesis in the Drosophila ovary (Silver and Montell 2001; Denef and Schupbach 2003; Silver, Geisbrecht et al. 2005) and fertility and early embryonic development in Holstein cattle(Khatib, Huang et al. 2009). Over activation of this pathway causes oncogenic transformation, for instance in many human cancers persistent activation of Stat 3 factor was identified (Bromberg 2001).

3.5.4. Wingless-type MMTV integration site (Wnt) signaling

The importance of Wnt signaling for the female fertility was first recognized when a study from Wnt4 null mice shows oocyte depletion and female to male sex reversal. Wnt4, Fzd4 (Frizzed) and Ctnnb1 (β-catenin) are critical components of this pathway and among the several (19) members of this family, Wnt2 and Wnt4 are expressed in granulosa cells of all stages of follicles (Boyer, Goff et al. 2010). In several recent studies, Wnt4/Ctnnb1 has been identified to increase FSH mediated granulosa cell proliferation, antral and preovulatory follicular growth and expression of steroidogenic enzyme genes such as *Star*, *Cyp11a1*, and *Cyp19* (Parakh, Hernandez et al. 2006; Fan, O'Connor et al. 2010). However,

overactivation of Wnt4/Ctnnb1 leads to repression of LH induced oocyte maturation, ovulation, luteinization and progesterone synthesis both *in vivo* as well as *in vitro* (Fan, O'Connor et al. 2010).

3.5.5. Phosphatidylinositol 3 kinase (Pik3r1) signaling

PI3K is a family of lipid kinases, responsible for the production of second messenger, phosphatidylinositol - 3, 4, 5 triphosphate(PIP3). PI3K can be activated by several ways through either tyrosine kinase or G-protein coupled receptors. Primarily Growth factors and hormones are important mediators that trigger PI3K activation. Activated PI3K converts phosphatidylinositol 4, 5-bisphosphate (PIP2) to PIP3, which results in Akt (alpha serine/threonine protein kinase otherwise known as protein kinase B/ PKB) activation. This in turn phosphorylates multiple proteins on serine-threonine residues including Tsc 1/2, Foxo3, IKK , Gsk3 , caspases and other kinases to regulate cell growth, survival and metabolism (Liu 2006).

In the ovary, PI3K/Akt pathway has been shown to be induced for the following process; Kitl/Kit mediated suppression of Foxo3 for primordial follicle activation (Reddy, Shen et al. 2005), Igf-1 mediated granulosa proliferation and survival (Richards, Russell et al. 2002), FSH stimulated granulosa differentiation (Alam, Maizels et al. 2004) Gonadotropin induced cumulus expansion and oocytes maturation *in vitro* (Shimada, Ito et al. 2003) , granulosa and luteal cell survival and life span by *in vivo* (Fan, Liu et al. 2008).

Recent studies in mutant mouse models reveal that many other intra ovarian signaling cascades including TGF-β/SMAD, human R-spondin homolog (*Rspo1*) gene, and the transcription factors forkhead box (FOXO/FOXL2) and CCAAT/enhancer-binding protein β (Cebpb) are determinants of female gonad and regulates ovarian follicle development and gonadotropin action in stage and contact specific manner ((Richards and Pangas 2010).

While multiple metabolic signals affect normal ovarian development and follicular function, exactly how these signals are mediated to the ovary is not clear. One of the pathways that serve to integrate various metabolic signals within the ovary could be of the

Mechanistic target of Rapamycin (Mtor) kinase to regulate cell responses to environmental cues.

4. Mechanistic Target of Rapamycin (Mtor)

TOR, Target of rapamycin (TOR) is a serine/threonine protein kinase that controls cell growth, size, survival and metabolism (Yang, Yang et al. 2008) in many types of cells from yeast to mammals in response to nutrients, growth factors, cellular energy, mitogens , phosphatidic acid(PA) and stresses. TOR was first identified following experiments selecting for rapamycin-resistant strains of *Saccharomyces cerevisae* (Yang, Yang et al. 2008; Bai and Jiang 2010).

The yeast homolog of TOR in mammals is termed as Mammalian (Mechanistic) target of rapamycin (Mtor), which acts as a master integrator of metabolic/nutrient signals to regulate diverse cellular processes (Bai and Jiang 2010). Mtor is also named in several ways as follows; FRAP (FKBP12-rapamycin-associated protein), RAPT (rapamycin target), RAFT (rapamycin and FKBP12 target) or SEP (sirolimus effector protein) (Fingar and Blenis 2004; Yang, Yang et al. 2008)

Rapamycin binds to its receptor FKBP12 (FK506-binding protein of 12 kDa) and this FKBP12-rapamycin complex then binds to FRB (FKBP12-rapamycin binding) domain of the Mtor to result in TOR inhibition (Bai and Jiang 2010; Dowling, Topisirovic et al. 2010). However, since the recognition of rapamycin as a highly effective Mtor inhibitor and rapamycin treated cells become resistant to growth factors and nutrient stimulation, several applications of this drug are currently being explored. Particularly rapamycin and rapamycin analogs (rapalogs) are being tested for the treatment of cancer (Fingar and Blenis 2004) or as a preventative measure against graft rejection (Soliman and Ghada 2005). Other therapeutic uses of rapamycin are remedy for diabetic nephropathy in both type 1 and 2 diabetic model (Inoki 2008),obesity and autoimmune diseases such as idiopathic and lupus membranous nephropathy (Tsang, Qi et al. 2007). Additionally, since the drug is capable of inhibiting vascular smooth muscle cells proliferation, it was approved as an anti-restenosis drug for coronary artery stents by the FDA (Tsang, Qi et al. 2007).

4.1. Structure of Mtor

Mtor is a 289-kDa multi-domain protein, which belongs to the family of the phosphatidylinositol 3-kinase (PI3-K)-related kinases (PIKK), composed of 2549 amino acids and various structurally conserved domains, as shown in Figure 2. The two blocks of N-terminal tandem repeats denoted as HEAT (Soliman and Ghada 2005). The HEAT motifs form alpha-helices involved in protein-protein interactions. Downstream of these blocks is the FAT domain, which is common to the PIKK family proteins and is important interaction with other proteins. The C-terminal kinase domain is structurally related to PI3-K and is responsible for the catalytic activity of Mtor (Bai and Jiang 2010). However, unlike PI3K proteins, Mtor functions as a Ser-Thr protein kinase. Flanked by the FAT and kinase domain lies the FRB domain where the FKBP12 (Fkbp1a) and rapamycin complex binds. Finally, another FAT domain is located at the C-terminus is very important for Mtor kinase function (Fingar and Blenis 2004; Hay and Sonenberg 2004; Soliman 2005; Yang, Yang et al. 2008; Bai and Jiang 2010) and the redox potential characteristic of this domain is responsible for structural and cellular stability of Mtor (Dames, Mulet et al. 2005).

Mtor phosphorylation appears to activate its kinase activity, and thus the phosphorylation status can be used as an indicator of Mtor activity. Mtor is Phosphorylated by p70 ribosomal S6 kinase (Rps6kb1), autophosphorylation and cAMP activated protein kinase (AMPK) at Ser 2448, Ser 2481 and Thr 2446 respectively (Cheng, Fryer et al. 2004; Chiang and Abraham 2005; Copp, Manning et al. 2009). In addition, MTORC1 activity is promoted by another phosphorylation site denoted as Ser 1261 that is essential for autophosphorylation of Mtor at Ser 2481(Acosta-Jaquez, Keller et al. 2009).

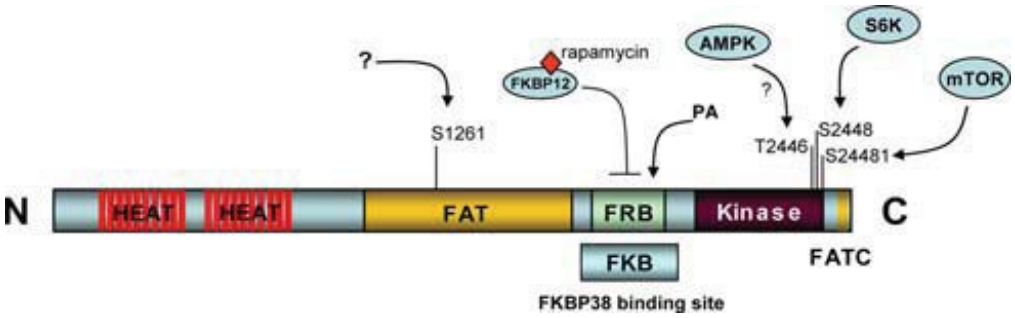

Figure 2: The structure and domains on Mtor. The HEAT-repeats allow for protein-protein interactions, and the FRB (*F*KBP12-*r*apamycin *b*inding region) is the inhibitory site. The FAT and FATC domains are common to the family of PIKK family that the serine-threonine kinases belongs to (Bai and Jiang 2010).

4.2. Components of mTOR complexes

Mtor occurs in two different complexes namely mTOR complex 1(MTORC1) and mTOR complex 2(MTORC2). However, both complexes require the presence of other proteins in order for the functional kinase activity to occur. The MTORC1 is sensitive to rapamycin and consists of Mtor, Rptor (regulatory associated protein of Mtor), Mlst8 (mammalian lethal with sec13 protein 8, also known as G protein beta subunit-like protein GβL) and PRAS 40 (Proline rich PKB substrate of 40 KD). The MTORC1 has been implicated in many growth related processes including translation, ribosome biogenesis, transcription, cell division, autophagy and oxygen adaptation (Tsang, Qi et al. 2007; Hall 2008; Bai and Jiang 2010).

MTORC2 is resistant to rapamycin and its components are Rictor (rapamycin-insensitive companion of Mtor), Mlst8, mSIN1 (mammalian stress-activated protein kinase-interacting protein 1) and Prr5 (proline-rich repeat protein-5) or Prr5-like (also known as protor1 and protor 2). Regulation of Akt and organization of actin cytoskeleton have been two major functions ascribed to MTORC2 (Bhaskar and Hay 2007; Meric-Bernstam and Gonzalez-Angulo 2009). The function of most of the components like G*β*L, rictor, mSIN1 and PRR5 are not known at the moment. In general, studies tend to focus on MTORC1

rather than MTORC2, since MTORC1 is affected by the administration of rapamycin (Yang, Yang et al. 2008).

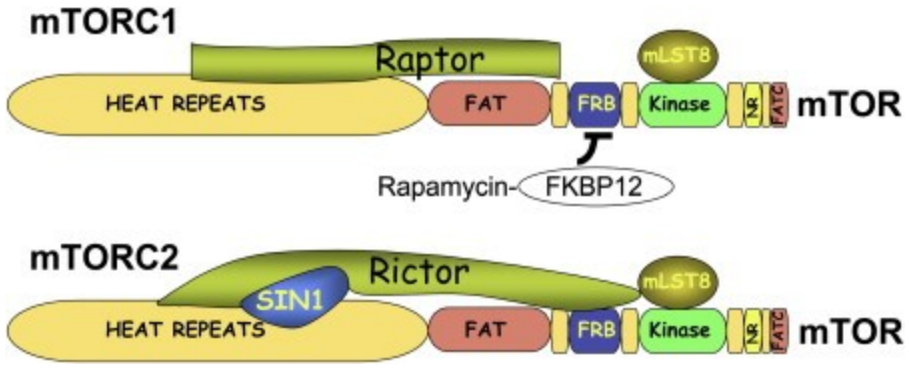

Figure 3: Mtor complexes (Bhaskar and Hay 2007).

4.2.1. Raptor (Rptor)

Rptor is a 150 kDa large protein, contains an N-terminal RNC domain, three HEAT repeats and C- terminally located seven WD-40 repeats. Raptor is the unique component of MTORC1 and it functions as an adaptor protein in order to bring various protein substrates to Mtor for their MTORC1 dependent phosphorylation (Kim, Sarbassov et al. 2002; Hall 2008). While mTORC1 has many other substrates, Rps6kb1/2 and 4E-BP1 are the two well-known downstream substrates. Other downstream targets of MTORC1 include Irs1, Cyclin D3 , p53, Pparg, Stat3 and hypoxia inducible transcription factor 1α(HIF1α) etc (Alam, Maizels et al. 2004; Kim and Chen 2004; Soliman 2005; Dowling, Topisirovic et al. 2010). Raptor binds to TOR signaling motif (TOS), which is a sequence motif present in Rps6kb1and 4E-BP1. In addition, RAIP and SAIN are other raptor interacting motifs found respectively in 4E-BP1 and insulin receptor substrate -1(Irs1).

Autophosphorylation of raptor at multiple sites is influenced by several kinases including AMPK, p90 ribosomal S6 kinases 1 and 2 (Rps6ka 1 and Rps6ka 3) and MTORC1 (Carriere, Cargnello et al. 2008). This results in alteration of MTORC1 kinase activity. From the above findings, it is clear that raptor can integrate various signals for Mtor regulation and thereby regulating cellular functions such as growth related process, oxygen adaptation etc.

4.2.2. Rictor

Rictor is a 200 kDa large protein, contains seven domains with sequence conservation, plays important role for the regulation of Akt phosphorylation at Ser473 (PKB) (Sarbassov, Ali et al. 2005). The FoxO transcription factor is one of the prime candidates most affected by MTORC2 deficiency (Bhaskar and Hay 2007). Another role of rictor is to regulate phosphorylation of protein kinase C (Prkca) for actin cytoskeleton (Sarbassov, Ali et al. 2004; Meric-Bernstam and Gonzalez-Angulo 2009). Prkca is ubiquitously expressed in mammalian cells and is important for growth related cellular processes and the regulation of cell shape and mobility. From gene targeting studies rictor is identified essential during mid and late embryogenesis, because knockout embryos found dead at midgestation (Bhaskar and Hay 2007).

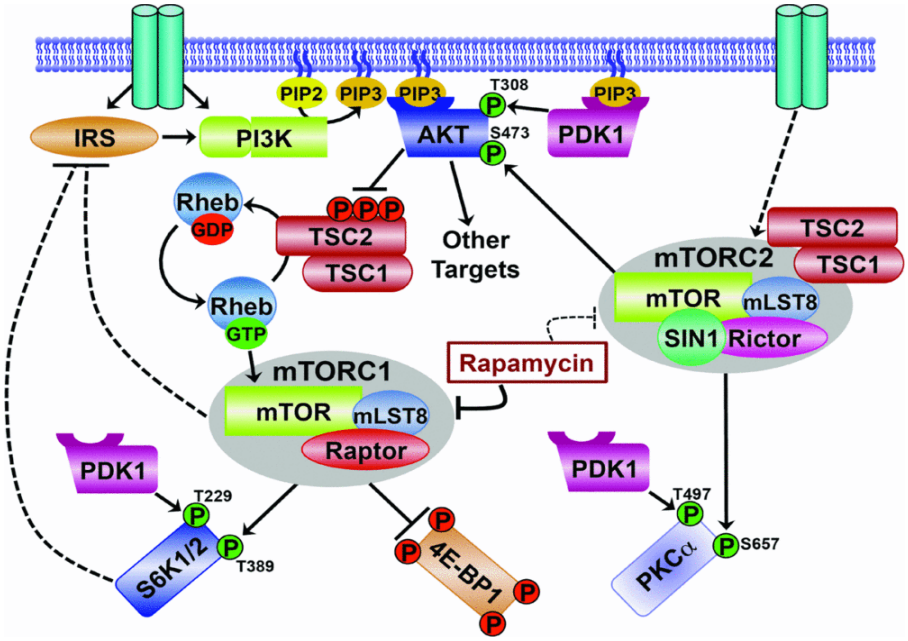

Figure 4: Relationship between Akt, the Tsc1-Tsc2 complex and the two Mtor complexes (Huang and Manning 2009).

4.3. The signaling network of Mtor

The mammalian physiological status can be inferred from various metabolic signals generated by hormones, growth factors, nutrient and energy availability, and stress factors. Being an intracellular kinase sensor, Mtor integrates with those metabolic signals and ultimately mediates many growth related processes including Translation, ribosome biogenesis, transcription, autophagy and oxygen adaptation in the case of MTORC1 or actin rearrangement throughout the cell cycle, in the case of MTORC2, (Wang, Fonseca et al. 2008; Yang, Yang et al. 2008; Bai and Jiang 2010).

4.3.1. Mtor regulators

4.3.1.1. Upstream regulators

i) Tuberous sclerosis complex 1/2(Tsc1/Tsc2)

Studies on Mtor led to the discovery of the tumour suppressor genes, tuberous sclerosis complex (Tsc) 1/2 as the negative regulator of Mtor. Inactivation or mutation in either of the two Tsc gene products leads to benign tumour formation such as hematomas, in several tissues (Soliman 2005; Bai and Jiang 2010). The heterodimeric Tsc complex exhibits GTPase-activating protein (GAP) activity on small GTP–binding protein, Rheb for Mtor activation. Genetic studies in *Drosophila* helped clarify the role of Tsc1/Tsc 2 in cell growth (Radimerski, Montagne et al. 2002; Sarbassov, Ali et al. 2005). Though, Tsc1 does not have GAP activity, it seems to be obligatory for the function of Tsc 2 that has GAP activity. Tsc 2 phosphorylation is linked with multiple kinases such as Akt and AMPK, Rps6ka1 and Mapk1/3, involved in wide variety of signaling pathways (Ma, Chen et al. 2005; Ma and Blenis 2009; Bai and Jiang 2010).

Akt directed phosphorylation of Tsc 2 in the case of insulin and growth factors mediated signaling results in up-regulation of Mtor activity. In general, Akt pathway mediates Mtor up regulation either through activation of Rheb-GTPase or through inhibition of PRAS40 (Ma and Blenis 2009). Similarly, Rps6ka1 and Mapk down-regulate Tsc function and thus up-regulate Mtor in response to mitogen stimulation and Ras activation. In contrast, Mtor is down-regulated when the cellular energy level is low and in hypoxic

conditions (Yang, Yang et al. 2008). In the both cases, Tsc1/2 complex is activated and thereby inhibiting Mtor via Rheb.

ii) Rheb (Ras-homology enriched in brain)

This small GTPase was originally identified in a rat brain and is more structurally similar to Ras than to other GTPases, thus it was named Ras-homology enriched in brain (Rheb) (Bai and Jiang 2010). As mentioned earlier, Rheb activity is increased by the Tsc1/2 complex. Although direct binding between Rheb and Mtor has not been demonstrated, it is known that Rheb is an activator for Mtor activity (Soliman and Ghada 2005; Bai and Jiang 2010). However, if the negative regulator, Fkbp1a is bound by positive regulator Rheb, Mtor inhibition is prevented (Bai and Jiang 2010).

iii) FKBP38 (Fkbp8)

Fkbp8 is a member of the FK506-binding protein (FKBP) family, which also includes Fkbp1a. This family of chaperones are involved in protein folding and all possess a common FKBP-C domain, which enables binding between Fkbp8 and Mtor. Interestingly, Fkbp8 binding site on Mtor is the same as that of the Fkbp1a-rapamycin complex, and thus inhibits Mtor activity. Much like the Fkbp1a-rapamycin complex, Fkbp8 only interacts and interferes with MTORC1 activity (Nielsen, Mitchelmore et al. 2004; Bai and Jiang 2010).

4.3.1.2. Downstream targets or effectors

i) **S6K1 (Ribosomal protein S6 Kinase 1 - Rps6kb1)**

Rps6kb1is phosphorylated by MTORC1 through PI3K/Akt pathway in response to growth factors and hormones. A linear pathway of Rps6kb1 activation starts from growth factor receptor activation and then involvement of the activities of PI3K, Akt, Tsc1/Tsc2, Rheb and then finally from MTORC1 to Rps6kb1.

Mtor activated Rps6kb1 then phosphorylates the major ribosomal protein S6, (Harris and Lawrence 2003). Phosphorylation of ribosomal S6 leads to increased mRNA translation. More specifically, this phosphorylation effects increased translation of the mRNA's that possess the tandem oligopolypyridimine tract (tract of pyramidine-TOP) (Hay and Sonenberg 2004; Soliman 2005) motif, which consists of a cytosine and 4-14 pyramidines

(Cytosine, Thymine and Uracil). TOP motif is present all the mRNA's that code the known ribosomal proteins, as well as several elongation factors. Hence, Mtor activation of Rps6kb1 leads to increased translation and higher protein levels in cells (Harris and Lawrence 2003; Fingar and Blenis 2004; Yang, Yang et al. 2008). However, Rps6kb1 is not the sole regulator of effective translation of TOP messages (Harris and Lawrence 2003).

Apart from this, another important activity of Rps6kb1 in Mtor regulation is that it phosphorylates Irs-1, which is responsible for the activation of phosphatidylinositol 3-kinase (PI3K) in insulin/IGF-1 mediated signaling to Mtor. In consequence to the phosphorylation, Rps6kb1forms a significant negative feedback that eventually down regulates downstream components of the insulin/PI3K pathway to Mtor (Bai and Jiang 2010).

ii) 4E binding protein 1 (4EBP1)

Similar to Rps6kb1, the 4EBP1 first identified as a protein in adipocyte cells, is also stimulated by insulin but is a negative regulator of Mtor. In quiescent cells, 4EBP1 acts as a translational repressor by binding tightly to the 7-methylguanosine (m7G) cap-binding protein. Insulin stimulation leads to higher phosphorylation events of 4EBP1, potentially due to Mtor kinase activity, release from its bound state with eIF4E, and consequently, inactivation of 4EBP1. This inactivation allows the eIF4E-eIF4G complex to form, allowing cap-dependent mRNA translation to occur, thereby increasing the effects of Mtor on protein translation (Harris and Lawrence 2003).

4.3.2 MTORC1 signaling network

4.3.2.1. Hormones and Growth factors

Among the various signal inputs, growth factors and hormones (insulin, leptin, etc) induced Mtor activation is the best characterized and is mediated by the activation of PI3K. Hormone regulation of Mtor signaling is mediated via their receptor and signal transduction pathway.

A. Insulin: In case of insulin receptor (Insr), its activation leads to phosphorylation activation of its substrate, Irs1. This helps PI3K to initiate the signaling cascade and activate other downstream proteins from Akt to the Tsc1/Tsc2 complex. The activated Akt

phosphorylates Tsc2 and destabilizes the Tsc1/2 complex, thereby promoting the activation of Mtor by Rheb GTPase. Eventually, the hormone induced Mtor kinase activity results into Rps6kb1 activation, 4EBPI inactivation and increased protein synthesis (Harris and Lawrence 2003; Fingar and Blenis 2004; Hay and Sonenberg 2004; Yang, Yang et al. 2008). Interestingly, as mentioned earlier, Mtor-activated Rps6kb1 can act to suppress Irs1 and PI3K and as a result, decrease the effects of insulin on Mtor (Harris and Lawrence 2003; Fingar and Blenis 2004; Yang, Yang et al. 2008). Both leptin (Mori, Inoki et al. 2009; Morris and Rui 2009) and insulin (Ortega, Rey et al. 2010) hormones mediate cellular effects through the Mtor signaling pathway and the hormonal activation of Mtor and Rps6kb1 is dependent on PI3K/Akt pathway.

B. Leptin: Though molecular steps related with leptin signaling mediated through long form of leptin receptor (Lepr) is not very clear at present, leptin has been shown to involve in the regulation of Mtor pathway. Recent findings of leptin increases Mtor signaling and Mtor regulates synthesis and secretion of leptin from adipose cells suggesting that Mtor has central role in whole body energy (Cota, Proulx et al. 2006; Morris and Rui 2009).

In the hypothalamus, leptin treatment stimulates Rps6kb1 phosphorylation (Blouet, Ono et al. 2008), and inhibition of leptin anorexigenic effects due to Rapamycin and deletion of Rps6kb1 are the evidence for Mtor contribution in Leptin signaling (Cota, Matter et al. 2008). In mice, leptin resistance, hyperphagia, and obesity could be observed when Mtor, Rps6kb1and PI3K signals are activated chronically by Rip2- Cre mediated deletion of Tsc2 (Mori, Inoki et al. 2009).

4.3.2.2. Nutrition

A. Energy status:

Changes in the ratio of AMP:ATP modulate Mtor signaling (Hay and Sonenberg 2004). Mtor activation is triggered when the intracellular ATP concentrations are higher than the homeostatic level, thereby stimulating cell growth and proliferation. Conversely, inactivation of Mtor signaling pathway occurs when ATP concentrations have dropped considerably relative to AMP concentrations, in an attempt to prevent cell growth. Mtor itself is believed to serve as an ATP sensor as the signaling is influenced by intracellular

adenosine triphosphate, *in vitro* (Dennis, Jaeschke et al. 2001). During low energy, AMPK that functions as an intracellular sensor of AMP: ATP ratio is phosphorylated by LKB-1. The phosphorylated AMPK proceeds to phosphorylate the Tsc1/Tsc 2 complex, and eventually Mtor is inhibited (Harris and Lawrence 2003; Yang, Yang et al. 2008). In general, reduced cell size and cell proliferation and onset of autophagy are reported due to decreased Mtor activity during the periods of compromised nutrition and in stress (Yaba, Bianchi et al. 2008).

B. Amino acids:

The exact mechanism by which the Mtor signaling pathway senses amino acid availability is not yet well-understood. Amino acids are currently thought to affect Mtor activity through four different mechanisms (Wang, Fonseca et al. 2008; Yang, Yang et al. 2008). In the first proposed mechanism, changes in amino acid availability stimulate the upstream regulator Tsc1/Tsc2 complex either directly or indirectly. Secondly, using a class III PI3-K (otherwise known as human vacuolar protein sorting 34 (hvps 34) amino acids can directly or indirectly activate MTORC1 through FYVE (*F*ab1, *Y*OTB, *V*ac1p, *e*arly endosome antigen 1 domain-containing proteins) and phox homology domain-containing proteins. These two proteins are believed as Rab effectors (Nobukuni, Kozma et al. 2007). But in contrast, (Gulati, Gaspers et al. 2008) reported that rise in intracellular Ca2 / calmodulin (cAM) signaling is the mechanism by which amino acid signals to hvps 34 and induces MTORC1. Thirdly, it has been suggested that eIF4G (Bolster, Vary et al. 2004) and tRNA aminoacylation are involved in Mtor signaling. Lastly, MTORC1 signaling can be influenced by mitogen-activated protein kinase kinase kinase kinase 3 (Map4k3). More recently, aminoacid was found to utilise class 1 PI3-K/Akt signaling pathway to activate MTORC2 (Tato, Bartrons et al. 2010).

More importantly, it is well known that leucine significantly up-regulates Mtor signaling, and thereby increases phosphorylation of Rps6kb1 and 4EBP1. It has been hypothesized that this is due to fact that leucine is a common precursor to protein synthesis (Soliman 2005; Cota, Proulx et al. 2006). In the absence of amino acid leucine, a decrease in Rps6kb1activity in response to insulin and vice versa, signify the link between amino acid and insulin in the regulation of Mtor mediated translation process (Proud, Wang et al. 2001).

C. Stress:

Hypoxia-inducible factor 1 (Hif1) induces the expression of REDD1 (regulated in development and DNA damage responses protein 1) due to cellular stress through hypoxia. In turn, REDD1 increases Tsc2 activity, thereby inhibiting Rheb, Mtor (Yang, Yang et al. 2008).

4.4. Abnormal Mtor signaling

Lethality is reported in mice that have complete deletion of Mtor gene (loss of TOR function) (Gangloff, Mueller et al. 2004), whereas in drosophila, dysfunction is associated with the formation of smaller cells and autophagy in all kind of tissues (Montagne, Stewart et al. 1999). The rats maintained on a high fat diet have been shown to develop obesity, cancer and diabetes owing to increased Mtor action in the liver and skeletal muscle (Khamzina, Veilleux et al. 2005). Hyper activation of Mtor signaling in cancer cells is caused by either over expression of Akt or a mutation in Pten (phosphatase and tension homolog deleted on chromosome 10) (Tsang, Qi et al. 2007)

4.5. Mtor inhibitors

The immunosuppressant macrolide rapamycin and its derivatives selectively inhibit the activity of MTORC1 (Dancey 2006). Inhibition of Mtor has been shown to cause G1 phase arrest of the cell cycle (Schmelzle and Hall 2000) and induce apoptosis through inhibition of survival factors signaling (Huang and Houghton 2003).

4.6. Mtor Regulation of metabolism and growth

4.6.1 Metabolic tissues

Metabolic tissues such as liver, muscle and adipose tissue are most sensitive to insulin and nutrients and thus control body energy homeostasis. The activation of the Mtor pathway is markedly elevated in the liver and skeletal muscle of insulin resistant obese rats maintained on high fat diet (Khamzina, Veilleux et al. 2005). Mtor is a key regulator of skeletal muscle development by governing distinct stages of myogenesis (Sun, Ge et al. 2010). The data from (Kim and Chen 2004) study suggest that Mtor acts as a molecular link

between nutrients and adipogenesis by a mechanism involves positive regulation of PPAR-γ (Pparg)activity in 3T3-L1 adipocytes.

4.6.2 Mtor senses energy status

In mammalian peripheral cells, Mtor controls growth and development by integrating nutrient signals with hormone signals (Schmelzle and Hall 2000). Mtor performs as a cellular fuel sensor and regulates energy intake through the hormones such as, ghrelin (Xu, Li et al. 2009), insulin (Proud, Wang et al. 2001; Zhang, Huang et al. 2009) and leptin (Cota, Proulx et al. 2006; Morris and Rui 2009).Studies in Chinese hamster ovary (CHO) cells suggest that Mtor mediated translation process is determined by the availability of nutrients such as glucose, amino acid and calcium (ca^{2+}) concentration within the cellular microenvironment (Proud, Wang et al. 2001).

In rats fasted for 48 h, a significant decrease in the expression of pMtor, pRps6kb1 (pS6K1) and pS6 in the arcuate nucleus of the hypothalamus suggesting that Mtor senses energy status and is responsible for central neuronal control of nutrient intake (Cota, Proulx et al. 2006). Increase hypothalamic Mtor signaling accompanied with decrease food intake and body weight gain in leucine administered rats suggests that amino acid regulates food intake through Mtor. However, another aminoacid L- valine does not have any effect on Mtor signaling (Cota, Proulx et al. 2006).

A previous report from gastric mucosa with fasting down regulates Mtor activity and up regulates ghrelin (a gastric hormone that provide hunger signal to the brain) and vice versa in fat mice represents that Mtor regulates Ghrelin production in reciprocal manner during energy changes.(Xu, Li et al. 2009)

5. Role of Mtor in the ovary

There are 3 important process in the ovary regulated by Mtor, which includes granulosa cell proliferation, Oocyte maturation (ie again controlled by ovarian follicular growth) and Steroidogenesis.

5.1. Mtor expression and Regulation

5.1.1. Follicular growth

Follicular development and oocyte growth are achieved through PI3K signaling cascade mediated from the granulosa cell-produced stem cell factor (SCF/Kitl) to the oocyte surface SCF receptor/Kit. Upon binding of SCF and Kit, Kit undergoes auto-phosphorylation and develops PI3K/Akt signaling cascade, which proceeds until Mtor senses the signal (Liu 2006). This leads to activation of Rps6kb1 thereby increased mRNA translation and protein synthesis.

According to (Yaba, Bianchi et al. 2008), reduction in granulosa cell proliferation and follicle growth due to Mtor inhibition *in vitro* supporting the hypothesis of ovarian follicle growth may be directly influenced by nutritional cues and stress via Mtor. Recent reports indicate that involvement of Mtor in FSH–mediated granulosa cell proliferation and differentiation (Alam, Maizels et al. 2004; Kayampilly and Menon 2007) In the former, FSH mediated Mtor is required for Ccnd2 expression (D type protein that regulates cell cycle progression from G1 to S phase), while, in the later, Mtor induces expression of several follicular differentiation markers including Lhcgr, inhibin-α, MAP2D and PKA regulatory subunit II b in granulosa cells. According to Alam et al, FSH activation of hypoxia inducible factor 1, a protein molecule associated with granulosa cell differentiation, is mediated by the PI3K/Akt/Rheb/Mtor pathway. In contrast, Mapk1/3 pathway is reported to involve in the regulation of FSH mediated activation of Mtor signaling in proliferating granulosa cell (Kayampilly and Menon 2007).

5.1.2. Oocytes

Tsc/MTORC1 and PTEN/PI3K signaling in oocytes regulate the quiescence and activation of primordial follicles in a synergistic way in order to achieve reproductive ability and longevity. In mutant mice null for Tsc1 gene in oocytes, the entire pool of primordial follicle is activated due to enhanced MTORC1 activity and accordingly results in premature ovarian failure (POF) and infertility (Adhikari, Zheng et al. 2010).

5.1.3. Corpus luteum

Studies with LH treated cultured bovine corpus luteal cells show that LH stimulates Mtor substrates Rps6kb1and 4EBP1 activity by inhibiting the activity of glycogen synthase kinase 3B (Gsk3b) and AMPK (Hou, Arvisais et al. 2010). In contrast to the activation of Mtor signaling in granulosa cells (Alam, Maizels et al. 2004; Kayampilly and Menon 2007), LH mediated Mtor signaling in luteal cells is not dependent of MAPK kinase 1 (Map2k1)/ MAPK or PI3K/Akt signaling pathways (Hou, Arvisais et al. 2010).

However, PGF2α, a hormone that governs CL regression, is found to activate Mtor signaling in cultured bovine luteal cells via Erk dependent (PKC-Raf-MEK-Erk pathway), Akt independent mechanism. Upon binding with G-protein coupled receptor(GPCR), PGF2α activates phospholipase β(PLC β) and generates second messengers diacylglycerol and inositol trisphosphate, which ultimately activates extracellular signal-regulated kinase (Erk) family of mitogen-activated protein kinases(Mapk) through a mechanism of Protein kinase C(PKC)-dependent activation of Raf (Arvisais, Romanelli et al. 2006). Reduction in PI3K/Akt signals, which is thought to influence survival of ovarian cells, is the mode of action through which PGF2α regulates luteal cell regression via Mtor (Arvisais, Romanelli et al. 2006).

5.1.4. Steroidogenesis

While LH induced Mtor increases translation machinery for normal cell growth in luteal cells, the role of Mtor on steroidogenesis (progesterone synthesis) is not known clearly at present as rapamycin treatment does not inhibit LH regulated progesterone secretion and steroidogenic enzymes gene expression (*Hsd3b* or *Cyp11a*). However, changes in AMPK and Gsk3b phosphorylation due to LH is believed to have an influence on progesterone synthesis (Hou, Arvisais et al. 2010).

5. 2. mRNA expression of Mtor pathway

Reverse transcription polymerase reaction analysis for key components of Mtor pathway genes at the mRNA level demonstrated that Mtor and its cofactors, Rptor and Rictor are expressed both in whole ovary as well as in isolated granulosa cells (Yaba, Bianchi et al. 2008).

A Significant decrease in FSH induced Ccnd2 mRNA expression in rapamycin treated cultured granulosa cells is an indicative of FSH stimulates granulosa proliferation using Mtor pathway in addition to cAMP-PKA-Erk pathways (Kayampilly and Menon 2007).

5. 3. Protein expression

Mtor is ubiquitously expressed in the mouse ovary with greater cytoplasmic expression than nuclei of all kind of ovarian cells including granulosa, theca, oocytes, and the ovarian stroma as well as surface epithelium throughout the follicular growth. However P-Mtor in this study was shown specific to granulosa cells with high level of expression during the Mitotic phase of the cell cycle. (Yaba, Bianchi et al. 2008).

Time course study to identify the phosphorylated form of p70S6K (Rps6kb1) in FSH treated cultured rat granulosa cells showed increase in the phosphorylation by 2-fold at 15 minutes of treatment to reduction to initial level at 60 minutes (Kayampilly and Menon 2007). Similarly, LH (Hou, Arvisais et al. 2010) and PGF2α (Arvisais, Romanelli et al. 2006) have been shown to increase in the phosphorylation of the Rps6kb1 in time and concentrate dependent manner and thereby stimulate Mtor directed translation responses in bovine luteal cells *in vitro*.

Immunohistochemical analysis for localization of different proteins involved in insulin signaling pathway in the sheep ovary showed that higher expression of Mtor in the cytoplasm of granulosa cells than theca and stromal cells across all follicular stages (Ortega, Rey et al. 2010).

6. Conclusion

In summary, there are strong pieces of evidence that Mtor and their down-stream substrates are involved in the regulation of cell growth and metabolism in many tissues because of their ability to integrate nutrient, metabolic and hormonal signals. From various *in vitro* studies, it is understood that Mtor is expressed in the ovary and appears to have major role in the regulation of ovarian processes such as granulosa proliferation, steroidogenesis, luteal growth and regression. However, *in vivo* expression profile of Mtor and its relationship with gonadotropins and ovarian dynamics remain not known. Hence, present study was planned to understand the specific role of Mtor in the regulation of ovarian function and

altered energy status at the ovarian level in order to provide *in vivo* evidence of Mtor in reproduction.

As the ovary is a complex structure and the function of ovarian somatic cells changes according to the developmental stage of the follicles, we decided to use Laser microdissection (LMD) technique for collection of ovarian somatic cells such as granulosa and luteal cells. Moreover, the expression of Mtor signaling genes could vary depending on the developmental stage of ovarian follicle and corpus luteum, we felt this study requires LMD for the homogenous cell collection. LMD is an example of novel method that can be used to enhance tissue or cell specific analysis and hence it helps to avoid the weakness of whole tissue analysis such as nonspecific results, misinterpretation etc in molecular biology (Sakurada, Shirota et al. 2006; Vega 2008)

Because of the wide variety of granulosa functions, and the signal cascades in granulosa cells are comparatively more important than other cell types, our primary interest was to study the role of Mtor on granulosa function of growing follicles (FSH function). **Hence, the objectives of the present study were:**

1) **To develop Laser Micro Dissection (LMD) technique as a new in vivo approach to collect pure population of granulosa and luteal cells from ovarian sections.**
2) **To characterize the expression pattern of Mtor signaling genes in granulosa and luteal cells in the mouse ovary using the techniques of super ovulation of the immature mice, LMD, real-time PCR and western blotting approaches.**
3) **To characterize the post translational modifications of Mtor proteins, an indicator of their function, during ovarian dynamic stages.**
4) **Examine the effect of reduced energy intake on ovarian Mtor pathway in order to identify how external metabolic signals are mediated to the ovary. We chose fasting as an approach to manipulate metabolic signals to the ovary.**

The results of the proposed study will enhance how the master integrator of metabolic signals, Mtor, regulates ovarian function and female fertility. Moreover, this study will be basis for further research on the granulosa specific Mtor conditional knockout mice being developed in our laboratory.

III. MATERIALS AND METHODS

1. Animals

The inbred C57BL/6NCrl mice (from Charles River) were used for all experiments in the present study. Mice were housed in standard plastic rodent cages and maintained on a 12-h light and 12-h dark cycle with *ad libitum* regular feed (Rodent Diet from Harlan Teklad, Canada) and water unless specified otherwise. The animal protocol was approved by the Animal Care and Use Committee, McGill University (Protocol # 5800). All mice were sacrificed by overdose of CO_2 inhalation.

2. Experimental design

2.1. Experiment 1: Development of Laser Microdissection Technique (LMD) for collection of pure populations of ovarian somatic cells

Validation of LMD **:** The relative mRNA level of three genes that have been reported earlier namely *Cyp19a1, Star* and *Fshr* was evaluated at specific time-points using real - time PCR with the purpose of validating LMD technique for purification of ovarian somatic cells.

2.2. Experiment 2: The expression pattern of Mtor pathway genes in the granulosa and luteal cells at mRNA and protein level

This experiment was conducted to identify the expression pattern of Mtor-pathway genes (mRNA and protein) and phosphorylation status of Mtor proteins in the ovary. We hypothesized that the expression of Mtor signaling genes and their phosphorylation status varies according to the developmental stage of the ovarian follicle and corpus luteum.

2.3 Experiment 3: Effect of fasting on Mtor pathway genes in the granulosa cells

In this experiment, the effect of fasting was studied to examine whether Mtor in the granulosa cells senses energy intake or not. The hypothesis was that acute fasting or reduced energy intake down regulates Mtor activity in the granulosa cells.

3. Ovary sample collection

3.1. Superovulation

The animals were superovulated with exogenous gonadotropins (Sigma) namely equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG). Immature mice were first administered with eCG @ 5 IU intra peritoneal (i.p) at the age of 23d to stimulate preovulatory follicle development and followed by 48h later with hCG @ 5 IU i.p. to induce ovulation and luteinization (Duggavathi, Volle et al. 2008). The superovulation protocol was used for all of the three experiments to collect ovaries at specific stages of follicle / CL development. Of the two exogenous gonadotropins, the former performs action similar to Follicle stimulating hormone (FSH), while the later has luteinizing hormone (LH) like action. This is the standard protocol used to mimic endogenous follicular and luteal growth and this paradigm is used extensively in several species such as mice (Wu, Gonzalez-Robayna et al. 2000; Richards, Hernandez-Gonzalez et al. 2005; Duggavathi, Volle et al. 2008) heifers (Fortune and Hansel 1985), cow (Fortune, Hinshelwood et al. 1991) sheep (Forcada, Amer-Meziane et al. 2010) goat (Kiessling, Hughes et al. 1986) and all farm animals (Driancourt 2001) since many years.

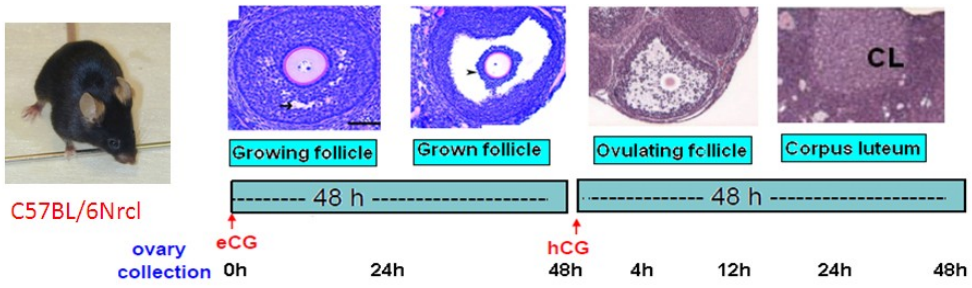

Figure 5: Collection of ovaries at specific time-points during the superovulatory regimen.

3.2. Experiment 1

Ovaries were collected at specific time-points (N=5/group; eCG0h, 48h and hCG 48h) through superovulation to represent small follicles, fully grown follicles and corpus luteum respectively for validation study. After collection, the ovaries were embedded in optimum cutting temperature (OCT) compound (Electron microscopy) and submerged in the liquid nitrogen to freeze the embedded sample and then stored at - 80°C until laser micro dissection (LMD) was followed.

3.3. Experiment 2

Ovaries were collected at specific time-points (N=5/group; eCG0h, 24h, 48h, hCG 4h, 12h, 24h and 48h) through superovulation to represent specific stages of follicular/ luteal growth and ovulation. The collected ovary samples were preserved and stored as described in previous experiment for LMD.

3.4. Experiment 3

A) 12h fasting: Six numbers of 23 old immature mice were superstimulated with eCG @ 5 IU i.p. and divided randomly into two groups as follows: control and fasted group. The control group (N=3) was fed *ad libitum* and the fasted group (N=3) was not fed from 36h post-eCG. Ovaries were collected at 12 h after fasting and stored as before for LMD.

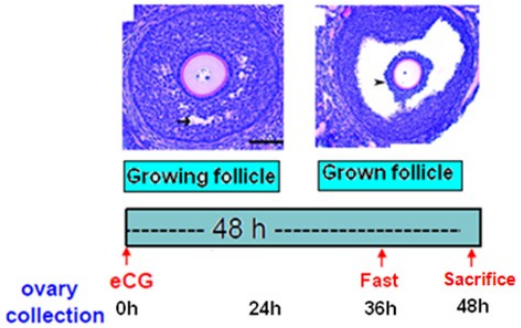

Figure 6: Schematic details of Fasting experiment (12h).

B) Preliminary study with 24h fasting: similar as previous experiment, Four 23d old immature mice were injected with eCG @ 5 IU i.p. and divided randomly into Fed and Fasted group. The Fed group (N=2) was fed *ad libitum* and the fasted group (N=2) was not fed from 24h post-eCG. Ovaries were collected at 24h after fasting and protein extracted from whole ovary to carry out western blot using 40μg/well.

4. Granulosa and Luteal cell collection by Laser microdissection(LMD)

Laser microdissection(LMD) was used to collect granulosa and luteal cells at specific stages of follicular and CL growth. It involved cryosection, toludine blue(Richa chemicals/ Fisher scientific) staining and LMD followed by extraction of macromolecules of interest for further molecular profiling.

The ovaries were cut with a thickness of 25 μm at -16 ^{o} C using cryostat(Leica)and the frozen sections were thaw - mounted on polyethylene naphthalate(PEN)slides(Leica). Then the slides were air dried and stained with 0.5%Toludine blue. The procedure for toludine blue staining was as follows; The slides were first washed with DEPC (Diethylpyrocarbonate) PBS for 3 min to remove OCT/cryomatrix , stained with 0.5% Toluidine Blue and then dipped in DEPC PBS to wash off extra stain. Subsequently, the slides were dipped 2 to 3 times each in 70% and 100% ethanol, followed by incubated at 37 °C for 30 min to 1 h for dehydration (Duggavathi, Volle et al. 2008).

After staining, the slides were placed with section facing towards the collection cap in the LCM microscope (a laser coupled microscope from Leica) and the targeted area (granulosa and luteal cells from the respective groups) was marked with the help of computer generated line in order to cut the tissue along the marked line by UV laser. Finally the cut sections were collected in the collection tube that contains extraction buffer (Duggavathi, Volle et al. 2008). One ovary from each animal of all groups was utilized for mRNA study using Real - time PCR and another one for protein study by western blotting.

5. Relative mRNA expression by Quantitative Real - time PCR (q PCR)

Total RNA was isolated from LMD samples using picopure RNA isolation kit (Catalog # kit 0202/kit 0204 from Arcturux Biosciences) according to the manufacturer protocol and RNA concentration was measured using the Nanodrop (Thermo Fisher ND 1000) as well as Bioanalyzer (Agilent). Subsequently, cDNA was synthesized using 250 ng of total RNA using iScript reverse transcriptase (Bio-Rad) according to the manufacturer protocol. Synthesized cDNA was then used to determine abundance of genes related with Mtor signaling pathway.

In order to perform q-PCR, each primer set was optimized such that the correlation coefficient was 0.95–1.0 and the PCR efficiency was 90–100%. Q-PCR was performed in duplicate using CFX ^{TM} Real–time system (Bio-Rad) and the power SYBR Green master mix (Applied bio systems) with 2-step amplification plus melt curve for 39 cycles. Thermo cycler conditions were: 95°C for 15 min, 95°C for 15s, annealing temperature 60°C for 30 s for 39 x 2 cycles; and 95°C for 10s. For the data analysis, the average starting quantity (SQ)/threshold cycle (CT) of each set of duplicates was calculated. Relative mRNA expression was quantified using the standard curve method. To normalize the data, expression levels of two reference genes (*B2m* and *Sdha*) were first determined. Average value of these two genes was used for normalization.

The expression pattern of genes encoding Gonadotropin receptors *(Fshr* and *Lhcgr*), Steroidogenic enzymes (*Cyp19a1, Star*, *Cyp11a1* and *Hsd 3b),* Cell cycle regulators (*Ccnd2 and Cdkn1b*) and Mtor-pathway (*Mtor, Rptor, Rictor, Rps6ka2* and *Rps6kb1*) was analyzed by real-time PCR. The primers used in the study were as follows:

Experiment 1

Primers	Forward	Reverse
mCyp19a1	TGGAGAACAATTCGCCCTTTC	CCGAGGTGTCGGTGACTTC
mStar	CAATACCATTGACCTGCCGAT	GAGCGACTCAAACTGCCCT
mFshr	GTGCTCACCAAGCTTCGAGTCAT	AAGGCCTCAGGGTTGATGTACAG

Experiment 2

Primers	Forward	Reverse
mFshr	GTGCTCACCAAGCTTCGAGTCAT	AAGGCCTCAGGGTTGATGTACAG
mLhcgr	AGCTAATGCCTTTGACAACC	GATGGACTCATTATTCATCC
mCyp19a1	TGGAGAACAATTCGCCCTTTC	CCGAGGTGTCGGTGACTTC
mStar	CAATACCATTGACCTGCCGAT	GAGCGACTCAAACTGCCCT
mCyp11a1	AGGTCCTTCAATGAGATCCCTT	TCCCTGTAAATGGGGCCATAC
mHsd3b	CAGTTGTTGGTGCAAGAGGA	CAATGATGGCAGCAGTATGG
mCcnd2	GAGTGGGAACTGGTAGTGTTG	CGCACAGAGCGATGAAGGT
mCdkn1b	TCAAACGTGAGAGTGTCTAACG	CCGGGCCGAAGAGATTTCTG

Experiment 3

Primers	Forward	Reverse
mMtor	TTGGAGTGGCTGGGTGCTGA	AAGGGCTGAACTTGCTGGAA
mRptor	GCCATCACAGATACCATCGC	CTGCTTACTGGGGTGCAGTT
mRictor	GAGAACGTCCCGCTCGATCT	TGGCCCAGCTTTCTCATATT
mRps6ka2	GCAGGTTCTTCTCCGTGTACC	GAGGGGTCTGCCTTCTCAAA
mRps6kb1	AGACACAGCGTGCTTTTACTT	GTGTGCGTGACTGTTCCATCA

6. Protein detection

6.1 Antibodies:

Primary antibodies used in this study were polyclonal anti-mTOR (1:1000, Cell Signaling Technology), polyclonal anti-phospho-mTOR (Ser 2448)(1:1000, Cell Signaling Technology), polyclonal anti-Raptor (1:1000, Cell Signaling Technology), anti-Rictor (1:1000, Cell Signaling Technology), Monoclonal anti-Cyclin D2 (1:1000, Cell Signaling Technology) and anti-β actin (1:1000, Abcam). Secondary antibody was horse–radish peroxidase conjugated goat anti-rabbit (1:10,000, Abcam).

6.2. Protein detection by Western blot:

For protein analysis, the LMD samples were collected directly in 70 µl of laemmli buffer (Bio-rad) with the addition of DDT and phosphatase and protease inhibitors (G Biosciences PN#786-331) to extract the protein. After collection, the samples were subjected to vortex and boiled at 95°C for 1mt each of the process and then stored at - 80 ^{o} C until western blots were performed. The collection was sufficient for 3 SDS-PAGE's.

After boiling the samples at 95°C for 5min, proteins were separated by electrophoresis using 7% SDS-PAGE gel (as given in the table) loaded with 25 µl of sample per lane and transferred to nitrocellulose membranes.

Resolving Gel 7 %	**Qty**	**Stacking Gel 5%**	**Qty**
40% Acrylamide/Bis	1.75ml	40% Acrylamide/Bis	1.25 ml
ddH_20	5.59ml	ddH_20	1.1ml
1.5M Tris buffer, pH 8.8	2.5ml	0.5M Tris buffer, pH 6.8	7.5 ml
20% SDS	50µl	20% SDS	50µl
10% APS	100µl	10% APS	100µl
TEMED	10µl	TEMED	5 µl
Total	10ml	Total	10ml

` The membranes were then incubated for 1 h at room temperature with 5% fat-free milk in phosphate-buffered saline containing 0.1% tween 20(PBS-T). Followed by, the

membranes were incubated overnight at 4°C with the primary antibodies. After washing 3 times for 10mts each in PBS-T, the membranes were incubated for 1hour at room temperature with secondary antibody conjugated with horse radish peroxidase. Then following 3 washing with PBS-T, the immunoblotted proteins were detected using enhanced chemi luminescence reagents (Immun-Star Western C Chemi luminescent Kit (HRP) (Bio-Rad) and the membranes were exposed on radiographic films (Kodak Biomax) in the dark room and developed the film for protein detection. After protein detection, the membranes were stripped with Restore western blot stripping buffer as specified below (Thermo scientific) and re-blotted with another targeted primary antibody if necessary. Antibody β-actin was used as a loading control (house keeping gene) for western blot.

Western blot Membrane stripping

1. The stripping buffer was prepared in the fumehood as follows:

10 % SDS	–	20 ml
0.5 M Tris – Hcl, pH 6.8	–	12.5 ml
DEPC H_2O	–	67.5 ml
2-Mercaptoethanol	–	0.8 ml

2. The buffer was warmed to 50° C in the incubator and then added to membrane.
3. The membrane was incubated at 50° C for 1.5h with agitation for every few mts.
4. Then the membrane was washed for 2 h using fresh PBS–T (0.05% tween 20) every 10 mts and blocked with PBS–T (0.05 %Tween 20) + 5% fat-free milk for 45 mts.
5. After blocking, the membrane was washed 3 times in PBS–T (0.1% Tween 20) for 10mts each wash.
6. Then the membrane was exposed to visualization protocol as described in western blot (WB) in order to check the presence of bound secondary antibody.
7. After ascertaining the absence of bands on the film and washing 3 times in PBS–T (0.1% Tween 20), the membrane was blotted with secondary antibody and visualized as per WB protocol to check the presence of bound primary antibody.
8. Once confirmed the absence of previous primary antibody, the membrane was re-blotted with different primary antibodies of protein of interest for the present study.

6.3. Localization of protein by Immunofluorescence:

1. The ovaries from eCG 48h and hCG 12h group were cryosectioned into 12 μm thickness at -16 ° C and the frozen sections were thaw - mounted on microscopic slide.
2. Then the slides were allowed to dry for 15- 20 minutes at room temperature and rehydrated in phosphate-buffered saline containing 0.1% tween 20 (PBS-T) for 10 mts.
3. After that, the sections were incubated for 45 mts in blocking solution otherwise known as antibody diluting buffer (3% Bovine serum Albumin (BSA) + 5% Normal goat serum (NGS)).
4. Followed by, the slides were incubated overnight at 4°C with respective primary antibodies (1:200 dilution of each Mtor, Rptor and Rictor) diluted in blocking solution.
5. Negative controls were exposed to normal rabbit serum (NRS) with similar concentration as that of primary antibodies.
6. After washing 3 times in PBS–T, the slides were incubated for 1h at room temperature with fluorescent dye labeled secondary antibody (1:500, Alexa flour 488 goat anti-rabbit - Dylight® 488, Vector Laboratories Inc. Burlingame, CA).
7. Finally, the sections were washed 3 times for 10 mts each in blocking solution followed by counterstained with DAPI and examined under fluorescent microscope (Nikon Eclipse 80i (Nikon,Tokyo,Japan).
8. Images were individually recorded and captured using a Retiga 2000R monochrome digital camera (Qimaging, BC,Canada) and saved in Tiff format.

7. Statistical Analysis

Experiment 1:

Data from Experiment 1 were analyzed by one way ANOVA using PROC GLM of SAS (SAS 1989, SAS Inst. Inc., Cary, NC.) with the following model:

$$Y_{ij} = \mu + \text{Time}_i + e_{ij}$$

Where Y_{ij} represents the observations for dependent variables, μ is the overall mean, Time_i is the fixed effect of the i^{th} time on the gene expression and eij is the random residual effect associated with the j^{th} animal's ovary from the i^{th} time period. The significance was declared when $P < 0.05$.

Experiment 2:

Similar as Experiment 1, the data were analyzed using PROC GLM of SAS (SAS 1989). The level of significance was set at $P< 0.05$. Relative mRNA expression pattern of the genes considered for this study were analysed by one - way ANOVA:

The proposed model is $Y_{ij}= \mu + Time_{i} + e_{ij}$

Y_{ij} = Measured /observed gene expression pattern of the j^{th} animal's ovary of the i^{th} time period, μ = the overall mean of Y, $Time_{i}$ is the fixed effect of the i^{th} time on gene expression and eij is the random residual effect associated with the j^{th} animal's ovary from the i^{th} time period. Once the overall significance is identified, the significance difference between time points was determined using Scheffe analysis.

Experiment 3:

For the fasting trial, the effect of fasting on the expression pattern of Mtor pathway genes was analyzed by one - way ANOVA with three replications using PROC GLM of SAS (SAS, 1989). The significant difference between treatments (control versus fasting group) was given at $P< 0.05$.

The model is $Y_{ij} = \mu + Trt_{i} + e_{ij}$

Y_{ij} represents Measured /observed Mtor gene expression pattern of the j^{th} animal on the i^{th} treatment. Where i= 1, 2. j=1, 2, 3, μ is the overall mean of Y, Trt_{i} is the fixed effect of the i^{th} treatment on Y and eij is the random residual effect associated with the i^{th} treatment.

The Data for all the three experiments were tested using the residuals for Normality using PROC UNIVARIATE of SAS and Normal distribution was declared when $P > 0.05$ (5% probability) for W statistics.

IV. RESULTS

1. Experiment 1. Development of laser microdissection technique for collection of pure populations of ovarian somatic cells

1.1. Laser microdissection:

Ovary is a complex tissue with many cells types at different physiological status. In order to establish a technique to collect pure population of ovarian somatic cells from follicles and CL, we performed laser microdissection (LMD) as first experiment. As shown in Fig. 1, LMD can be successfully used to collect granulosa and luteal cells from the mouse ovaries at specific developmental stages of follicular/CL growth, as determined by light microscopy.

The typical time required to perform various steps for sample from one ovary was as follows: i) Cryosection - 45 min to 1 h to collect 50 sections from one ovary (25 sections per slide); ii) Staining–15 min; iii) Drying at 37° C–1h; iv) Cell collection -1h; v) RNA extraction–30 min. In general, LMD samples were collected from 2-3 ovaries per day (7 to 8h).

1.2. Validation of LMD for downstream molecular analysis:

A typical collection of granulosa or luteal cells from one ovary yields sufficient RNA or protein for downstream molecular phenotyping. Our analysis using the Nanodrop (Thermo Fisher ND 1000), the total RNA yield was ranged from 600 to1000 ng per pooled cells from one ovary. The 260/280 ratio, indicative of the purity of RNA, ranged from 1.7 to 1.9 (Table 1). Additional analysis of representative RNA samples using the BioAnalyzer (Agilent) showed RIN of 5–8 and the ratio for 28S/18S peaks and 260/280 were 1.28 and 1.64 respectively.

In order to examine the possibility of LMD samples to generate authentic mRNA expression data, we chose to determine the expression patterns of genes that have already been reported using other techniques (e.g. follicle puncture). The genes included for this analysis, using real-time PCR, were *Cyp19a1, Star* and *Fshr*. Average value of two reference genes namely *B2m* and *Sdha* were used for normalization. The data for all genes included in this analysis were normally distributed ($P>0.05$ for W statistics).

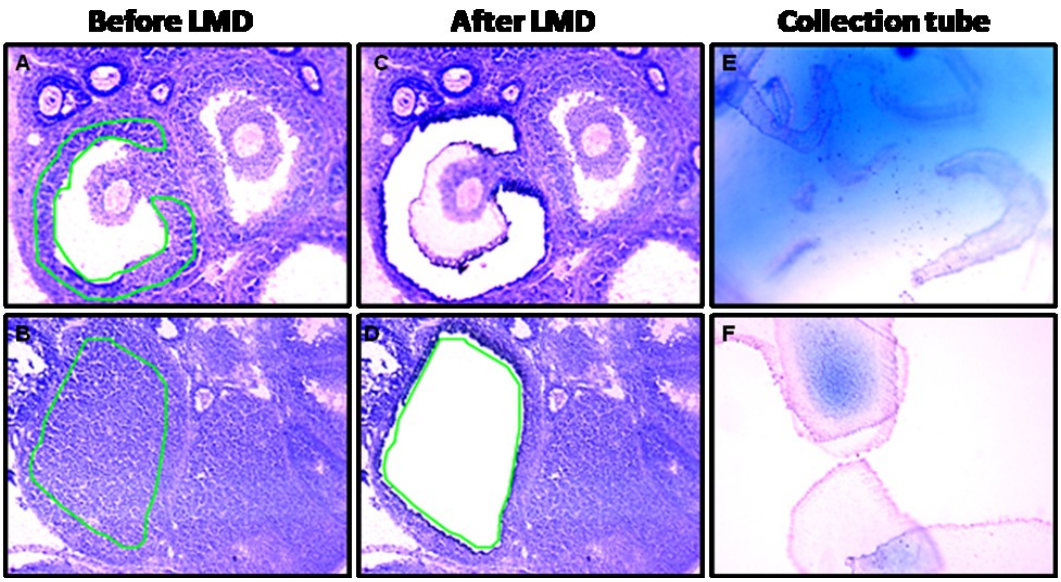

Figure 1: Laser microdissection of granulosa and luteal cells from cryosection of the mouse ovary. **A** and **B** represent, microscopically identified granulosa and luteal cells respectively for LMD collection. **C** and **D** represent respective parts after the cell collection. **E** and **F** are the collection tube with several representative cut sections of specific cell types.

Sample	RNA quantity ng/µl	RNA quality (260/280 ratio)
1	91.7	1.82
2	114.7	1.87
3	92	1.76
4	131.1	1.94
5	69.3	1.88

Table 1: Analysis of RNA quantity and quality in representative laser microdissected samples using Nano drop Spectrophotometer (Thermo Fisher ND 1000).

Cyp19a1: The relative mRNA level of *Cyp19a1*, an enzyme required for conversion of testosterone to estradiol, was up regulated significantly in the granulosa cells of fully grown follicles at 48 h post-eCG ($P<0.01$) and down regulated after hCG treatment in the corpus luteum (Fig. 2A).

Star: *Star* is a rate-limiting enzyme of steroidogenesis, essential for transportation of cholesterol from cytoplasm into mitochondria. Abundance of *Star* mRNA was low in the granulosa cells of both immature and fully grown follicles, and was induced significantly ($P<0.01$) in luteal cells after hCG treatment (Fig. 2B).

Fshr: The profile of *Fshr* gene expression was similar as that of *Cyp19a1* with a significant up regulation at eCG 48h ($P<0.01$) as compared to other time points (Fig.2C).

2. Experiment 2: Expression pattern of Mtor pathway genes in the ovarian granulosa and luteal cells at mRNA and protein level

The mRNA data for all the genes considered in the present study were normally distributed as the probability associated with "W statistics" was greater than 0.05 ($P>0.05$) for each of them.

2.1. mRNA expression pattern of Gonadotropin receptors, Steroidogenic enzymes and Cell cycle regulator genes in the ovarian granulosa and luteal cells:

We used superovulation in immature mice to obtain ovaries at specific stages of follicular and CL development. To confirm the specific stages of development of the granulosa/luteal cells collected, we first examined the expression pattern of genes encoding gonadotropin receptors (*Fshr* and *Lhcgr*), steroidogenic enzymes (*Cyp19a1, Star, Cyp11a1* and *Hsd3b*), and positive and negative regulators of cell cycle (*Ccnd2* and *Cdkn1b* respectively).

2.1.1. Gonadotropin receptors

Fshr: The relative mRNA level of *Fshr* was high during follicular growth with the highest expression ($P<0.01$) observed at 48h post-eCG. Conversely, the mRNA level decreased ($P<0.05$) during pre-ovulatory differentiation of ovulating follicles following hCG treatment.

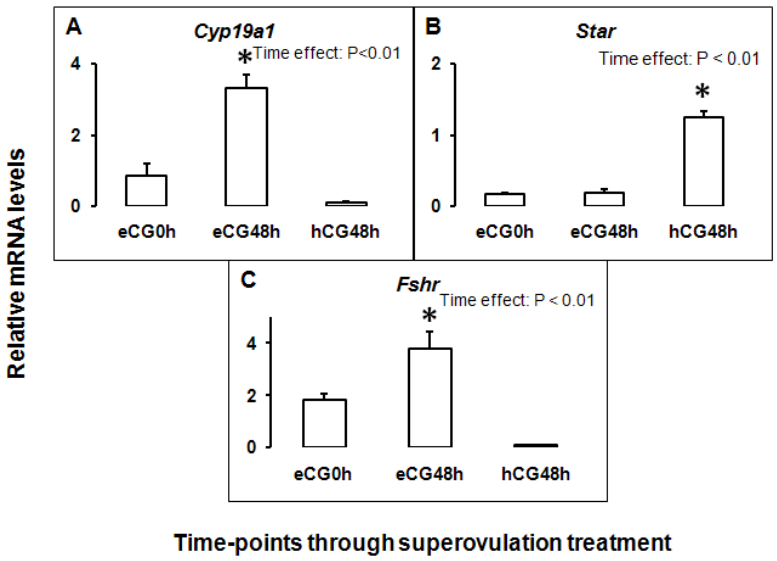

Figure 2: Abundance of mRNA for *Cyp19a1***(A)**, *Star* **(B)** and *Fshr* **(C)** relative to average value of *B2m* and *Sdha* in the granulosa and luteal cells using real-time PCR. Ovaries were collected at indicated time points (n=5 per time point) during the superovulation protocol, and the granulosa and luteal cells were isolated by LMD. Bars represent mean mRNA values of respective gene and (*) $P < 0.01$.

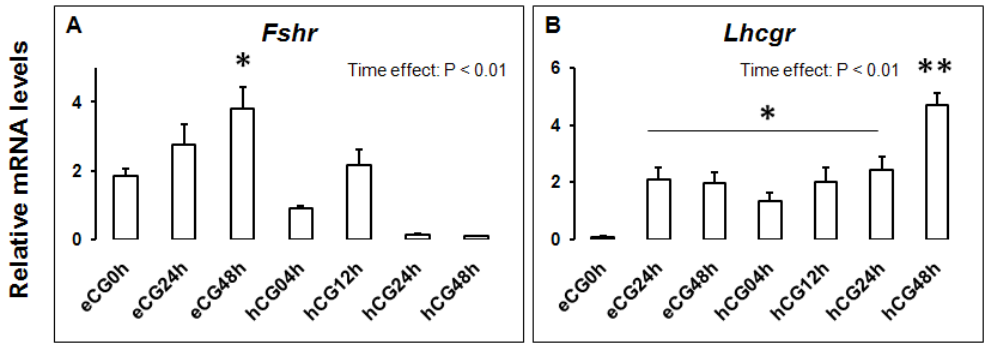

Figure 3: Abundance of mRNA for gonadotropin receptors (*Fshr* **(A)** and *Lhcgr* **(B)**) relative to average value of *B2m* and *Sdha* in the granulosa and luteal cells using real-time PCR. Ovaries were collected at indicated time points (n=5 per time point) during the superovulation protocol, and the granulosa and luteal cells were isolated by LMD. Bars represent mean mRNA values of respective gene and (*) $P < 0.01$ for *Fshr*, whereas (**) $P < 0.01$ for *Lhcgr*.

The expression of *Fshr* mRNA was almost non-detectable in luteal cells from CL at 24h and 48h post-hCG (Fig. 3A).

Lhcgr : When compared to eCG 0h, *Lhcgr*, the receptor for LH, was significantly up regulated at 24h post-eCG. The mRNA levels remained high through follicular growth, pre-ovulatory differentiation and CL formation until 24h post-hCG, after which there was further increase ($P<0.01$) at 48h post-hCG (Fig. 3B).

2.1.2. Steroidogenic enzymes

Cyp19a1 : The expression pattern of *Cyp19a1* was almost similar to that of *Fshr*. The mRNA abundance of *Cyp19a1* was high during follicular growth with the highest expression ($P<0.01$) observed at 24 and 48h post-eCG. Conversely, the mRNA level decreased during pre-ovulatory differentiation of ovulating follicles following hCG treatment. The expression of *Cyp19a1* mRNA was almost non-detectable in luteal cells from CL at 24h and 48h post-hCG (Fig.4A).

Star: The relative abundance of *Star* mRNA was low in granulosa cells of growing follicles, but was then highly up regulated by hCG treatment. However, the peak expression ($P<0.01$) was specifically restricted to the preovulatory follicles at 4h post - hCG, after which it sharply declined (Fig. 4B) to low levels.

Cyp11a1: Similar to that of *Lhcgr*, the *Cyp11a1* mRNA was induced at 24 h post-eCG. However, the mRNA abundance continued to increase through follicular and CL development to reach peak levels in luteal cells at 48 h post-hCG (Fig.4C).

Hsd3b: The mRNA levels of *Hsd3b* did not show statistically significant difference ($P>0.05$) among the different time points through gonadotropin stimulated follicular and CL development (Fig.4D).

2.1.3. Cell cycle regulators: *Ccnd2*: The mRNA abundance of *Ccnd 2*, a positive regulator of cell cycle in granulosa cells, was significantly increased by eCG treatment at 24h post-eCG. Then, there was a further significant induction of *Ccnd2* mRNA expression in response to hCG treatment at 4h post-hCG before significantly decreasing at the time of ovulation (12h post-hCG). The levels of *Ccnd2* mRNA remained low in luteal cells.

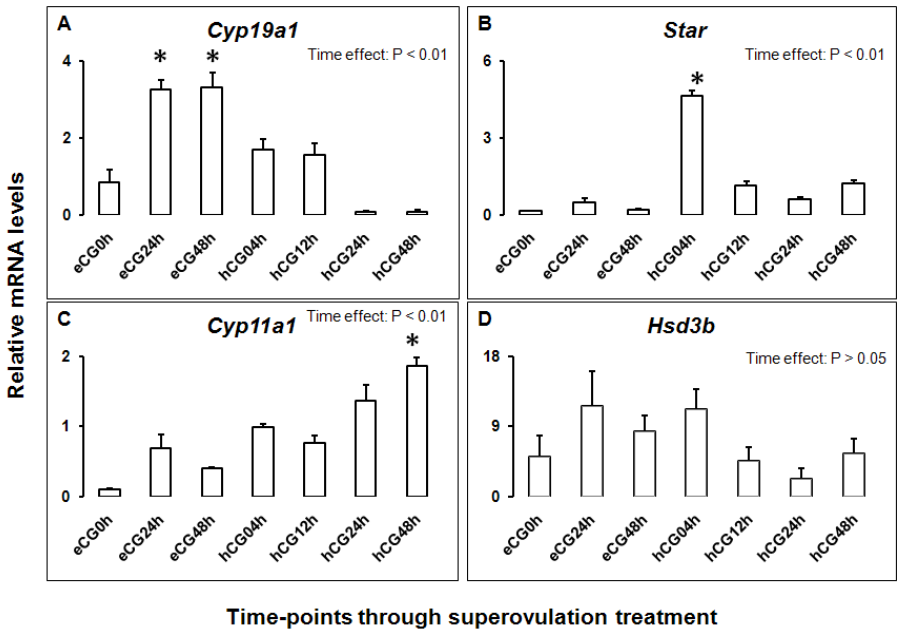

Figure 4: Abundance of mRNA for steroidogenic enzyme genes (*Cyp19a1* **(A)**, *Star* **(B)**, *Cyp11a1***(C)** and *Hsd3b* **(D)**) relative to average value of *B2m* and *Sdha* in the granulosa and luteal cells using real-time PCR. Ovaries were collected at indicated time points (n=5 per time point) during the superovulation protocol, and the granulosa and luteal cells were isolated by LMD. Bars represent mean mRNA values of respective gene and (*) $P < 0.01$.

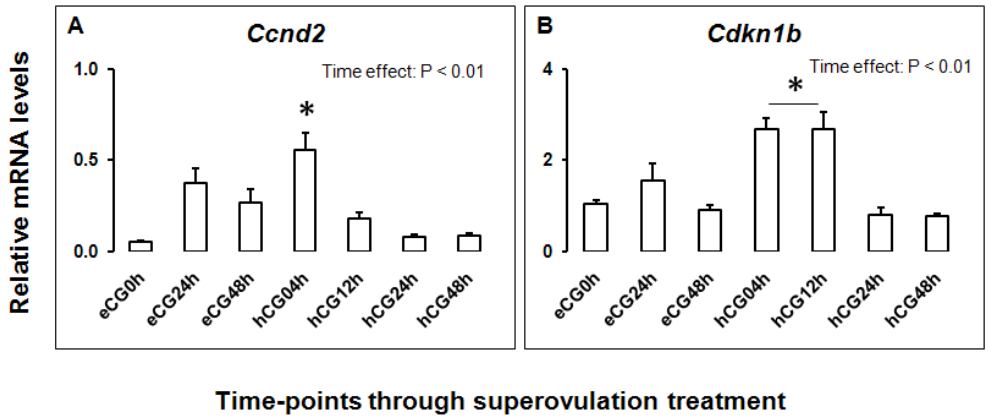

Figure 5: Abundance of mRNA for cell cycle regulator genes, *Ccnd2* **(A)** *and Cdkn1b* **(B)** relative to average value of *B2m* and *Sdha* in the granulosa and luteal cells using real-time PCR. Ovaries were collected at indicated time points (n=5 per time point) during the superovulation protocol, and the granulosa and luteal cells were isolated by LMD. Bars represent mean mRNA values of respective gene and (*) $P < 0.01$.

Cdkn1b: *Cdkn1b*, a negative regulator of cell cycle in granulosa cells, showed high expression in granulosa cells undergoing preovulatory differentiation in response to hCG treatment ($P<0.01$) (Fig.5B). Its expression also reduced to low levels in luteal cells at 24h and 48h post-hCG.

2.2. mRNA expression pattern of Mtor pathway genes in the ovarian granulosa and luteal cells:

The mRNA expression pattern of genes involving in Mtor pathway considered for the present study was: *Mtor, Rptor, Rictor, RpS6Ka2* and *RpS6Kb1*.

Mtor: *Mtor* expression profile at mRNA level (Fig. 6A) showed that it is constitutively expressed throughout the gonadotropin stimulated follicular and CL development ($P>0.05$). In fact, the mRNA levels remained constant between eCG 0h and hCG 48h time-points, without any alteration in mRNA abundance in response to eCG or hCG treatment.

Rptor: The expression of *Rptor* (Fig.6B), a critical component of MTORC1, was maximum in growing follicles including those at eCG0h, and gradually declined to reach a nadir at 24h post-hCG. This low mRNA level was followed by a significant increase at 48h post-hCG in luteal cells.

Rictor: The relative mRNA levels of *Rictor*, a component of MTORC2, showed a unique pattern through gonadotropin stimulated follicular and CL development. The mRNA expression of *Rictor* was down regulated in granulosa cells of growing follicles in response to eCG treatment ($P>0.05$; Fig. 6C). Though hCG treatment increased ($P>0.05$; Fig. 6C) Rictor expression at 4h post-hCG, the mRNA abundance decreased in ovulating follicles (12h post-hCG) and luteal cells of CL ($P < 0.01$; Fig. 6C).

Rps6ka2: The mRNA expression of *Rps6ka2*, an upstream activator of MTORC1 (Fig. 7A) was dramatically induced at hCG 4h similar to that of Star ($P<0.01$). However, unlike Star, there appeared to be significant expression of *Rps6ka2* mRNA in granulosa cells of growing follicles.

Rps6kb1: Similar to Mtor, *Rps6kb1*, a well characterized downstream target of *Mtor*, showed (Fig: 7B) no significant difference ($P>0.05$) in expression at mRNA level throughout the follicular and CL development.

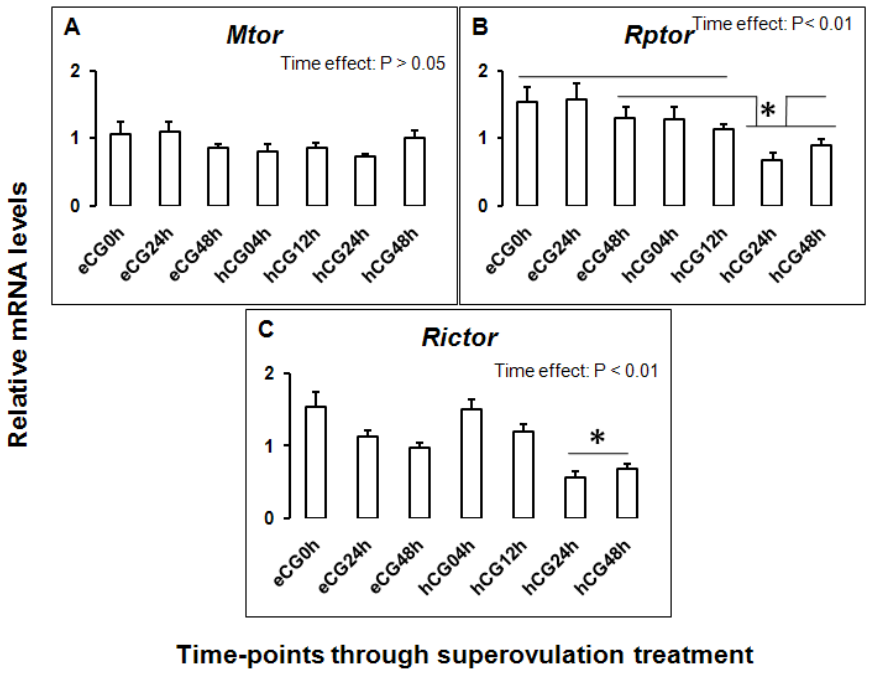

Figure 6: Abundance of mRNA for *Mtor* **(A)**, *Rptor* **(B)** *and Rictor* **(C)** relative to average value of *B2m* and *Sdha* in the granulosa and luteal cells using real-time PCR. Ovaries were collected at indicated time points (n=5 per time point) during the superovulation protocol, and the granulosa and luteal cells were isolated by LMD. Bars represent mean mRNA values of respective gene and (*) $P < 0.01$.

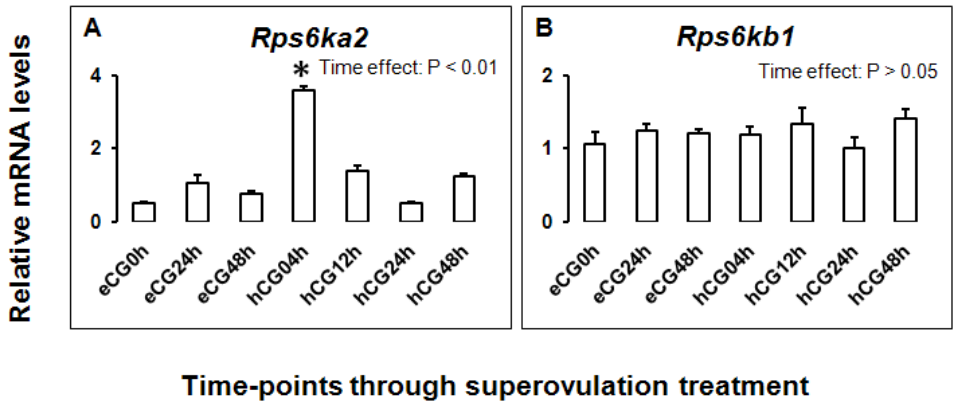

Figure 7: Abundance of mRNA for *Rps6ka2* **(A)** and *Rps6kb1* **(B)** relative to average value of *B2m* and *Sdha* in the granulosa and luteal cells using real-time PCR. Ovaries were collected at indicated time points (n=5 per time point) during the superovulation protocol, and the granulosa and luteal cells were isolated by LMD. Bars represent mean mRNA values of respective gene and (*) $P < 0.01$.

2.3. Protein Expression

2.3.1 Identification of proteins in LMD samples by western blot:

Actb: We used β Actin (Actb) as loading control. Our western blot analysis with Actb-antibody showed that the protein was abundantly expressed both granulosa and luteal cells of all the stages. More importantly when the equal volume of the protein extract per well was used, the Actb levels remained constant through follicular and CL development (Fig. 8A to C).

Mtor: Mtor protein in LMD-purified granulosa and luteal cells at specific time points revealed constitutive expression with little variation at different stages of follicular and CL development (Fig.8A).

Rptor: Our efforts to detect the expression pattern of Rptor protein in LMD-purified granulosa and luteal cells were not successful. This appeared to be due to the amount of total protein present in LMD-purified samples, as there was a faint band for Rptor in luteal cells at 48h post-hCG(Fig: 8B) and a clear band could be seen if 40μg of total protein (possible when protein extract from whole ovary is used) per well was used (Fig 8D)

Rictor: There was no detectable level of Rictor protein in LMD-purified granulosa and luteal cells, even though, a clear band could be seen when 40 μg of total protein from whole ovary was used (Fig 8D).

Ccnd2: The protein levels of Ccnd2 increased at 48h post-eCG though the protein levels were not maintained at 4h post-hCG. Surprisingly, there was a strong band indicative of increased expression of Ccnd2 protein in luteal cells at 48h post-hCG (Fig.8C).

2.3.2. Localization of Mtor pathway proteins in the ovary by Immunofluorescence (Preliminary study)

Immunofluorescent detection of Mtor and Rptor using specific antibodies on cryosection of ovaries collected at specific time-points shows ubiquitous expression in the ovary (Fig.9). Rictor, another partner of Mtor, appears to be expressed specifically in theca cells of follicles (Fig 9).

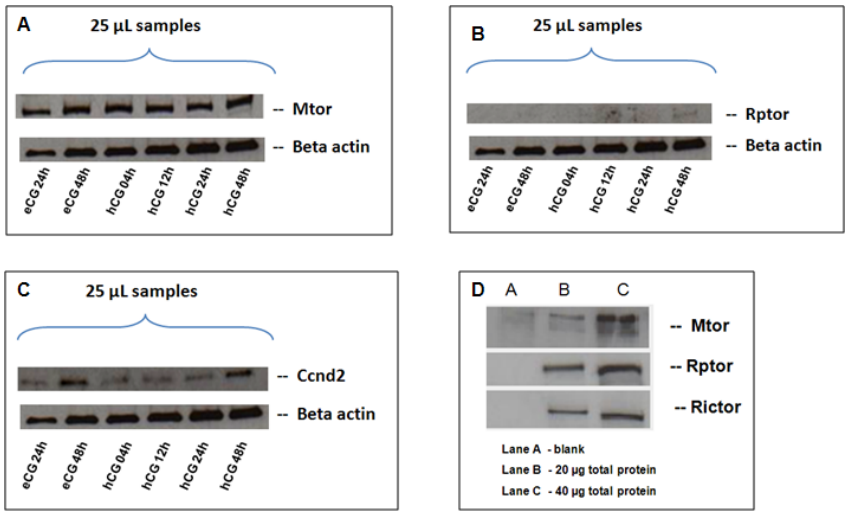

Figure 8: Western-Blot analysis for Mtor **(A)**, Rptor **(B)** and Ccnd2 **(C)** using LMD purified granulosa and luteal cells at specific time-points (n=3 per time point) during superovulation protocol. Beta actin was used as loading control. **D)**. Validation of antibodies Mtor, Rptor and Rictor for western blotting with ovarian protein extract.

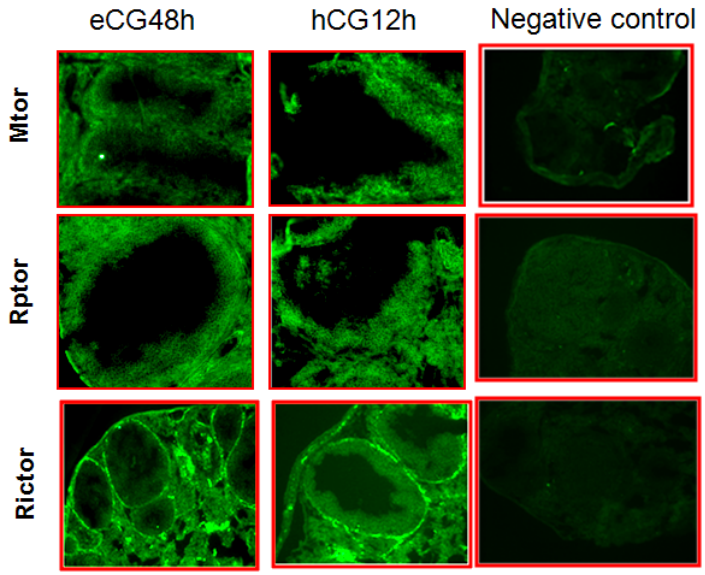

Figure 9: Localization of Mtor signaling proteins in the mouse ovary. Immunofluorescence detection of Mtor, Rptor, Rictor proteins and respective negative controls in mouse ovaries during gonadotropin stimulated follicular growth (eCG 48h) and ovulation (hCG 12h). Mtor and Rptor show ubiquitous expression, whereas Rictor expression is higher in theca cells than granulosa cells. The respective negative controls (primary antibody was not used) demonstrate the specificity of the primary antibodies.

3. Experiment 3: Effect of fasting on Mtor signaling in the granulosa cells

3.1. Effect of 12h fasting on mRNA levels in granulosa cells

Effect of fasting on the mRNA levels for the genes indicative of granulosa cell proliferation and function was studied using LMD-purified granulosa cells from fasted or control (fed ad libitum) mice that were superstimulated with eCG. The representative data for all genes were normally distributed ($P > 0.05$ for W statistics). No significant difference ($P>0.05$) was observed in the mRNA abundance of *Cyp19a1, Ccnd2, Fshr* and *Lhcgr* genes (Fig. 10) in the granulosa cells of mice that were fasted for 12h starting from 36h post-eCG as compared with control mice. Similarly, the mRNA abundance of Mtor pathway genes including *Mtor, Rptor, Rictor, Rps6ka2* and *Rps6kb1* (Fig. 11) did not differ significantly between control and fasted groups.

3.2 Effect of 12h fasting on protein expression

In order to assess the effect of fasting on MTORC1 activity, we determined the phosphorylation status of Mtor in LMD - purified granulosa cells from control and fasted mice. Contrary to our expectations, there was no reduction in the phosphorylation of Mtor at serine-2448 (S2448). However, surprisingly there was a marked reduction in Ccnd2 protein in the granulosa cells of fasted mice as compared to those from control mice (Fig.12b).

In order to resolve this discrepancy, we performed an additional 24h fasting experiment. Our preliminary results show that phosphorylation status of the ovarian Mtor, at both S2448 and S2481, was not affected. However, the phosphorylation status of Rps6kb1 (T389), a regulator of mRNA translation and a well characterized downstream target of MTORC1, was dramatically reduced in the ovaries of 24h fasted mice as compared to control mice.

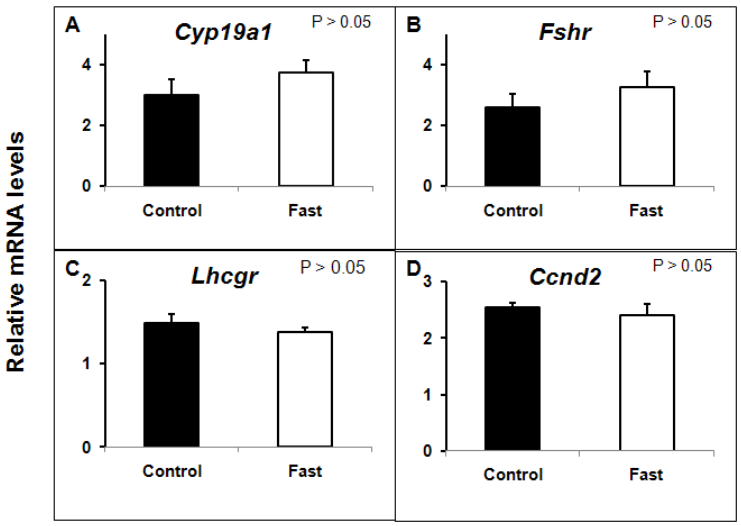

Figure 10: mRNA abundance of *Cyp19a1* **(A)**, Gonadotropin receptors (Fshr **(B)** and Lhcgr **(C)**)and *Ccnd2* **(D)** relative to average value of *B2m* and *Sdha* in the granulosa cells of control versus fasted (12h) group using real-time PCR. Ovaries were collected from respective group (n=3 per group) during the superovulation protocol and the granulosa cells were isolated by LMD.

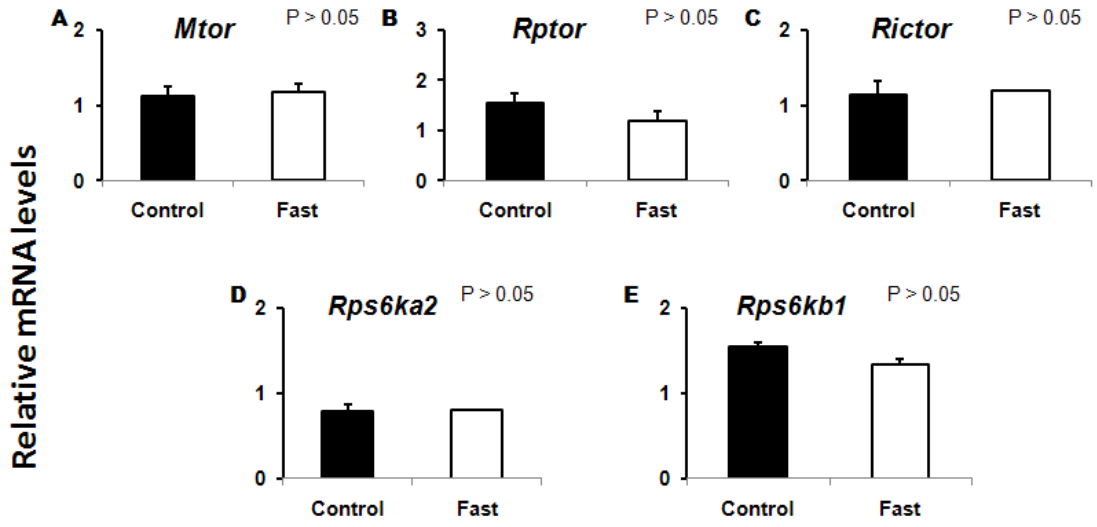

Figure 11: mRNA abundance of Mtor pathway genes (*Mtor* **(A)**, *Rptor* **(B)**, *Rictor* **(C)** *Rps6ka2* **(D)***and Rps6kb1***(E)**) relative to average value of *B2m* and *Sdha* in the granulosa cells of control versus fasted (12h) group using real-time PCR. Ovaries were collected from respective group (n=3 per group) during the superovulation protocol and the granulosa cells were isolated by LMD.

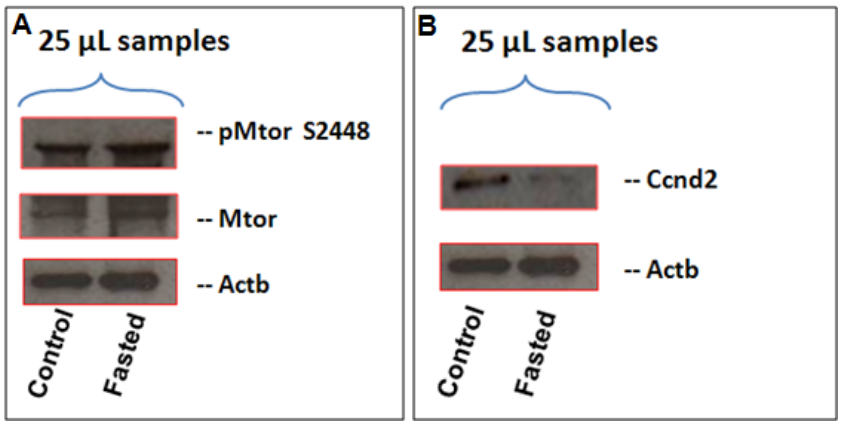

Figure 12: Western blot for pMtor S2448 **(A)** and Ccnd 2 **(B)** proteins in the granulosa cells from control versus fasted groups for 12h. Beta actin was used as loading control. Ovaries were collected from respective group (n=3 per group) during the superovulation protocol and the granulosa cells were isolated by LMD.

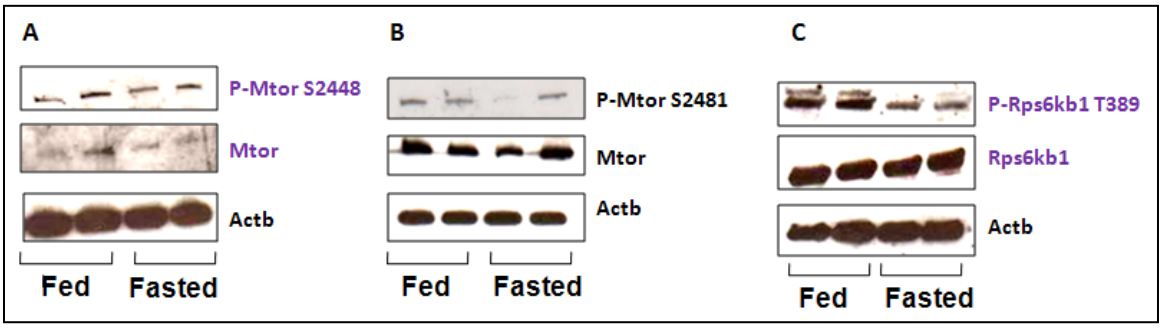

Figure13: Western blot for pMtor S2448 **(A)** pMtor S2481 **(B)** and pRps6kb1 T389 **(C)** proteins using mouse whole ovarian extract from fed versus fasted groups (24h). Beta actin was used as loading control. Ovaries were collected from respective group (n=2 per group) during the superovulation protocol.

V. DISCUSSION

1. Experiment 1: Development of laser microdissection technique (LMD) for collection of pure populations of ovarian somatic cells

Obtaining pure populations of granulosa cells has been a requirement for ovarian biologists investigating *in vivo* molecular pathways regulating these cells. The large animal models (e.g., cow, mare, etc.) have been used to collect granulosa cells from follicles at specific stages of development using techniques such as ultrasound guided follicle aspiration (Evans, Adams et al. 1994; Evans and Martin 2000; Rivera, Chandrasekher et al. 2001). Large animal models allow for granulosa cell collection from follicles at the beginning of gonadotropin dependent growth through to ovulation. However, these models are expensive and not suitable for genetic manipulation. As mice offer remarkable genetic recourse, it is always desirable to purify granulosa cells from follicles at specific stages of development. While many studies indiscriminately use whole ovaries to represent granulosa cells at specific developmental stage (Alam, Maizels et al. 2004; Ortega, Rey et al. 2010) some researchers have used technique of "follicle puncture" to collect pure population granulosa (Kayampilly and Menon 2007; Yaba, Bianchi et al. 2008; Liu, Fan et al. 2010) or cumulus (Adriaenssens, Segers et al. 2010) cells or cumulus oocyte complexes (Liu, Fan et al. 2010; Salhab, Tosca et al. 2011). The major drawback of this approach is that it can only be used to collect granulosa cells from large antral follicles. For example, follicle puncture technique is used to collect granulosa cells from follicles typically at time-points between eCG48h to hCG12, thus restricting the studies to address peri-ovulatory period only. This technique will not be useful if granulosa cells are to be collected from small antral follicles or luteal cells from CL (since CL's cannot be punctured). Thus, our primary objective was to develop a technique that would allow us to study ovarian somatic cells through a wide range of development (small follicles to CL). Though, very few studies have used LMD to purify specific cells from mouse ovaries (Duggavathi, Volle et al. 2008; Mouzat, Volat et al. 2009), there has been no report on systematic validation of LMD for ovarian studies. Our present study demonstrated very clearly that it is possible to collect granulosa and luteal cells at specific developmental stages by LMD. The RNA quantity (up to 1 μg) and quality (260/280 ratio >1.7) as analyzed by Nanodrop were of high standard. Interestingly, we got higher amount of RNA than most of the earlier published reports in ovary and other organs (Mikulowska-Mennis, Taylor et al.

2002; Niklaus, Babischkin et al. 2002; Quennell, Stanton et al. 2004; Volle, Duggavathi et al. 2007), where the RNA quantification was not even possible as the total RNA yield after LMD was extremely low. However, the quantity is comparable with the previous data showing adequate concentration (0.1μg/μl) in other tissues (Sanchez-Carbayo, Saint et al. 2003).

As far as purity of RNA is concerned, the absorbance ratio from our study agrees with the data (Sanchez-Carbayo, Saint et al. 2003) suggesting >1.8. This suggests the RNA isolated from LMD purified granulosa/luteal cells were of adequate quantity and sufficient quality. Moreover, the RIN value of 5 - 8 obtained through Bioanalyzer is a further indication that the integrity of the extracted RNA was adequate (Schroeder, Mueller et al. 2006).

In order to validate the applicability of the LMD technique for mRNA analysis, we analyzed three candidate genes (*Cyp19a1, Star* and *Fshr*) that are known to be expressed in granulosa cells in a stage specific manner. In agreement with the previous reports (Fitzpatrick, Carlone et al. 1997; Sakurada, Shirota et al. 2006; Duggavathi, Volle et al. 2008; Lavoie and King 2009) our present study showed significant up regulation of *Cyp19a1* at 48h post eCG and down regulation after hCG treatment. This confirms *Cyp19a1* is an FSH responsive gene, expressed in the granulosa cell. Abundance of *Star* mRNA was induced significantly in luteal cells after hCG treatment (Duggavathi, Volle et al. 2008; Lavoie and King 2009), which supports Star is an LH responsive gene. In correlation with *Cyp19a1*, the induction of *Fshr* gene at 48h post - eCG as evidenced in previous reports (Richards 1994; Hillier 2001) suggests that *Fshr* is a granulosa receptor, expressed in response to FSH.

These characteristic stage specific expressions further prove the RNA obtained from LMD samples was well preserved, which allowed us to achieve more accurate downstream molecular phenotyping in a cell type-specific manner within complex ovary. Besides, through comparisons with the mRNA expression pattern of those genes in the LMD purified granulosa and luteal cells, this study provided evidence that LMD technique is a proficient and precise in vivo approach for collection of pure populations of ovarian somatic cells. Thus, as evidenced in various research fields (Sluka, O'Donnell et al. 2002; Vega 2008; Liu 2010); LMD approach could be a useful platform and is highly recommended for the purpose of purifying desired somatic cell population from the ovary.

2. Experiment 2: Expression pattern of genes of ovarian function and Mtor pathway in the granulosa and luteal cells at mRNA and protein level

2.1 Expression of genes of ovarian function

Gonadotropin receptors

In accordance with previous rat ovarian studies, High *Fshr* expression during follicular growth as compared to luteal growth indicates that *Fshr* is a granulosa cell receptor that is regulated by FSH to induce granulosa cell proliferation, differentiation and estrogen production (Hillier 2001). Afterwards, due to LH surge the *Fshr* expression was undetectable during luteal growth (Richards 1994).

The significant induction of *Lhcgr* by eCG in our study is consistent with the earlier in vivo and in vitro data in rat ovaries showing that FSH induction of *Lhcgr* on granulosa cells (Hillier 2001). Subsequently, the high mRNA levels until hCG 24h and further increase at 48h post hCG indicate *Lhcgr* is crucial for the action of LH, by which LH regulates ovulation, reprograms granulosa cell for luteinization during preovulatory stage and induces CL formation (Richards 1994; Fitzpatrick, Carlone et al. 1997; Hillier 2001).

Steroidogenic enzymes

High mRNA levels of *Cyp19a1* during follicular growth and low levels during luteal growth support the earlier evidence in rat ovaries (Fitzpatrick, Carlone et al. 1997) indicating that FSH increases and LH decreases the steady-state levels of Cyp19 mRNA. As per previous reports in mice (Duggavathi, Volle et al. 2008; Lavoie and King 2009), the present results confirm that *Cyp19a1* is an FSH responsive gene in granulosa cells and is known to be involved in the regulation of estrogen synthesis.

Star expression is induced by hCG treatment indicating that *Star* is an LH responsive gene, specifically in terminally differentiating granulosa cells during preovulatory period. This observation concurs with the *in vivo* data in rodent (Rat and mouse) studies suggesting LH regulates *Star* gene expression and this robust *Star* expression at 4h post-hCG is tightly coupled with progesterone production (Ronen-Fuhrmann, Timberg et al. 1998; Duggavathi, Volle et al. 2008; Lavoie and King 2009) .

In agreement with the previous published data in rat ovarian follicles and CL (Goldring, Durica et al. 1987; Lavoie and King 2009), in the present study, *Cyp11a1* mRNA was induced by both eCG and hCG with peak expression in luteal cells. This result suggests although both FSH and LH involve in the regulation of *Cyp11a1*, more specifically, the later controls progesterone secretion by up-regulating the levels of *Cyp11a1* in the CL (Christenson and Devoto 2003).

In the present study, gonadotropin stimulation did not alter *Hsd3b* expression through follicular and CL growth. The result is not consistent with previous reports suggesting *Hsd3b* expression nearly correlates with *Cyp11a1* upon superstimulation as it is involved in progesterone synthesis (Christenson and Devoto 2003; Lavoie and King 2009). However, the result is supported by (Duggavathi, Volle et al. 2008), where Lrh1 KO mice had down regulation of *Star* and *Cyp11a1*, but not *Hsd3b* indicating that *Hsd3b* regulation may not be critically required for the regulation of progesterone synthesis.

Cell cycle regulators

The relative mRNA levels of *Ccnd2* were high during follicular growth and low during CL growth in the present study. These results are in accordance with the observations in rat and human (Sicinski, Donaher et al. 1996; Cannon, Cherian-Shaw et al. 2007; Kayampilly and Menon 2007) suggesting that in the ovarian granulosa cells, *Ccnd2* is an FSH responsive gene, which is specifically induced by FSH and thereby regulates granulosa cell proliferation. The down regulation of *Ccnd2* mRNA from 12h post-hCG indicates the termination of follicular growth by LH (Robker and Richards 1998). The protein level of Ccnd2 increased in granulosa cells of growing follicles. However, in contrast to increase in mRNA in response to hCG, the Ccnd2 protein level fell down indicating that hCG/LH begins the cell cycle exit of the granulosa cells of the ovulating follicles (Robker and Richards 1998). Surprisingly, there was another increase in the level of Ccnd2 protein in luteal cells at 48h post-hCG. This unusual increase in Ccnd2 remains to be further investigated.

Low *Cdnk1b* expression in eCG treated small follicles and high expression in granulosa cells undergoing preovulatory differentiation after hCG are suggestive of termination of follicular growth by LH surge in rodent ovaries (Robker and Richards 1998).

However, in contrast (Robker and Richards 1998), the expression was down regulated in luteal cells from 24h - 48h post-hCG in our study.

Overall, our result with the typical expression profile of the genes encoding gonadotropin receptors, steroidogenic enzymes and cell cycle confirmed that the ovaries used for this study represented specific stages of follicular and CL development.

2.2. Expression of Mtor pathway genes and proteins

Mtor

There was no significant difference in the mRNA abundance of *Mtor* among different time points throughout the gonadotropin stimulated follicular and luteal growth. In fact, the expression was high even in the granulosa cells of small follicles (eCG0h). This result indicates *Mtor* is expressed *in vivo* in the granulosa and luteal cells of the mouse ovary and the expression at the mRNA level does not vary with the developmental stage of the ovarian follicle and corpus luteum. The *in vivo* expression across follicular and luteal stages was also confirmed at protein level through western blot. In agreement with the mRNA data, the uniform protein expression profile upon superstimulation with gonadotropins provided additional proof that the expression of Mtor may not be developmentally regulated in the ovary *in vivo*. This was further supported by our immunofluorescent data, where granulosa cells express Mtor irrespective of the development of associated follicles.

Our data of constitutive expression of Mtor in follicles is supported by the *in vitro* (Yaba, Bianchi et al. 2008) and *in vivo* (Ortega, Rey et al. 2010) data, in which Mtor expression has been shown in the granulosa compartments of the ovarian follicles. Further, in the latter study, the expression at protein level did not vary among primordial, primary, preantral and antral follicles in fetal ovaries of sheep. However, in contrast, *in vitro* reports have shown that FSH (Kayampilly and Menon 2007) and LH (Hou, Arvisais et al. 2010) stimulates the expression of Mtor in cultured rat granulosa and bovine luteal cells respectively. Nonetheless, the results of the present study along with other in vivo observations clearly demonstrate that Mtor is constitutively expressed in granulosa / luteal cells in a gonadotropin independent manner.

Rptor

In correlation with that of *Mtor*, the mRNA expression of *Rptor* did not change significantly in response to eCG or hCG, albeit there was a slight decline in mRNA abundance at hCG24h. Since there was a significant level of its mRNA in granulosa cells obtained at eCG0h, this indicates that Rptor, like Mtor, is not developmentally regulated in the granulosa cells of the ovarian follicles. This inference is plausible, because similar correlation between the expression of Mtor and Rptor has also been seen in several human tissues (e.g. brain, heart, skeletal muscles, liver, lung, thymus, spleen, small intestine, kidney and placenta) as demonstrated by Northern blot analysis (Kim, Sarbassov et al. 2002). Further, there was no difference in the immune reactivity to Rptor antibody in granulosa cells of eCG48h (fully grown) and hCG12h (ovulating) follicles confirming that Rptor protein levels remain constant during different stages of granulosa cell differentiation. Whereas, the expression at protein level could not be detected successfully using western blotting technique, the reason for the failure of western blot might be due to amount of total protein that was available from LMD -purified samples as there was a weak band for *Rptor* in luteal cells at 48h post-hCG.

Rictor

The presence of significant levels of *Rictor* mRNA in granulosa cells from unstimulated follicles (eCG 0h), is supportive of the hypothesis that Mtor pathway is constitutively active in granulosa cells. Further, observation that the mRNA levels do not vary in response to eCG and hCG treatment confirms this idea. However, our data showed that *Rictor* mRNA levels significantly decrease in luteal cells of the CL. This means that Rictor expression is down regulated in terminally differentiated luteal cells. Similar decrease in Rictor expression in cultured muscle cells in response to "differentiating medium" was observed in a previous report (Shu and Houghton 2009). Our western blot analysis did not detect Rictor protein in LMD-purified granulosa cells, probably suggestive of low levels of Rictor in these cells. This was confirmed by our immunofluorescent data, which showed that Rictor was expressed at higher levels in theca cells as compared to granulosa cells. Nonetheless, our mRNA data along with a previous report (Yaba, Bianchi et al. 2008), clearly demonstrate that Rictor is expressed in the granulosa cells and thus provide evidence for the presence of MTORC2 in these cells.

Rps6ka2

We examined the expression pattern of Rps6ka2, as it is one of effectors of Mapk pathway that is known to crosstalk with Mtor pathway (Caron, Ghosh et al. 2010). The dramatic up regulation of *Rps6ka2* mRNA at hCG4h clearly demonstrated that it is a LH driven gene. These results, along with the *in vitro* data implicating Rps6ka2 in the regulation of ovulation (Adriaenssens, Wathlet et al. 2010), suggest that LH stimulated Mtor pathway may be important for the preovulatory differentiation of granulosa cells.

Rps6kb1

The unaltered stable mRNA expression of *Rps6kb1* during gonadotropin stimulated follicular and CL growth is supportive of the hypothesis that the expression of Mtor pathway genes are not regulated developmentally in ovarian granulosa cells. The result does not agree the *in vitro* data showing FSH (Kayampilly and Menon 2007) and LH (Hou, Arvisais et al. 2010) stimulates Rps6kb1 activity.

Overall, our results suggest genes encoding for Mtor signaling pathway are expressed *in vivo* in the mouse ovary. However, the gene expressions are not developmentally regulated across follicular and luteal stages of ovarian growth, because those genes (except *Rps6ka2)* had no responsiveness to gonadotropin stimulation both at mRNA and protein level. Given that our LMD purification at specific stages were very specific as demonstrated by the expression pattern of Gonadotropin receptor, steroidogenic enzymes and cell cycle genes, this study confirms Mtor pathway genes are constitutively expressed; however, these data are suggestive of regulation of Mtor activity in granulosa cells as evidenced by the significant up-regulation of *Rps6ka2* by hCG.

Experiment 3: Effect of fasting on Mtor pathway genes in the granulosa cells

This experiment was designed to investigate whether constitutively expressed Mtor within the granulosa cells performs the role of a metabolic sensor at the ovarian level. We chose acute fasting as an approach to manipulate metabolic signals to the ovary and this would allow us to test our hypothesis that lower energy intake down-regulates Mtor activity thereby affecting ovarian function.

mRNA and protein expression

Fasting for short period of 12 h did not significantly affect the mRNA expression pattern of gonadotropin receptors (*Fshr* and *Lhcgr*), *Cyp19a1, Ccnd2* and Mtor signaling genes including *Mtor, Rptor, Rictor, Rps6ka2* and *Rps6kb1*. The unaltered *Fshr* and *Lhcgr* gene expression is consistent with a previous study (Yan, Zhou et al. 2008) showing that acute fasting for 48h after eCG treatment did not down regulate the mRNA expressions of those genes. However, these ovarian data are in contrast to what was observed in the hypothalamus (Cota, Proulx et al. 2006) and liver (Martinova, Vasiliev et al. 2009). In those studies, Mtor expression was significantly reduced in response to 48h-fasting and was promptly restored in refed mice.

Although there was no change in the mRNA expression of *Ccnd2*, the Ccnd2 protein level was remarkably reduced in the granulosa cells of the fasted mice. This observation indicated that Mtor may be involved in the translation of Ccnd2 as Mtor is mainly associated with the control of translation (Howell and Manning 2011). It also indicated that the inhibition of Mtor in response to reduced metabolic input may underlie the marked reduction in Ccnd2 in fasted mice. This notwithstanding, phosphorylation of Mtor at S2448 was not reduced in the granulosa cells of 12h fasted mice. Therefore, the data of Experiment 3 are not sufficient to confirm the hypothesis that fasting reduces Ccnd2 protein through down-regulation of Mtor activity and therefore requires further investigation.

Consistent with the results of our 12h-fasting study, the preliminary results of our additional study revealed that the ovarian extract from 24h fasted mice did not show reduction in the phosphorylation of Mtor at both S2448 and S2481. However, there was a dramatic reduction in the phosphorylation of Rps6kb1 (T389) in the ovarian extract of 24 h fasted mice. As phosphorylation of Rps6kb1 at T389 is the most commonly used indicator of Mtor activity, our preliminary results suggest Mtor signaling is functional and therefore participates in metabolic sensing in the granulosa cells of the mouse ovary. Of the multiple phosphorylation residues, Rps6kb1 is directly phosphorylated at T389 site by Mtor as the part of the MTORC1, which allows complete activation. Therefore, phosphorylation of Rps6kb1 (T389) could be used as best readout of Mtor activity as compared to other phosphorylation sites (Alessi, Kozlowski et al. 1998; Pullen, Dennis et al. 1998; Averous and Proud 2006). Moreover, Mtor is more known for it effects on translation rather than

transcription in mammalian cells. Hence, we suggest/speculate the down regulated phosphorylated Rps6kb1 (T389) is an indicative of regulation of functional status of Mtor (Mtor activity) in the granulosa cells of the mouse ovary.

Overall, based on the observations from the three experiments of the present Master's project and also from our preliminary results from 24h-fasted mice, we concluded that:

1. Mtor signaling genes are expressed *in vivo* in the ovary
2. The expressions of Mtor and its downstream target, *Rps6kb1,* were found to be stable both in the granulosa and luteal cells. These data provided evidence that Mtor signaling genes are not developmentally regulated in the ovary. However, these data are suggestive of regulation of Mtor activity in granulosa cells as indicated by increased *Rps6ka2* expression by hCG.
3. No effect of fasting on mRNA expression of Mtor pathway and granulosa genes, and the reduced Ccnd2 protein expression in the granulosa cells of fasted mice suggest that Mtor may mediate its effect at translational level.
4. A dramatic down-regulation of the phosphorylated form of Rps6kb1, the best indicator of Mtor kinase activity, in fasted mice is suggestive of Mtor within the granulosa cells responds to nutrient availability. Thus, ovarian Mtor may act as a fuel sensor and its activity in ovarian granulosa cells is controlled by changes in energy status.

VI. CONCLUSION

The results of the present study show that the Mtor and its signaling pathway genes were expressed constitutively in the granulosa cells across all stages of the gonadotropin stimulated follicular and CL development. However, the regulation of Mtor function during ovulation was evidenced from increased expression of Rps6ka2, an upstream activator of Mtor signaling pathway, in terminally differentiating granulosa cells in ovulating follicles. Contrary to our expectation, there was no significant effect of fasting on the mRNA expression of Mtor pathway genes, granulosa-function genes (Ccnd2, Cyp19, Fshr, etc.) and phosphorylation of Mtor at S2448 and S2481. Nevertheless, absolute reduction of Ccnd2 protein in the granulosa cells of fasted mice is suggestive of regulation of Mtor activity, because Mtor is a candidate molecule that mediates cellular translation by sensing energy status. Further, a dramatic down regulation of phosphorylated form of Rps6kb1, a downstream Mtor target and a marker of MTORC1 activity, in the ovaries of 24h-fasted mice in our preliminary study, supported the hypothesis of the regulation of Mtor activity in response to nutrient availability.

Therefore, these data collectively suggest that the expression of Mtor pathway genes is not developmentally regulated in the ovary, despite this, the ovarian Mtor appears to function as a fuel sensor and its activity in the granulosa cells is controlled by changes in energy status. Eventually, this study suggests Mtor signaling genes are required for regulation of normal ovarian function. On top of this, this study proved that LMD is an efficient in vivo method of approach to purify homogenous populations of ovarian somatic cells, and the quantity and quality of RNA obtained from LMD purified granulosa/luteal cells were of adequate and sufficient for subsequent downstream molecular analysis.

However, further knockout mouse model would be required to confirm the functional role of Mtor in granulosa cells and to examine whether Mtor signaling pathway is required for normal ovarian dynamics. It is also essential to identify the functional links between Mtor pathway genes and other glycoprotein/peptide hormones in the granulosa cells. The development of granulosa specific Mtor knock out mice is one of the ongoing researches in our laboratory and the results of the present studies will serve as the basis for the systematic phenotyping of the Mtor knockout mice.

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